

**Investigating the involvement of FHA domain lateral
surfaces in DNA damage and cell cycle checkpoint responses
in *Saccharomyces cerevisiae***

by

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Statement of Contributions

Exception to sole authorship of materials are as follows:

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Dr. Brendan McConkey assisted with the bioinformatics experimental design and analysis. Ema Hazra and Allison Guitor generated the pJG4-6 *DUN1 FHA*, pEG202 *DIF1*, and pEG202 *SML1* plasmid constructs. Damir Mingaliev and Aaron Robertson contributed to the initial bioinformatics analysis of FHA domains.

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Dr. Brendan McConkey assisted with the bioinformatics experimental design and analysis. The yeast two-hybrid analysis for the Dun1-Dif1 interaction looking at the H77A and C64A mutations was conducted by Senior Honours Thesis student Renessa Gomes.

Chapter 4.

Aaron Neiman (Stony Brook University, New York) performed the Alpha Fold modelling for the Mek1 FHA domain interaction with Ndt80 “bc” domain (Figure 4.3). The figure was provided by Nancy Hollingsworth (Stony Brook University, New York). Lihong Wan conducted yeast two-hybrid spotting and liquid assays for Mek1 loop residue mutants as well as sporulation efficiency assays, spore viability testing in the *DMC1* background and immunoblotting (Figures 4.4-4.6). Data was provided by both Lihong Wan and Nancy Hollingsworth (Stony Brook University, New York). Intellectual input from Nancy Hollingsworth contributed to the interpretation of the data.

Abstract

Since the discovery of the Forkhead-associated (FHA) domain in 1995, research groups have pursued the characterization of FHA domains and the interactions that they regulate through the lens of their pThr-epitope binding specificity. Over the past two decades, isolated cases of FHA domains that were not just relying on the pThr-epitope binding site for the establishment of interactions have been reported. One case from the Duncker Lab reported that the interaction between the FHA1 domain of Rad53 and the H-BRCT domain of Dbf4 did not use the Rad53 FHA1 pThr-epitope binding site for the establishment of the interaction. Instead, a conserved patch of residues located on the distal lateral surface was responsible for the interaction between Rad53 and Dbf4. Since the publication of that discovery, the prevalence of such non-canonical interactions has been a persistent question. This thesis aimed to address instances of additional cases where an FHA domain was responsible for mediating a protein-protein interaction in a way that was independent of the pThr-epitope binding site. Specifically, the goals were to: (1) explore whether the entire list of budding yeast FHA domains had been collected, (2) characterize the known FHA domains for lateral surface conservation via the identification and distinguishment of candidate residues on the lateral surfaces that could be involved in protein-protein interactions as well as the pThr-epitope binding site, (3) explore whether other H-BRCT domains besides that of Dbf4 and Sir4 could be identified, in case the non-canonical nature of the Rad53-Dbf4 interaction was found to be a consequence of the H-BRCT domain structure, and (4) demonstrate instances of other yeast FHA domain-containing proteins that utilize the lateral surface(s) of the FHA domain as an interface to mediate protein-protein interaction.

Yeast two-hybrid assays looking at the interactions between Dun1 and two of its ligands Dif1 and Sml1 showed that the Dun1 FHA domain utilizes its distal lateral surface in conjunction with the pThr-epitope binding site to mediate ligand binding and efficiency. Loss of interactions with Dif1 and Sml1 in response to mutations of the pThr-epitope binding site and distal lateral surface resulted in hypersensitivity to methyl methanesulfonate (MMS), a DNA alkylating agent, and hydroxyurea (HU), which depletes dNTP pools, as well as sensitivity to DNA double strand break (DSB)-inducing agents bleomycin and phleomycin. Analysis of the sequence and structural conservation of all known budding yeast FHA domains revealed two ‘motifs’, the first being the ‘GR motif’ for the identification of the conserved arginine residue of the pThr-epitope binding site. The second motif was an ‘SxxH motif’ located downstream of the ‘GR motif’. When the conserved histidine residue

was mutated to alanine, the Dun1-Dif1 interaction was significantly reduced. Comparing the FHA domains of budding yeast paralogs and a human homolog: Rad53, Dun1, Mek1, and Chk2, a different secondary motif was found that consisted of a highly conserved cysteine and proline residue. Mutation of the cysteine residue also resulted in a significant reduction in the Dun1-Dif1 interaction, although not to the same extent as mutation of the conserved histidine. Alignment of the FHA domain structures of the Rad53 FHA1, Dun1, Mek1, and Chk2 FHA domains showed positional conservation of residues that are known to be involved in FHA domain-mediated protein-protein interactions, indicating evolutionary conservation of lateral surface regions that could also be related to protein function(s). Novel FHA and H-BRCT domains were not identified, although further investigation could lead to a better understanding of the difference and evolutionary development of tandem BRCT domains and the alpha-helical linker region that can be found between tandem BRCT domains. Characterization of the meiosis specific Rad53 paralog, Mek1, revealed that the proximal lateral surface of the Mek1 FHA domain is involved in the establishment of the Mek1-Ndt80 interaction. Loss of interaction as a result of mutating residues on the β -strands of the proximal lateral surface led to compromised interhomolog repair of Spo11-induced DSBs and Mek1 kinase activity whereas mutation of residues on an α -helical loop connecting those β -strands specifically abrogated the Mek1-Ndt80 interaction without impairing Mek1 kinase activity.

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In memory of my loving mother, June Allison Dian Straker, who passed away during the first year of my PhD program. Her passing both broke my heart and created the circumstances for God to show me just how well He can put broken pieces back together. Thank you for being such an amazing demonstration of selfless love. I pray that I was able to make you proud.

Dedication

To my Heavenly Father, who, during my pursuit of this degree, took my broken pieces and crafted them into a version of myself that I did not have the courage or capacity to imagine. May my life be a testimony and witness of His goodness. Without Him, the completion of this degree and the depth of knowledge and provision that carried me through would not have been possible. Truly He is an awesome God.

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List of Abbreviations

BLAST: basic local alignment search tool
BRCA1: Breast Cancer gene 1
Cdc: Cell division cycle
CDK: Cyclin dependent kinase
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
DDK: Dbf4 dependent kinase
DNA: Deoxyribonucleic acid
dNTP: Deoxyribonucleoside triphosphate
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
FHA: Forkhead-associated
FL: Full-length
GAL/RAF: Galactose/Raffinose
gRNA: guide RNA
HA: Hemagglutinin
H-BRCT: Helix-BRCA 1 Carboxy-terminal
HO: Homothallism
HU: Hydroxyurea
Kb: kilobases
kDa: kilodalton
MAT: Mating type
MMS: Methyl methanesulfonate
MYC: Myelocytamatosis
ONPG: 2-Nitrophenyl- β -D-galactopyranoside
PCR: Polymerase chain reaction
pHMM: profile Hidden Markov Model
PI3K: phosphatidylinositol 3' kinase
PIKKs: phosphatidylinositol 3' kinase (PI3K)-related kinases

PMSF: Phenylmethanesulphonyl fluoride

pThr: phosphothreonine

RNA: ribonucleic acid

RNR: Ribonucleotide reductase

SC: Synthetic complete

SDS: Sodium dodecyl sulfate

WCE: Whole cell extract

WT: Wild type

YPD: Yeast extract, peptone, dextrose

Chapter 1

Literature Review and Research Objectives

1.1 *Saccharomyces cerevisiae* and the Cell Cycle

The biological processes that govern life utilize an intricate network of interacting proteins and signal transduction pathways that continue to require intensive study. Due to the complexities of “higher level” organisms like humans, many research techniques were first established in and applied to model organisms such as *Saccharomyces cerevisiae*, baker’s/brewer’s yeast. The *Saccharomyces cerevisiae* model organism, more commonly known as budding yeast, has been and continues to be a fundamental tool for the advancement of our understanding of the cell cycle and cell cycle checkpoint responses, key biological processes that contribute to the development of many human diseases such as cancer when they malfunction and/or are not properly regulated.

1.1.1 Budding Yeast as a Model Organism

Budding yeasts are single-celled eukaryotic micro-organisms that belong to the fungal kingdom. These facultative anaerobes use fermentation for cellular respiration, producing carbon dioxide and ethanol (reviewed in Mortimer, 2000). The ease with which yeast cells can be grown and maintained under optimal laboratory conditions of sugar and nitrogen-based media, with a typical doubling time of 90 minutes, makes yeast very easy to utilize and manipulate (Mortimer and Johnston, 1986, reviewed in Duina et al., 2014, Gershon and Gershon, 2000 and Herskowitz, 1988). Budding yeast is one of two main yeast species used for cell cycle and aging studies and was used throughout the research presented in this thesis. The ‘budding yeast’ common name is a description of the distinguishing feature of this yeast species where cell proliferation presents as an asymmetrical division of the ‘mother cell’, producing a smaller ‘bud’ daughter cell that needs to increase in size prior to chromosomal duplication (reviewed in Duina et al., 2014 and Herskowitz, 1988).

Budding yeast has a relatively small and fully sequenced genome of just over 12,000 kilo bases (Kb) assembled into 16 linear chromosomes in the haploid genome for which there are approximately 6000 genes (Goffeau et al., 1996, reviewed in Gershon and Gershon, 2000). Budding yeast cells can exist as one of three distinct cell types, two of which are haploids (reviewed in Lee and Haber, 2015 and Duina et al., 2014). Haploid cell types proliferate mitotically and can be classified based on yeast cell mating type, *MATa* or *MAT α* (reviewed in Duina et al., 2014, Gershon and Gershon, 2000, Mortimer, 2000, and Herskowitz, 1988, Figure 1.1). Mating *MATa* and *MAT α* haploid cells generates the third cell type,

MATa/a diploid cells which are capable of mitotic proliferation and meiotic spore formation, in response to nutrient limitation (reviewed in Lee and Haber, 2015 and Duina et al., 2014, Figure 1.1).

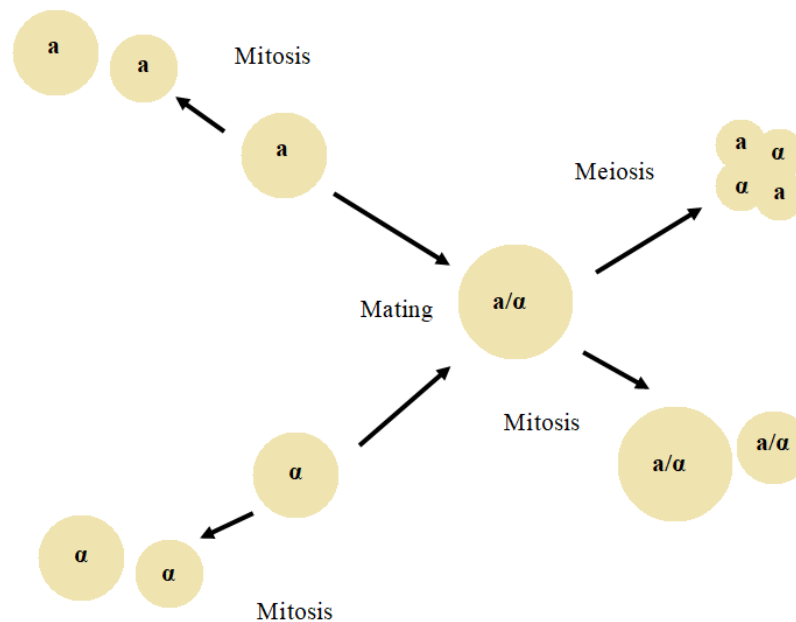


Figure 1.1 Budding Yeast Cell Types

Illustration of simplified budding yeast life cycle and cell types showing mating type distinctions for the *MATa* or *MATα* haploids (*a* or *α*, respectively) and *MATa/a* diploids (*a/a*). Sporulation of a *MATa/a* diploid cell via meiosis produces a 4-spore tetrad (adapted from Duina et al., 2014).

The *MAT* locus is in the middle of the right arm of chromosome III and contains the genetic information for mating type determination (reviewed in Lee and Haber, 2015 and Duina et al., 2014, Figure 1.2). The two non-homologous mating type alleles, *MATa* or *MATα*, encode proteins that lead to the expression of allele-specific genes such as the mating pheromones, *a*-factor and *α*-factor, as well as trans-membrane receptors for the pheromone of the opposite mating type (reviewed in Lee and Haber, 2015). Recognition of mating factor from the opposite mating type triggers cell cycle arrest in G1 phase allowing haploid cells to prepare for cellular and nuclear fusion to generate diploid cells (reviewed in Lee and Haber, 2015 and Duina et al., 2014). Also located on chromosome III are two silent genetic cassettes, *HMLα* and *HMRa*, which carry the genetic information of the *MATα* and *MATa* alleles, respectively (Figure 1.2). However, due to their location within heterochromatic regions of the left and right arms, both cassettes are transcriptionally silent (reviewed in Lee and Haber, 2015 and Duina et al., 2014).

Functional HO endonuclease, encoded by the *HO* (“Homothallism”) gene, can facilitate the transposition of either the *HML α* or *HMR α* gene cassette from its location in the heterochromatin to the *MAT* locus where it will be expressed. Therefore, homothallic haploid cells which possess functional HO endonuclease can easily switch between the two mating types. In the absence of functional HO endonuclease, haploid cells are heterothallic and unable to switch mating types (Mortimer and Johnston, 1986, reviewed in Lee and Haber, 2015 and Duina et al., 2014).

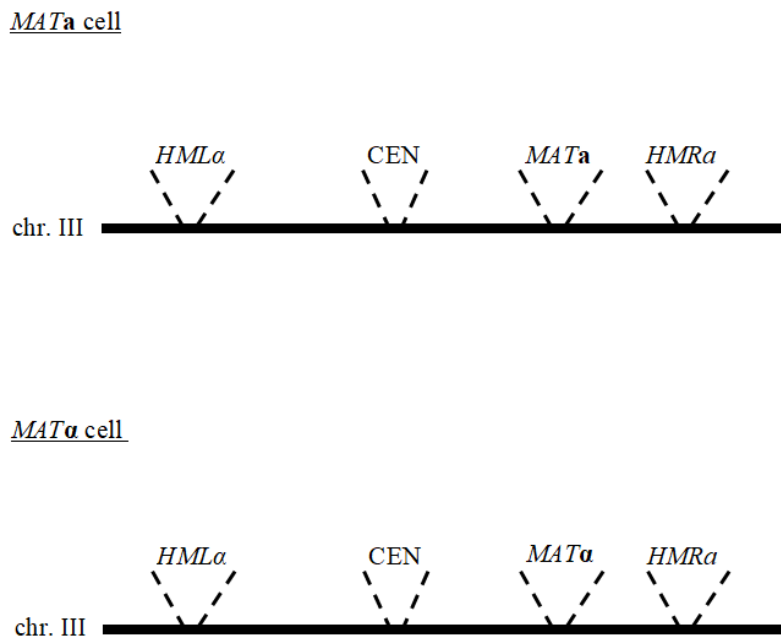


Figure 1.2 *Saccharomyces cerevisiae* chromosome III

Simplified graphic representation of chromosome III illustrating the key genetic elements for mating type determination based on the budding yeast cell type, *MAT α* or *MAT α* respectively. The location of the centromeric region (*CEN*) is indicated as well as the *HML α* or *HMR α* genetic cassettes. Loci are not necessarily drawn to scale (adapted from Duina et al., 2014).

Yeast geneticists Ojvind Winge and Carl Lindergren used heterothallic and homothallic yeast strains found on rotting figs during the late 1800s-early 1900s to pioneer the use of yeast for research (Mortimer and Johnston, 1986, reviewed in Duina et al., 2014 and Mortimer, 2000). Yeast strains used for research today are mostly derived from a heterothallic strain isolated by Emil Mrak in 1891 which allows for the generation of stable yeast strains for research (Mortimer and Johnston, 1986, reviewed in Duina et al., 2014 and Mortimer, 2000). The nature of cell cycle progression in budding yeast has allowed for the

use of chemicals such as the mating factor pheromones to synchronize cell cultures by triggering cell cycle arrest (reviewed in Gershon and Gershon, 2000). Additionally, the proficiency of genetic recombination mechanisms in budding yeast have allowed for the development of a vast and easily accessible collection of genetic knockout strains that can be used to study yeast genes as well as a collection of techniques for highly efficient genetic manipulation (reviewed in Gershon and Gershon, 2000). The budding yeast genome also has many orthologs for genes in humans that are involved in pathological events for similar, if not highly conserved, biological pathways (reviewed in Calhoun et al., 2019).

1.1.2 Cell Cycle Progression

Cellular division refers to the process by which cells grow and divide or proliferate. The typical eukaryotic cell cycle consists of two growth phases, Gap 1 (G_1) and Gap 2 (G_2), which flank the Synthesis (S) phase, during which DNA is replicated, followed by the mitotic or mitosis (M) phase (Figure 1.3). The G_1 phase allows for preparatory growth of cells prior to DNA replication which occurs in S phase (reviewed in Harashima et al., 2013 and Herskowitz, 1988) as well as the regulated association of DNA replication initiation factors at sites of DNA replication referred to as origins of replication. The G_0 phase is a quiescent stage where cells can rest if optimal conditions do not exist for DNA replication and cellular division (reviewed in Herskowitz, 1988; Figure 1.3). The START point of the cell cycle is a highly regulated point during late G_1 at which cells commit to entering S phase based on and influenced by nutrient availability, presence of compatible mating pheromones, and/or cell size (reviewed in Harashima et al., 2013 and Herskowitz, 1988; Figure 1.3). DNA content is duplicated via semi-conservative replication at 'licensed' origins of replication during S phase and then the G_2 phase is available as a secondary period of growth for most eukaryotic cells to prepare for the separation of their cellular components and cytoplasmic contents (reviewed in Harashima et al., 2013 and Herskowitz, 1988). The M phase features the physical segregation of replicated genetic material over the span of four stages: Prophase, Metaphase, Anaphase and Telophase. The actual separation of the daughter cell(s) and their cytoplasm occurs after mitosis and is referred to as cytokinesis (reviewed in Harashima et al., 2013 and Herskowitz, 1988). Cytokinesis is established via the formation of a contractile actomyosin ring at the 'division plane' between the two poles formed by the spindle microtubules during mitosis (reviewed in Balasubramanian et al., 2004). The contraction of the actin ring between the two poles pinches the cytoplasm generating two distinct daughter cells (reviewed in Balasubramanian et al., 2004).

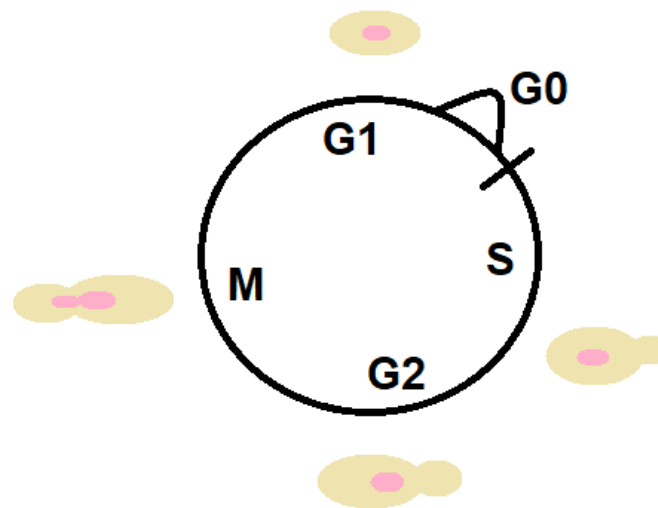


Figure 1.3 Eukaryotic Cell Cycle and *Saccharomyces cerevisiae*

Illustration of the typical eukaryotic cell cycle showing the order of cell division phases: Gap 1 (G₁), Synthesis (S), Gap 2 (G₂), and Mitosis (M). Bud formation of a single budding yeast cell (pale yellow cell with pink nucleus/nuclei) is illustrated alongside typical eukaryotic cell cycle. The solid line represents the START point for S phase entry commitment. G₀ indicates the quiescent stage.

The budding yeast cell cycle differs from the typical eukaryotic cell cycle in that it does not feature an actual G₂ phase due to the mitotic spindle formation during S phase (reviewed in Gershon and Gershon, 2000; Figure 1.3). Instead, bud size increases as the cell progresses into mitosis. An additional distinguishing feature of the budding yeast cell cycle is that due to the bud formation (shown in Figure 1.3), the resulting daughter cell is significantly smaller than the mother cell. As a result, the duration of the G₁ phase is extended to allow additional time for the daughter cell to grow to its ‘adult’ size prior to getting past the START point and successfully completing the cell cycle (reviewed in Gershon and Gershon, 2000). Budding yeast do not exhibit nuclear envelope breakdown. Instead, spindle pole bodies form within the nuclear envelope as well as a septum of cell wall material at the ‘division plane’ after the formation of the actomyosin ring (reviewed in Balasubramanian et al., 2004). Spindle pole body formation influences chromosomal segregation to opposite poles of the nucleus which generates two ‘daughter nuclei’ and the passage of one of the two ‘daughter nuclei’ through the ‘bud neck’ is indicative of mitotic exit and cytokinesis in budding yeast (reviewed in Balasubramanian et al., 2004).

Cell cycle progression and regulation is driven by two main modes of post-translational modifications: phosphorylation and ubiquitinylation. Protein kinases and phosphatases work in opposition to each other to regulate the phosphorylation states of various proteins involved in the cell cycle to modify protein activity and/or stability (reviewed in Ong and Torres, 2019). The E3 ubiquitin ligases and deubiquitylases function to control the ubiquitylation states of their substrates which targets or protects them from proteasomal degradation, respectively. Early studies assessing the DNA content, size, and doubling time of mutant strains of the fission yeast *Schizosaccharomyces pombe* led to the identification of genes that influence the cell cycle, referred to as cell division cycle or *cdc* genes (Hartwell et al., 1973; reviewed in Ong and Torres, 2019). The first *cdc* gene to be characterized was *cdc9-50* or *WEE1* (Nurse, 1975). The observation that *WEE1*-mutant yeast cells divided at a smaller size compared to WT cells, indicative of accelerated mitotic entry, led to the conclusion that Wee1 inhibits mitosis.

The overexpression of fission yeast *cdc25*, which encodes a protein phosphatase, resulted in increased rates of mitotic entry (Lee et al., 1992; Russell and Nurse, 1986). Together, Wee1, which was later determined to be a protein kinase (Russell and Nurse, 1986), and Cdc25 work together to regulate the mitotic entry via the phosphorylation and dephosphorylation of a common substrate, the Cdc2 protein kinase in *S.pombe* (Cdc28 in *S. cerevisiae* and CDK1 in humans) which is involved in DNA replication initiation. Wee1 is responsible for phosphorylating and inactivating Cdc2 whereas Cdc25 dephosphorylates and activates Cdc2 (reviewed in Ong and Torres, 2019). Alongside these studies done in fission yeast, the analysis of frog oocytes led to the identification of a substance in the cytoplasm, referred to as maturation-promoting factor (MPF), which was found to regulate entry into meiosis (Hara et al., 1980; Masui and Markert, 1971), with the levels of MPF oscillating in a phase-dependent manner (Gerhart et al., 1984). When purified (Lohka et al., 1988), MPF was determined to be a complex of a *S. pombe* Cdc2 homologue (Dunphy et al., 1988) and a larger protein, later identified as a member of the cyclin class of proteins, cyclin B (Draetta et al., 1989). The cyclin proteins were discovered in sea urchins and clams and named based on the observation that their protein levels cycled in accordance with the mitotic cell cycle (Evans et al., 1983).

Natural cell cycle progression is regulated by the association of various regulatory cyclins with cyclin-dependent kinases (CDKs) (Koivomagi et al., 2011). In budding yeast, there are six CDKs that can be divided into two groups (reviewed in Malumbres, 2014). The first group of CDKs consists of Cdc28 and Pho85 and can associate with multiple cyclins and regulate the cell cycle (reviewed in Harashima et

al., 2013, Malumbres, 2014, and Morgan, 1997). The second group, which contains Kin28, Srb10, Bur1, and Ctk1, can only each be activated by a single cyclin and are involved in regulating transcription (reviewed in Harashima et al., 2013, Malumbres, 2014, and Morgan, 1997). Regulation of G₁ phase activities is largely controlled by the Cln1-3 cyclins in association with Cdc28 (Figure 1.4; Koivomagi et al., 2011; reviewed in Nasmyth, 1996 and Morgan, 1997). The Clb5 and Clb6 cyclins associate with Cdc28 to coordinate S phase events while Clb1-4 associate with Cdc28 to regulate mitosis (Figure 1.4; Koivomagi et al., 2011; reviewed in Nasmyth, 1996 and Morgan, 1997).

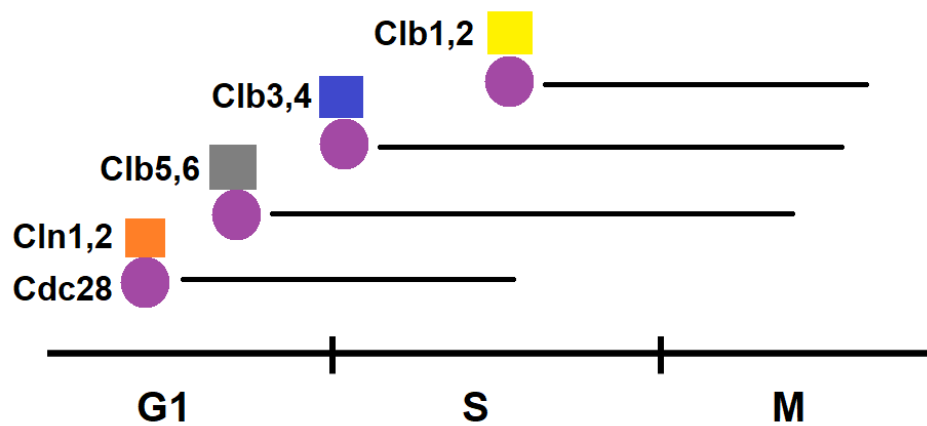


Figure 1.4 Cell Cycle Progression of Cyclin-CDK Complexes in Budding Yeast

Linear representation of the budding yeast cell cycle showing major cyclin-CDK complexes involved in the regulation of the cell cycle progression per stage. Solid black lines represent the main initiation and duration of effects for phase-specific cyclin-CDK associations. The purple circle represents the Cdc28 kinase, and the coloured squares represent various budding yeast cyclins (reproduced from Straker, 2018; adapted from Morgan, 1997).

1.1.3 Cell Cycle Checkpoints

The cell cycle checkpoint concept was proposed by Weinert and Hartwell in the late 1980s pertaining to how cells evaluate their preparatory and executory stages of cellular division to ensure the proper order of cell cycle events (Weinert and Hartwell, 1988; reviewed in Hartwell and Weinert, 1989 and Lanz et al., 2019). Studies done in yeast and mammalian systems during the 1990s identified a collection of evolutionarily conserved DNA damage signaling kinases that were found to be connected to cell cycle control and cell cycle checkpoints (reviewed in Lanz et al., 2019). During the late 1990s and early 2000s, these same kinases were found to also be involved in processes beyond cell cycle control

such as apoptosis, transcription, and DNA replication (reviewed in Lanz et al., 2019). These kinases include phosphatidylinositol 3' kinase (PI3K)-related kinases (PIKKs) and PIKK-regulated downstream kinases (reviewed in Lanz et al., 2019). A mass spectrometry (MS)-based proteomics analysis of these kinases revealed the complex nature of the system of phosphorylation events attributed to these DNA damage signaling kinases and the numerous substrates that they regulate (Matsuoka et al., 2007; Smolka et al., 2007; reviewed in Lanz et al., 2019). At the time that these DNA damage signaling kinases were discovered, there was insufficient data to connect the kinases with the known DNA repair machinery. Since then, however, numerous studies have shown that the DNA damage signaling kinases function to activate and/or regulate the DNA damage repair machinery which are often direct substrates of the DNA damage signaling kinases (reviewed in Lanz et al., 2019).

The DNA damage signaling kinases typically associate with DNA structures that are formed due to DNA damage or DNA replication stress and can be classified as either apical or effector kinases. Mec1 and Tel1 are the budding yeast apical PIKKs and ATR, ATM, and DNA-PKcs are the mammalian counterparts (Figure 1.5; reviewed in Lanz et al., 2019). Mec1 and ATR are known to associate with single-stranded DNA (ssDNA) whereas Tel1, ATM and DNA-PKcs are known to associate with double-stranded DNA (dsDNA) breaks (reviewed in Lanz et al., 2019). The apical kinases transfer the DNA damage 'signal' to downstream effector kinases, Rad53/Chk1/Dun1 in budding yeast and CHK2/CHK1 in humans, by phosphorylating the downstream effector kinases. In humans, this is a cascade mediated by adaptor proteins (Figure 1.5; reviewed in Lanz et al., 2019). The main adaptor proteins are Rad9 and Mrc1 in yeast and 53BP1, MDC1, and Claspin in humans (Figure 1.5; reviewed in Lanz et al., 2019). In budding yeast, Mec1/Tel1 phosphorylation of histone H2A and the 9-1-1 complex at sites of DNA damage leads to the recruitment and subsequent Mec1/Tel1-dependent phosphorylation of Rad9 (reviewed in Lanz et al., 2019). Rad9 phosphorylation leads to Rad9 oligomerization and the generation of Rad53 docking sites which leads to Rad53 recruitment at DNA damage sites for Mec1/Tel1-dependent Rad53 phosphorylation and activation (reviewed in Lanz et al., 2019). Active Rad53 leads to Dun1 phosphorylation and activation which influences transcriptional and functional control of the ribonucleotide reductase (RNR) enzyme responsible for the regulation of deoxyribonucleoside triphosphate (dNTP) production in budding yeast (Figure 1.5; Chen et al., 2007). Rad9 is also involved in mediating Mec1-dependent phosphorylation for Chk1 activation in yeast, but instead of relying on Mec1-dependent phosphorylation for the recruitment of Chk1 to Rad9 such as is seen with Rad53, Chk1 relies on cyclin-dependent kinase (CDK)-dependent phosphorylation for recruitment (Abreu et al., 2013; reviewed in Lanz et al., 2019).

Mrc1, which is also a natural component of the replisome, can mediate Rad53 activation, leading to a more rapid response to replication stress (Alcasabas et al., 2001; Osborn and Elledge, 2003; Pardo et al., 2017; Bacal et al., 2018; reviewed in Lanz et al., 2019).

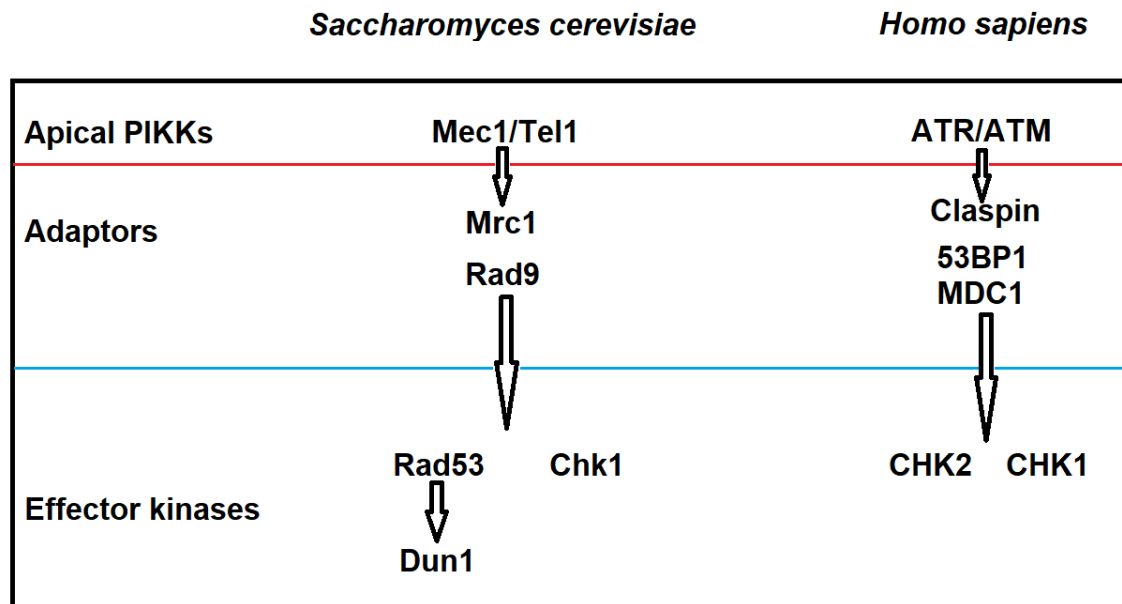


Figure 1.5 DNA damage and checkpoint proteins in *Saccharomyces cerevisiae* and *Homo sapiens*

This figure illustrates a simplified pathway of the main DNA damage sensor kinases in yeast and humans and the effector kinases that they influence for the DNA damage and checkpoint responses. Adaptor proteins that mediate the phosphorylation cascade for signaling are indicated.

The activated DNA damage signaling cascade leads to a plethora of responses that control, regulate and/or correct errors in DNA replication and cellular division. Such responses range from cell cycle arrest and regulating origin firing to replication fork processing and transcriptional regulation (reviewed in Lanz et al., 2019). Additional responses have also been identified that influence apoptosis and dNTP levels within cells (reviewed in Lanz et al., 2019). In more recent studies, researchers have observed that these DNA damage signaling kinases can also be attributed to regulating autophagy, gene gating, chromosome mobility, and transcriptional and replication processing (reviewed in Lanz et al., 2019). Despite the breadth of knowledge revealed over the past decades regarding these DNA damage

signaling kinases, there are still many mechanisms of DNA damage responses and cell cycle checkpoints that have yet to be fully elucidated (reviewed in Lanz et al., 2019).

1.2 Protein-Protein Interactions and the FHA Domain

Protein-protein interactions are essential to the biological processes that govern life. Such interactions allow for the signal transduction pathways that regulate cell cycle progression and cell cycle checkpoint responses (reviewed in Almawi et al., 2017 and Seet et al., 2006). Protein-protein interactions can be mediated by independently folded subunits of a protein, protein modules, that rely on phosphorylation and dephosphorylation events (reviewed in Pawson and Nash, 2000, Almawi et al., 2017, and Kuriyan and Cowburn, 1997). Phosphorylation modifications in eukaryotes typically occur at serine and threonine (Ser/Thr) amino acid residues and were the basis for the identification of families of phospho-epitope binding proteins (reviewed in Yaffe and Elia, 2001). First discovered in proteins involved in signal transduction, protein modules contribute to the evolution of complex organisms due to their ability to be shuffled within the genome (reviewed in Kuriyan and Cowburn, 1997 and Pawson and Nash, 2000). This allows organisms to develop more complex pathways for protein and ligand recognition (reviewed in Kuriyan and Cowburn, 1997 and Pawson and Nash, 2000). Protein modules can be divided into different classes based on structure and/or binding specificity. The 14-3-3 proteins were the first identified pSer/Thr-binding proteins (reviewed in Yaffe and Elia, 2001). F-box containing proteins recognize substrates of the Skp1-Cdc53/Cullin-F-box (SCF) ubiquitin ligases and possess WD40 or leucine-rich-repeat (LRR) domains which are thought to be pSer/Thr-binding modules (reviewed in Yaffe and Elia, 2001). WW domains are approximately 40 amino acids long and fold into three anti-parallel β strands which bind short proline-rich sequences (reviewed in Yaffe and Elia, 2001). Forkhead-associated (FHA) domains are the only known group with specificity for phosphorylated threonine (pThr) residues (Durocher et al., 2000).

1.2.1 FHA Domain Structure and pThr-binding Specificity

The FHA domain was originally identified as a 55-75 amino acid long module in a subset of the family of forkhead transcription factors with three conserved sequence blocks (reviewed in Yaffe and Elia, 2001). Since then, they have been more fully characterized as 100-120 amino acids in length with the topology of an 11-stranded β sandwich (Durocher et al., 2000; reviewed in Durocher and Jackson, 2002, Mahajan et al., 2008, and Mohammad and Yaffe, 2009; Figure 1.6). The most prominent feature of FHA domains is their specificity for phosphothreonine (pThr)-containing epitopes (Durocher et al., 1999,

2000; Li et al., 1999; Liao et al., 1999, 2000; reviewed in Li et al., 2000). Prior to the actual discovery of FHA domains in a 1995 bioinformatics study of the forkhead-type transcription factors by Hofmann and Bucher, two separate studies, investigating signal transduction in *Arabidopsis thaliana* and the DNA damage checkpoint response in *Saccharomyces cerevisiae*, demonstrated the phospho-dependent nature of the domain (Stone et al., 1994; Durocher et al., 1999). In 1994 the interaction between *Arabidopsis thaliana* kinase-associated protein phosphatase (KAPP) and receptor-like protein kinase (RLK5) was found to rely on phosphorylation via what was then called the kinase-interaction (KI) domain of KAPP (Stone et al., 1994). The plant receptor-like kinase CLAVATA 1 (CLV1) was later discovered to be negatively regulated by KAPP, exhibiting similarities to the SH2-containing tyrosine phosphatases (Stone et al., 1998). More striking was the discovery that conserved residues within KAPP's FHA domain were required for the activity of the KI domain (Li et al., 1999). This discovery indicated that the KAPP FHA domain mediates protein-protein interactions that rely on phosphorylation (Li et al., 1999).

In budding yeast, the discovery that the protein encoded by *RA*diation sensitive (*RAD53*), Rad53 checkpoint kinase, interacted with the phosphorylated form of the Rad9 adaptor protein demonstrated another case of phospho-dependence (Durocher et al., 1999; Sun et al., 1998; reviewed in Durocher and Jackson, 2002). More definitively, a pThr-containing version of p53 could compete with Rad9 for interaction with the first FHA domain of Rad53, not the unphosphorylated form, and this competitive binding was able to completely abrogate the interaction between Rad53 FHA1 and Rad9 (Durocher et al., 1999). A phosphothreonine-containing peptide of the p53 N-terminus was found to directly bind to Rad53 FHA1, and mutation of the pThr-binding site of Rad53 FHA1 completely abrogated the interaction (Durocher et al., 1999). The Rad53 FHA1 domain was able to protect the phosphorylated residue of Rad9 from phosphatase treatment, and the interaction between Rad53 FHA1 and Rad9 only existed with phosphorylated threonine, not serine, and the conservation of a specific residue at the pThr + 3 position which likely confers selectivity (Durocher et al., 1999; reviewed in Durocher and Jackson, 2002). Together, these results led to the classification of FHA domains as pThr-binding motifs (reviewed in Durocher and Jackson, 2002).

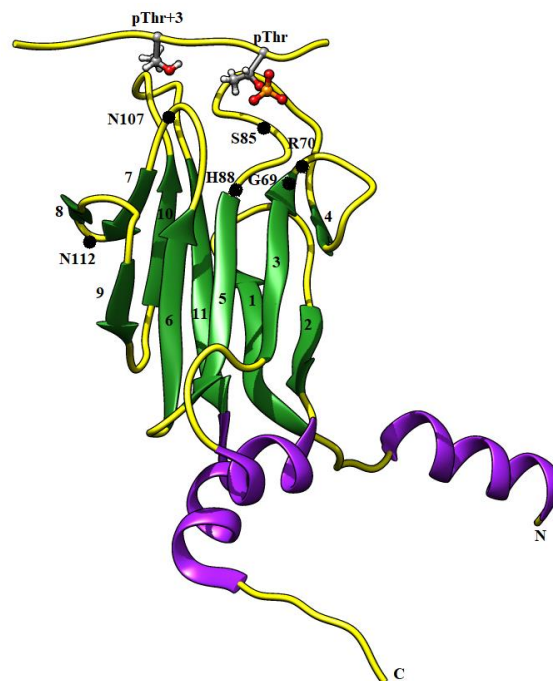


Figure 1.6 FHA Domain Topology.

Ribbon diagram representation of the Rad53 FHA1 as an example of the conserved FHA domain structure. The Rad53 FHA1 domain is shown in complex with a peptide (ball and stick model) that has a phosphorylated threonine residue (PDB ID: 2JQI). Neighbouring residues to the phospho-threonine (pThr) are indicated numerically based on proximity to the pThr residue. The core homology region for the FHA domain consists of the β 3- β 10 sheets coloured in green. Indicated residues are highly conserved in multiple sequence alignments and contribute to the interaction (adapted from Durocher and Jackson, 2002).

Despite sharing key features, FHA domains typically lack amino acid conservation (reviewed in Mahajan et al., 2008). Eight of the eleven strands comprise the core homology region on one face of the beta sandwich with the remaining three strands being more variable with respect to positioning (reviewed in Durocher and Jackson, 2002 and Mohammad and Yaffe, 2009). Residues required for the recognition of phosphorylated peptides are all located within the core homology region, and either are responsible for establishing direct contact with the phosphopeptide backbone, establishing hydrogen bonds to contact the phosphorylated threonine residue, or maintaining the integrity of the pThr-binding site (reviewed in Durocher and Jackson, 2002). The FHA domain has since been identified in over 200 eukaryotic and prokaryotic proteins (reviewed in Almawi et al., 2017) and are highly enriched in proteins contributing to and/or controlling gene expression, signal transduction, protein transport and degradation, DNA damage

repair, and checkpoint activation (Li et al., 1999; Durocher et al., 2000; reviewed in Durocher and Jackson, 2002).

Most research confirms the phospho-dependent nature of FHA domains. However, there are instances where FHA domains do not rely on the recognition of a phospho-epitope for protein and/or ligand binding (reviewed in Durocher and Jackson, 2002). One such example is the human homolog of budding yeast Rad53, Chk2. Mutation of a conserved isoleucine residue at position 157 of the Chk2 FHA domain to threonine does not prevent Chk2 from binding to phosphorylated epitopes nor does it impair kinase activity (Li et al., 2001). However, the I157T mutation can abrogate the interaction between Chk2 and several other proteins: Cdc25, p53 and BRCA1 (Li et al., 2001; Falck et al., 2001b; Falck et al., 2001a). These results suggest that the I157 residue resides in a non-canonical alternative interaction motif and that by focusing on the canonical phospho-dependence of FHA domains the scope of the research conducted on FHA domains is being limited, thereby hindering our understanding of the molecular processes driven by FHA domains.

1.2.2 Rad53 Non-canonical Lateral Surface Binding

In 1998, Rad53 was identified in budding yeast as a cell cycle checkpoint kinase whose characterization was central to understanding DNA damage-based checkpoint signaling (Shirahige et al., 1998; reviewed in Durocher and Jackson, 2002). DNA replication initiation and checkpoint activation are heavily studied pathways in budding yeast, and Rad53 acts in conjunction with Dbf4-dependent kinase (DDK) to regulate the licensing and firing of origins of replication during replication stress (Shirahige et al., 1998; Duncker et al., 2002). DDK subunits, Dbf4 and Cdc7, function as the regulatory subunit and kinase, respectively, and DDK-dependent phosphorylation of proteins recruited to licensed origins of replication triggers DNA replication (Weinreich and Stillman, 1999). Activated Rad53 delays cell entry into mitosis in response to genotoxic stress (Weinreich and Stillman, 1999). Rad53 uniquely possess two FHA domains, FHA1 and FHA2 (Durocher et al., 1999; Liao et al., 1999; Liao et al., 2000). Characterization of the Rad53-Dbf4 interaction and its contribution to checkpoint arrest identified FHA1 as necessary and sufficient for the Rad53-Dbf4 interaction (Duncker et al., 2002).

Characterization of the interaction between FHA1 and the minimal interacting region of Dbf4, a BRCA1 C-terminal (BRCT) domain consisting of an additional alpha-helix within its core domain (H-BRCT domain), demonstrated a novel mechanism for an FHA domain-based interaction (Matthews et al., 2014). Contrary to the logical assumption that the Rad53 FHA1 - Dbf4 H-BRCT interaction would rely on the recognition of a phosphorylated threonine residue within the H-BRCT domain of Dbf4 by the

pThr-binding site of FHA1, individual mutagenesis of each threonine residue within the H-BRCT domain of Dbf4 was unable to disrupt the interaction between the H-BRCT domain and FHA1. Instead, nuclear magnetic resonance, bioinformatics and yeast two-hybrid analyses identified a set of conserved residues on a lateral surface of the Rad53 FHA1 domain that mediates the Rad53-Dbf4 interaction that does not exist in the Rad53 FHA2 domain. Whether or not a similar mechanism of interaction exists between human homologs of Rad53 and Dbf4, Chk2 and Dbf4/Drf1, is unknown. This discovery illustrated the limitations of conducting research under the expectation that FHA domains function solely through pThr-binding, introducing the potential for discovering novel protein-protein interactions and functions that have previously been overlooked in DNA damage and checkpoint mechanisms.

In addition to Dbf4, Rad53 is known to interact with a multitude of other proteins (Sun et al., 1998; Leroy et al., 2003; Emili et al., 2001; Hu et al., 2001; Smolka et al., 2005; Smolka et al., 2006). Rad53 autophosphorylation and activation is instigated by its association with hyperphosphorylated Rad9 and Mrc1 adaptor proteins (reviewed in Lanz et al., 2019). Both FHA domains of Rad53 mediate the interaction between Rad53 and hyperphosphorylated Rad9 in response to DNA damage, although their binding specificities differ (Durocher et al., 2000). The chromatin assembly protein Asf1 and phosphatase Ptc2 were also identified as Rad53 FHA1 phosphorylation-dependent ligands (Emili et al., 2001; Hu et al., 2001; Leroy et al., 2003; Schwartz et al., 2003). Cdc7 interacts with the Rad53 FHA1 domain via the FHA1 pThr-binding site (Aucher et al., 2010) as do the Kap95, Yta7, and Mdt1 proteins (Smolka et al., 2005; Pike et al., 2004). The Dun1 checkpoint kinase interacts with a diphosphothreonine motif of Rad53 and is subject to Rad53-dependent phosphorylation in response to DNA damage (Bashkirov et al., 2003), and the active helicase component Cdc45 interacts with the FHA1 domain of Rad53 via the canonical pThr-binding site of FHA1, with mutation of the T189 and T195 Cdc45 residues to alanine preventing the interaction between Rad53 and Cdc45 (Aucher et al., 2010).

1.3 *DUN1* FHA Domain and the dNTP Regulation Pathway

DNA-damage uninducible1 (DUN1), a paralog of *RAD53*, encodes the Dun1 checkpoint kinase, a non-essential nuclear phosphoprotein involved in the DNA damage and replication stress response in budding yeast (Zhou and Elledge, 1993; reviewed in Lanz et al., 2019). The Mec1-Rad53-Dun1 checkpoint kinase cascade regulates the assembly and function of the ribonucleotide reductase (RNR) enzyme in budding yeast which is responsible for catalyzing the rate-limiting step of deoxyribonucleoside triphosphate (dNTP) synthesis (Zhou and Elledge, 1993; Zhao and Rothstein, 2002; reviewed in Lanz et

al., 2019; Figure 1.7). RNR consists of a homodimer of the Rnr1 large subunit, which houses the catalytic and allosteric sites, and a heterodimer of the Rnr2 and Rnr4 small subunits, which contain a di-iron centre for the tyrosyl radical necessary for RNR activity (Huang and Elledge, 1997; Nguyen et al., 1999; Chabes et al., 2000; Ge et al., 2001; reviewed in Kolberg et al., 2004, Nordlund and Reichard, 2006, and Cotruvo and Stubbe, 2011). Activated Mec1 sensor kinase leads to the hyperphosphorylation of Rad53 and subsequent Dun1 phosphorylation and activation (Lee et al., 2003; Bashkirov et al., 2003; Lee et al., 2008; Figure 1.7).

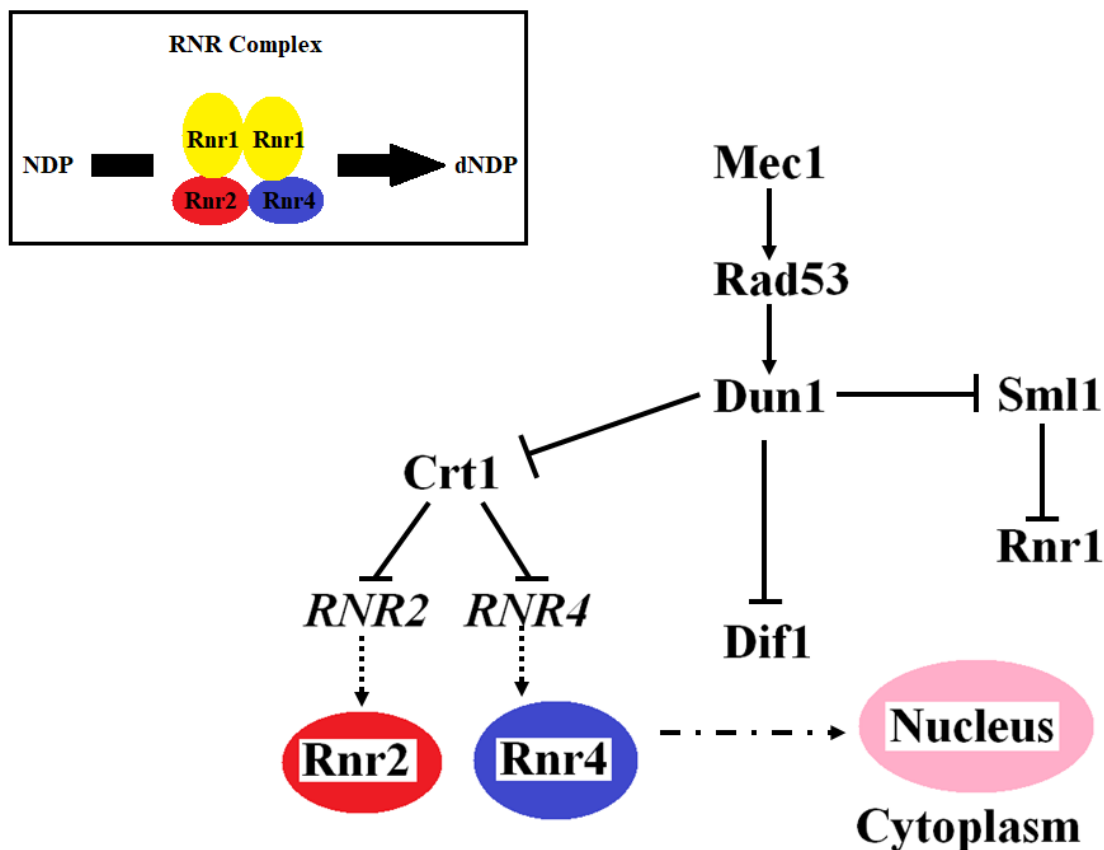


Figure 1.7 Dun1 dNTP Regulation Pathway

Schematic diagram illustrating the key Dun1 targets in the checkpoint response pathway for regulating dNTP synthesis in *Saccharomyces cerevisiae*. Dashed arrows illustrate gene expression for the *RNR2* and *RNR4* genes and the transport of the Rnr2 and Rnr4 subunits from the cytoplasm to the nucleus via Dif1.

Activated Dun1 hyper-phosphorylates several main targets in the dNTP regulation pathway.

Dun1-dependent phosphorylation of the Crt1 transcriptional repressor encoded by the *Constitutive RNR*

Transcription (CRT1) gene removes the transcriptional repression of the *RNR2* and *RNR4* genes, leading to increased *RNR2* and *RNR4* expression and the formation of the RNR holoenzyme (Huang et al., 1998; Figure 1.7). During S phase or after DNA damage, Dun1 phosphorylates and targets Sml1, encoded by the *Suppressor of mec1 lethality (SML1)* gene, for ubiquitylation and subsequent degradation by the 26S proteasome after being recognized by the Rad6-Ubr2-Mub1 E2/E3 ligase complex (Andreson et al., 2010). Sml1 negatively regulates dNTP synthesis via binding to the Rnr1 subunit and competing with the Rnr1 carboxyl terminus for association with the Rnr1 amino terminus. The association of the amino and carboxyl termini of Rnr1 is required for the regeneration of the Rnr1 active site for RNR catalytic function (Zhao et al., 1998; Chabes et al., 1999; Zhang et al., 2007). Dif1, encoded by the *Damage-regulated Import Facilitator (DIF1)* gene, is a paralog of *SML1* and is involved in dNTP regulation during S phase when protein levels of Dif1 peak (Lee et al., 2008). Dif1 functions as a nuclear import protein, transporting the Rnr2 and Rnr4 small subunits from the cytoplasm to the nucleus (Lee et al., 2008). Dun1-dependent phosphorylation targets Dif1 for degradation, resulting in Rnr2 and Rnr4 remaining localized in the cytoplasm and available for association with the Rnr1 homodimer for the formation of the RNR holoenzyme (Lee et al., 2008). The Wtm1 nuclear anchor protein contributes to dNTP regulation by anchoring the Rnr2 and Rnr4 subunits at the nucleus, however a Dun1-dependent mechanism for removing this negative regulation of dNTP synthesis has not been shown (Lee and Elledge, 2006; Zhang et al., 2006). Additionally, in a Rad53-dependent, Dun1-independent manner, the intrastrand cross-link recognition (Ixr1) transcription factor is required for expression of the *RNR1* gene which, in contrast to the *RNR2-4* genes, is not regulated by the Crt1 transcription factor (Tsaponina et al., 2011).

1.4 MEK1 and the Meiotic Recombination Checkpoint

Mitotic division requires the faithful replication of DNA and the segregation of sister chromatids to produce two identical daughter cells and is critical to the survival of vegetative cells. Meiotic division differs in that the segregation of homologous chromosomes precedes sister chromatid segregation, referred to as reductional division, producing four haploid cells carrying half of the original genomic content in preparation for fertilization events for sexual reproduction (reviewed in Lai et al., 2011). A unique feature of meiotic division that is conserved in all studied genera is the synaptonemal complex (SC) which is integral to the formation and processing of programmed meiotic DNA double strand breaks (DSBs) at recombination hotspots which influence homologous chromosome segregation and permit the

exchange of genetic material between homologs (Fawcett, 1956; Moses, 1956; reviewed in Lai et al., 2011). The SC consists of two axial/lateral elements held together by the transverse filaments/central element (Kleckner, 1996, 2006; Zickler and Kleckner, 1999; reviewed in Lam and Keeney, 2015; Figure 1.8). After DNA replication in yeast, cohesin complexes consisting of the meiosis-specific kleisin subunit Rec8 hold the sister chromatids together while they condense along protein cores of Rec8, Hop1 and Red1 to form the axial elements (Klein et al., 1999). The insertion of the transverse filament protein Zip1 by the ZMM proteins between the axial elements creates the tripartite synaptonemal complex during pachytene (Borner et al., 2004; Sym et al., 1993; Sym and Roeder, 1994).

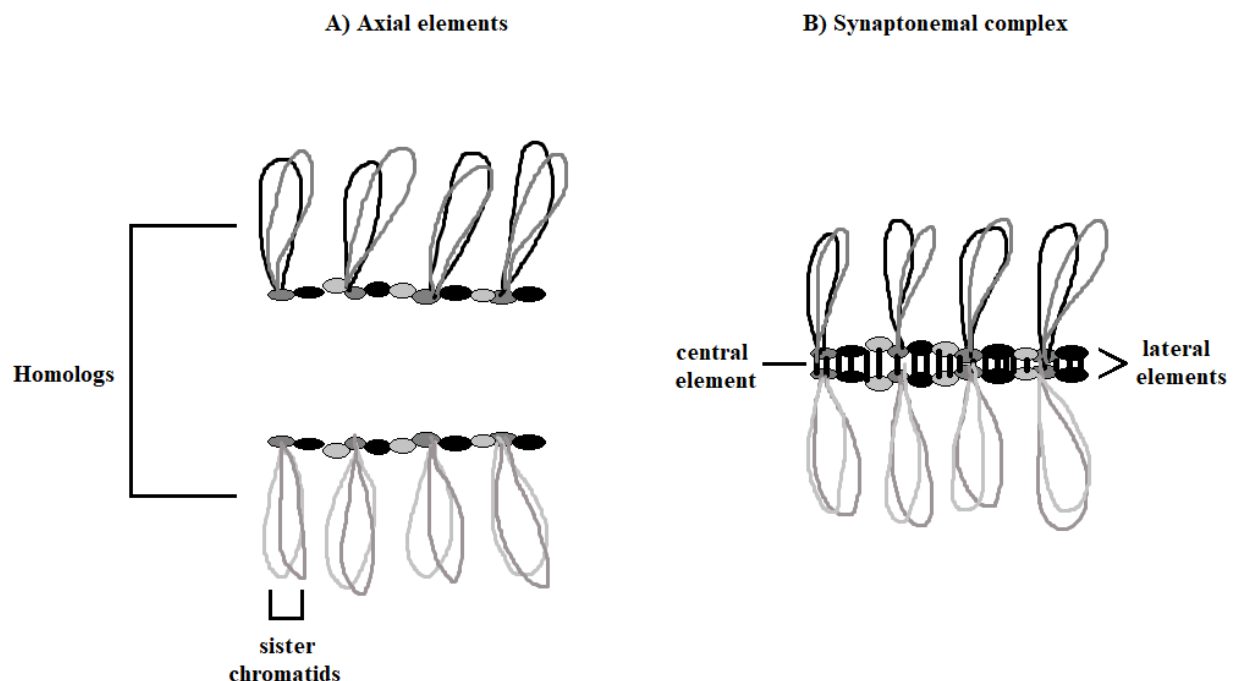


Figure 1.8 Components of the synaptonemal complex.

A) During meiosis chromatin loops for each homolog adhere to the proteins of the axial element. (B) Connection of the axial elements by the transverse filament proteins create the central element and the complete formation of the synaptonemal complex with the axial elements now referred to as the lateral elements. SC formation is completed by the pachytene stage (adapted from Lam and Keeney, 2015).

Meiotic DSBs initiate meiotic DNA recombination and are generated by the meiosis-specific type II topoisomerase, Spo11 (Keeney, 2001; reviewed in Lai et al., 2011). Upon endonucleolytic cleavage near the site of Spo11-generated DSBs, Spo11 is released from the covalent bonds adhering it to the 5' end of the break (de Massy et al. 1995; Keeney and Kleckner 1995; Liu et al. 1995; Neale et al. 2005;

Zakharyevich et al. 2010; Garcia et al. 2011). Following the release of Spo11, Mre11 exo-nuclease activity is responsible for 3' to 5' end resection towards the DSB (de Massy et al. 1995; Keeney and Kleckner 1995; Liu et al. 1995; Neale et al. 2005; Zakharyevich et al. 2010; Garcia et al. 2011) and Exo1 exonuclease conducts 5' to 3' end resection to expose the 3' single-stranded tails (Sun et al., 1991; Zakharyevich et al., 2010). Dmc1 and Rad51, members of the RecA family of strand exchange proteins, will bind to the exposed 3' tails and permit strand invasion for DSB repair (Chen et al., 2008; San Filippo et al., 2008).

Each pair of homologs should have at least one crossover (reviewed in Keeney et al., 2014). However, cells typically exhibit many more DSBs during meiotic prophase than are necessary (reviewed in Keeney et al., 2014). The meiotic recombination checkpoint uses meiosis-specific modifications to the mechanisms of the DNA damage checkpoint in mitotic cells to delay cells in prophase I while crossovers (COs) are executed and all DSBs are repaired (Lydall et al., 1996; Page and Hawley, 2004; Subramanian and Hochwagen, 2014). In vegetative cells, DNA DSBs trigger the recruitment and activation of the Mec1 and Tel1 checkpoint kinases in budding yeast which hyper-phosphorylate the Rad9 adaptor protein which is responsible for recruiting Rad53 (reviewed in Lanz et al., 2019). During meiosis, Mec1 and Tel1 phosphorylate the meiosis-specific adaptor protein and component of the axial element, Hop1, which then recruits the meiosis-specific paralog of Rad53, Mek1, encoded by the *MEiotic Kinase (MEK1)* gene, to sites of DSBs resulting in the oligomerization and activation of Mek1 via autophosphorylation in *trans* (Carballo et al., 2008; Leem and Ogawa, 1992; Niu et al., 2007; Rockmill and Roeder, 1991; Figure 1.9). Mek1 opposes inter-sister chromatid cohesion locally at the sites of DSBs to facilitate the interhomolog strand invasion required for meiosis and to regulate the choice between interhomolog recombination intermediates being repaired either as crossovers or non-crossovers based on Mek1-enabled DDK phosphorylation of Zip1 or Sgs1 helicase activity (Chen et al., 2015; Kim et al., 2010; Figure 1.9). Mek1 drives interhomolog bias by preventing the non-specific Rad51 recombinase from competing with the meiosis-specific recombinase Dmc1 (Cloud et al., 2012; Liu et al., 2014; Figure 1.9). Mek1 phosphorylates the Rad54 accessory factor protein which binds to Rad51, preventing Rad51-Rad54 association and inter-sister chromatid-based repair, and phosphorylates and stabilizes Hed1 which is a meiosis-specific protein that binds to Rad51 to prevent the Rad54-Rad51 interaction (Busygina et al., 2008; Callender et al., 2016; Niu et al., 2009; Tsubouchi and Roeder, 2006; Figure 1.9).

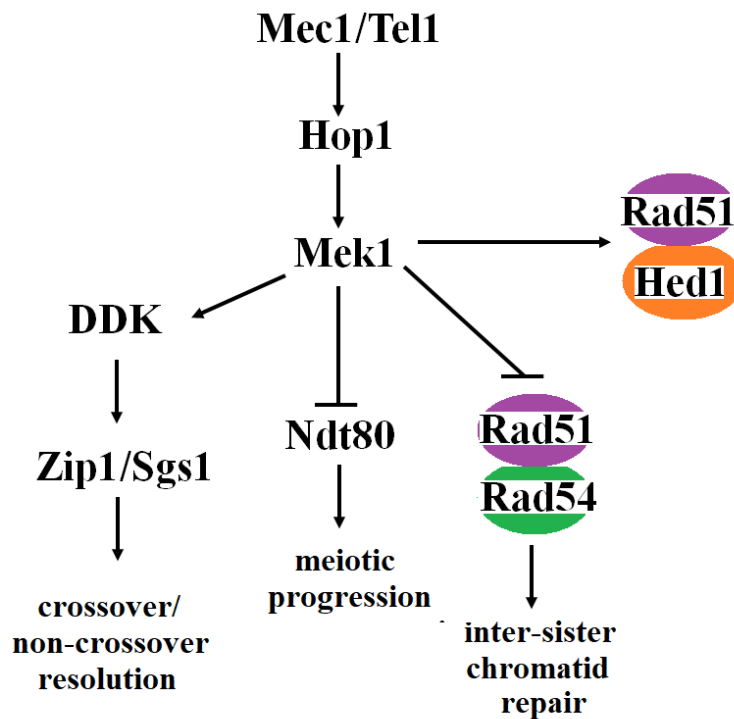


Figure 1.9 Meiotic Recombination Checkpoint Pathway

Schematic diagram illustrating key factors involved in the meiotic recombination checkpoint pathway where the checkpoint signal is propagated via Mec1/Tel1-dependent phosphorylation of the Hop1 adaptor protein.

During early meiotic prophase, a meiosis specific E3 ligase targets mitotic regulators for degradation (reviewed in Keeney et al., 2014). During the meiotic recombination checkpoint, the meiosis-specific transcription factor Ndt80 is inactivated (reviewed in Keeney et al., 2014). The Ndt80 transcription factor recognizes a nine-base pair sequence, the middle sporulation element (MSE), in the promoters of more than 300 target genes called the "middle" and "late" genes whose expression is required for both normal meiotic progression as well as checkpoint alleviation (Chu and Herskowitz, 1998; Hepworth et al., 1995; Ozsarac et al., 1997). Activated Mek1 interacts with Ndt80 via the Ndt80 'bc' domain located at amino acid positions 346-402 (Chen et al., 2018). A highly conserved RPSKR motif at positions 371-375 of Ndt80 is necessary for the Mek1-Ndt80 interaction in a manner independent of the pThr-epitope binding site of the Mek1 FHA domain (Chen et al., 2018). Mek1 phosphorylation of the Ndt80 DNA binding domain (DBD) prevents Ndt80 from binding to the MSEs of target genes thereby inhibiting Ndt80 activity, however there are likely additional mechanisms for Mek1 inactivation of Ndt80

(Chen et al., 2018). Active Mek1 also protects Red1 from efficient degradation in a mechanism currently unknown, thereby preventing efficient SC disassembly and the alleviation of the meiotic recombination checkpoint in the presence of Cdc5 which contributes to Red1 phosphorylation and degradation (Chen et al., 2018). The exact mechanism of Ndt80 subcellular localization is unknown, but it is speculated that phosphorylation might affect nuclear import or export (Chen et al., 2018).

1.5 Research Objectives and Significance

The objectives of the research presented in this thesis are centered around investigating the frequency of FHA domain-based protein-protein interaction that use a non-canonical mechanism to establish protein-protein interaction such as was observed with Rad53 (Matthews et al., 2014). To address this question yeast two-hybrid and spot plate assays were used to demonstrate the importance of the FHA domain lateral surface of the Rad53 paralog, Dun1, for interactions with two Dun1 ligands, Dif1 and Sml1. Simultaneously, a more comprehensive analysis of the FHA domain-containing proteins in *Saccharomyces cerevisiae* was conducted via the use of several bioinformatics tools to examine the sequence conservation of budding yeast FHA domain sequences and their structures. Multiple sequence alignments, amino acid sequence conservation mapping and protein modelling analyses were used to confirm the complete collection of FHA domain-containing proteins in budding yeast and to identify candidate lateral surface regions in known FHA domains that could participate in non-canonical FHA domain-ligand binding. Analyses of the protein-protein interactions of budding yeast Mek1 kinase involved in the meiotic recombination checkpoint was used to support evidence that non-canonical interaction mechanisms exist in other FHA domain-based interactions in various molecular pathways. Since Mek1 does not interact with the Ndt80 transcription factor via the canonical mechanism of the pThr-epitope binding site (Chen et al., 2018), yeast two-hybrid assays, sporulation assays, and tetrad dissection were utilized to investigate whether Mek1 might also utilize the lateral surface for mediating protein-protein interactions and what, if any, effects disruption of the lateral surface might have on the Mek1-Ndt80 interaction and the meiotic recombination checkpoint. Co-immunoprecipitation was used to investigate whether Mek1 might interact with the subunits of DDK. The research presented in this thesis extended beyond vegetatively dividing cells to also analyze meiotic recombination in diploid yeast strains and demonstrated applicability to human molecular pathways.

A complete understanding of the structure and function of FHA domains requires investigation into both canonical and non-canonical methods of protein-protein interactions. Until recently, researchers

have focused almost exclusively on the importance of the pThr-epitope binding site. This thesis demonstrates the relevance of looking beyond the canonical mechanisms of protein-protein interactions and considering additional surfaces of the FHA domain as contributors to ligand binding specificity and/or efficiency. The information gained from an unbiased approach to FHA domain research can contribute to a more wholistic knowledge and understanding of the cellular processes that utilize FHA domain-based interactions, processes that when disrupted can lead to errors that manifest as complex diseases such as cancer.

Chapter 2

A non-canonical Dun1 forkhead-associated (FHA) domain interaction surface contributes to ligand specificity and resistance to genotoxic stress.

2.1 Summary

DNA damage UNinducible (DUN1) encodes a cell cycle checkpoint kinase, Dun1, involved in regulating dNTP synthesis. In the budding yeast *Saccharomyces cerevisiae*, Rad53-dependent phosphorylation of Dun1 leads to the phosphorylation of downstream factors involved in the regulation of dNTP levels. Dun1-dependent phosphorylation of Dif1 and Sml1 targets them for degradation, thereby removing their inhibitory controls on the assembly and function of the ribonucleotide reductase (RNR) holoenzyme. The involvement of a non-canonical lateral binding surface of the Dun1 FHA domain, distinct from its recognition region for phosphothreonine (pThr)-containing epitopes, in mediating Dif1 and Sml1 interactions as well as the consequent contribution to cell growth and resistance to genotoxic stress was examined. Yeast two-hybrid assays revealed that the Dun1 FHA domain is necessary for both interactions with Dif1 and Sml1. However, the Dun1 FHA domain on its own retains only a partial interaction with Dif1 and exhibits no association with Sml1 at all. Bioinformatic analysis was used to identify candidate interaction residues on the Dun1 FHA domain lateral surface, based on sequence conservation as well as the position, size, and charge of the side chains and corresponding site-directed mutagenesis was used to alter these residues. Yeast two-hybrid assays revealed that the interaction with Dif1 relies primarily on the Dun1 FHA pThr-epitope binding site whereas the Dun1-Sml1 interaction requires both the Dun1 pThr-epitope binding site and the non-canonical lateral surface. Spot plate assays revealed that simultaneous disruption of the Dun1 lateral surface and pThr-epitope binding site resulted in sensitivity to methyl methanesulfonate (MMS), hydroxyurea (HU), bleomycin, and phleomycin, indicating that Dun1 checkpoint function, including dNTP level regulation, relies on the protein-protein interactions mediated by both regions of the Dun1 FHA domain, adding to the small but growing body of evidence supporting the functional importance of non-canonical FHA domain interactions.

2.2 Introduction

Forkhead associated (FHA) domains have been identified in over 2000 prokaryotic and eukaryotic proteins and are well known for their phosphothreonine (pThr)-containing epitope binding specificity (reviewed in Mahajan et al., 2008 and Almawi et al., 2017). Although very little amino acid sequence similarity is exhibited among FHA domains, they have a highly conserved structure of approximately 100-120 residues folded into an 11-stranded beta sandwich with connecting coiled loops (reviewed in Mahajan et al., 2008 and Almawi et al., 2017). FHA domain-containing proteins contribute to regulatory responses involved in the DNA damage response (DDR), cell growth, and cell cycle regulation (Almawi et al., 2017). One such protein is Rad53, encoded by *RA*diation sensitive (*RAD53*), a cell cycle checkpoint kinase in budding yeast that has been extensively studied to further our understanding of DNA damage-based checkpoint signaling (reviewed in Lanz et al., 2019). Rad53-dependent phosphorylation of Dbf4, the regulatory subunit of the Cdc7 kinase, DDK, and the Sld3 replication initiation factor inhibits origin firing during the checkpoint response (Shirahige et al., 1998; Duncker et al., 2002; Zegerman and Diffley, 2010). Sld3 is required for the recruitment of Cdc45 to origins of replication for the formation of the active CMG (Cdc45-MCM2-7-GINS) helicase complex, and DDK-dependent phosphorylation of the MCM helicase triggers DNA replication (reviewed in Fu et al., 2010 and Boos et al., 2011).

Previously, we determined that the minimal region of budding yeast Dbf4 required for the interaction with the FHA1 domain of Rad53 was within the N-terminus of Dbf4, consisting of a BRCA1 C-terminal (BRCT) domain with an additional N-terminal alpha-helix, which we termed H-BRCT (Matthews et al., 2012). Individual mutagenesis of each threonine residue of Dbf4 H-BRCT did not interrupt its interaction with Rad53 FHA1 in any case (Matthews et al., 2014). Nuclear magnetic resonance, bioinformatics analysis and yeast two-hybrid assays revealed a set of conserved residues along a lateral surface of the FHA1 domain that were responsible for mediating the interaction between Rad53 and Dbf4, identifying a novel, non-canonical method of an FHA domain mediating a protein-protein interaction (Matthews et al., 2014). Whether this non-canonical binding pattern is unique to Rad53 is of central importance in understanding FHA-mediated protein-protein interactions.

Budding yeast *DNA damage UN*inducible (*DUN1*), a *RAD53* paralog, is a non-essential gene that encodes a checkpoint kinase involved in signal transduction pathways for DNA damage, replication stress, and natural progression through S phase (Zhou and Elledge, 1993). Dun1 regulates the levels of

deoxyribonucleoside triphosphates (dNTPs) via the transcriptional, spatial, and functional regulation of ribonucleotide reductase (RNR), the tetrameric protein complex responsible for catalyzing the rate-limiting step of dNTP synthesis by converting ribonucleoside diphosphates into their deoxy form (Sanvisens et al., 2016; Yoshitani et al., 2008; Lee and Elledge, 2006; Zhao and Rothstein, 2002). Ribonucleotide reductase consists of two large subunits, a homodimer of Rnr1, and two small subunits, a heterodimer of Rnr2 and Rnr4 (Huang and Elledge, 1997; Tsaponina et al., 2011). RNR small subunits have a di-iron centre that holds and maintains a tyrosyl radical essential to the catalytic biochemical activity of the enzyme, and the large subunits possess the catalytic and allosteric sites (Huang and Elledge, 1997; Elledge and Davis, 1987; Hurd et al., 1987; Elledge and Davis, 1990).

In response to DNA damage, replication stress, or when cells enter S phase, the Mec1/Rad53/Dun1 kinase cascade leads to the assembly and activation of the RNR enzyme (Zhou and Elledge, 1993; Zhao and Rothstein, 2002). Activated Mec1 sensor kinase leads to the phosphorylation of Rad53 and the FHA domain of Dun1 recognizes a di-phosphothreonine motif in hyperphosphorylated Rad53, thereby activating it and leading to the Dun1-dependent phosphorylation of downstream targets essential to Dun1 function (Lee et al., 2003; Lee et al., 2008a; Bashkirov et al., 2003). Two such targets of Dun1 are Suppressor of mec1 lethality (Sml1) and Damage-regulated Import Facilitator (Dif1) (Zhao and Rothstein, 2002; Lee et al., 2008b). Sml1 is a small, largely disordered suppressor protein that negatively affects dNTP synthesis via its physical association with the Rnr1 subunit (Zhao et al., 1998; Andreson et al., 2010). Dun1-dependent phosphorylation of Sml1 triggers a conformational change that results in the dissociation of Sml1 from Rnr1 and the regeneration of the Rnr1 active site (Andreson et al., 2010). Phosphorylated Sml1 is then ubiquitinated and targeted for degradation by the 26S proteasome after its recognition by the Rad6-Ubr2-Mub1 E2/E3 ligase complex (Andreson et al., 2010). Dif1, a paralog of Sml1, is also a small and largely disordered protein which contributes to dNTP regulation in a cell cycle-dependent manner when its levels peak towards the end of S phase (Lee et al., 2008b). Dif1 association with the Rnr2-Rnr4 small subunits via its Hug domain allows Dif1 to import the small subunits from the cytoplasm to the nucleus by acting as an adaptor for the nuclear import of Rnr2-Rnr4 or by activating a dormant nuclear localization signal on the Rnr2-Rnr4 complex (Lee et al., 2008b). During S phase and in response to DNA damage when a rise in dNTP levels is required, Dif1 is phosphorylated and degraded to prevent Rnr2-Rnr4 import to the nucleus (Lee et al., 2008b). The Sml domain of Dif1 is a phosphodegron and the direct phosphorylation of serine residues within the Sml domain by Dun1 results in the degradation of Dif1 and the maintenance of Rnr2-Rnr4 localization in the cytoplasm where it can

associate with Rnr1 subunits to form a functional RNR holoenzyme for the synthesis of dNTPs (Lee et al., 2008b).

Here we show that, whereas interaction with Dif1 is entirely dependent on the Dun1 FHA domain pThr-epitope binding site, both the lateral surface of the Dun1 FHA domain and its pThr-epitope binding site contribute to the Dun1-Sml1 association, demonstrating another instance of such a ligand-specific non-canonical FHA domain interaction.

2.3 Methods

2.3.1 Yeast Strains

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology was used to generate genomic mutations for the *DUN1* gene sequence (Table 1). The Benchling web tool was used to determine the optimal Cas9 target sites for each point mutation (<https://benchling.com>). The pML104 CRISPR vector for *Saccharomyces cerevisiae*, which includes a selectable marker for the biosynthesis of uracil (*URA3*), was used to express the guide RNA (gRNA) and Cas9 (<https://www.addgene.org/67638/>). The sequence of *DUN1* was imported into Benchling from the Saccharomyces Genome Database and 20 base pair oligomer gRNAs were designed via the CRISPR feature using the single guide design type from the R64-1-1 *Saccharomyces cerevisiae* genome with NGG PAM sites. The selection of optimal gRNAs was determined based on the highest on-target and off-target scores as well as location of the gRNA relative to the site of mutation in the gene. The forward and reverse gRNA sequences were incorporated into the design of a forward and reverse single-stranded oligomer. The forward oligomer contained the 5' overhang for the BclI restriction enzyme, followed by the 20mer *DUN1* gRNA and the sgRNA sequence. The reverse oligomer contained the sgRNA sequence and the reverse complement of the 20mer gRNA sequence. Repair templates for homology-directed repair of the DNA breaks to be generated by Cas9, were designed to include 40 base pairs upstream and downstream, the left and right homology arms respectively, of the region in which the mutation would be made. The homology arms were also designed to encompass the PAM site. The repair template contained homology to the wild-type *DUN1*, except for also incorporating the altered nucleotide sequence corresponding to the genetic mutations desired as well as any necessary nucleotide substitutions required for the destruction of the PAM recognition site. The forward and reverse oligomers for the gRNA as well as the single-stranded repair templates were ordered from Eurofins Genomics.

Table 2.1 List of Yeast Strains.

Strain	Genotype	Source
DY-1	<i>MATa, ade2-1, can1-100, trp1-1, his3-11, ura3-1, leu2-3, leu2-112, pep4::LEU2</i>	(Duncker et al., 2002)
DY-30	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</i>	ATCC
DY-351	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1Δ</i>	GE Healthcare
DY-396	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1H36A, V38A</i>	This study
DY-397	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1L134A, K136A</i>	This study
DY-398	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1R60A</i>	This study
DY-399	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1H36A, V38A, R60A</i>	This study
DY-400	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1L134A, K136A, R60A</i>	This study
DY-401	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1H36A, V38A, L134A, K136A</i>	This study
DY-402	<i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dun1H36A, V38A, L134A, K136A, R60A</i>	This study

To clone the *DUN1* gRNA sequence, pML104 was digested with *Swa*I and *Bcl*I and the digestion reaction was then purified using a commercial PCR clean up kit (Geneaid). The forward and reverse gRNA oligomers were annealed using a PCR thermocycler. Using 100 μM solutions of the oligomers, 2 μg of each oligomer was added to a total volume of 50 μL of annealing buffer (10 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA). The gRNA mix was heated to 95°C for 2 minutes, cooled to 25°C over 45 minutes, and then cooled to 4°C. After annealing, 5 μL of the annealed oligos were diluted with 45 μL of nuclease-free water to a concentration of ~8 ng/ μL. The diluted annealed oligos were mixed with the purified and digested pML104 vector at a molar ratio of 1:6 in a standard ligation reaction incubated overnight at 4°C. Transformation into calcium chloride competent DH5α bacterial cells was completed using 3 μL of the ligation reaction and cells were plated on Luria-Bertani (LB) agar (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Plasmid DNA was sequenced (Sick Kids Sequencing Facility, Toronto, Ontario) to ensure successful ligation of the gRNA into the

vector. Upon successful cloning of the gRNA into pML104, ~0.4 µg of the pML104 constructs and ~5 µg of ssDNA repair template were co-transformed into the DY-30 or mutant yeast strains as described previously. Successful transformants were selected on synthetic complete (SC) agar plates lacking uracil, replica plated on 5-FOA plates to select for the loss of the pML104 plasmid to facilitate sequential transformations of pML104 containing different gRNAs for the addition of other mutations, and then streak plated onto YPD rich agar. Genomic DNA was isolated as described below and then used as the DNA template for PCR amplification of each full-length prospective *dun1* mutant gene sequence which were sequenced as above, to ensure successful editing.

2.3.2 Genomic DNA Isolation

Genomic DNA was isolated from the DY-1 yeast strain for use as template DNA in PCR reactions contributing to plasmid construction. Genomic DNA was also isolated from CRISPR-generated genomic mutants to determine the success of genomic editing via DNA sequencing. A working culture for each strain, inoculated from a saturated preculture, was grown to a concentration of $\sim 1 \times 10^7$ cells/mL in 10 mL of YPD (1% yeast extract, 2% peptone, and 2% dextrose) media at 30°C. Cells were centrifuged at 4000 rotations-per-minute (rpm) for 5 minutes (Thermo Scientific centrifuge CL2), resuspended in 500 µL of sterile water and transferred to a sterile 2 mL screw-cap tube. The cells were then centrifuged for 10 seconds at maximum speed (Eppendorf 5424 R centrifuge), the supernatant decanted, and then vortexed to loosen the pellet. A volume of 200 µL of 'genomic prep mix' (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8, and 1 mM EDTA) was added to the loosened pellet, followed by 200 µL of phenol:chloroform:isoamylalcohol (25:24:1) and 0.5 g of 0.5 mm glass beads. All components were mixed for 3-4 minutes using a vortex and then 200 µL of 1X TE pH 8 was added and mixed. Following a 5-minute centrifugation at maximum speed (Eppendorf 5424 R centrifuge), the top layer was transferred to a new sterile 1.5 mL Eppendorf tube with 1 mL of 100% ethanol and mixed by inversion. After another centrifugation for 2 minutes at maximum speed (Eppendorf 5424 R centrifuge), the pellet was completely re-suspended in 0.4 mL of 1X TE pH 8 to which 10 µL of 10 mg/mL RNase A (Sigma) was added before incubating at 37°C for 10 minutes. After incubation, 10 µL of 4 M ammonium acetate and 1 mL of 100% ethanol were added and mixed by inversion. After a 2-minute centrifugation, the supernatant was discarded, and the pellet left to air dry before completely re-suspending the pellet in 50 µL of 1X TE pH 8. Genomic DNA was then stored at -20°C.

2.3.3 Bioinformatics

The Dun1 amino acid sequence was submitted to the IUPred and PSIPRED online servers for the prediction of the disordered regions and protein secondary structure, respectively, to confirm the complete FHA domain boundaries (<https://iupred2a.elte.hu/>; <http://bioinf.cs.ucl.ac.uk/psipred/>). The peptide sequence corresponding to the FHA domain was entered as the query to search the non-redundant protein sequences (nr) database using the Position-Specific Iterated BLAST (PSI-BLAST) algorithm (NCBI) to establish a collection of homologs to assess amino acid conservation. Returned sequences exhibiting a percent identity of 25% or above to the query sequence were selected as homologs (Appendix A Table 1) and their sequence data exported for use in generating a multiple sequence alignment (MSA). Homolog sequence data was uploaded to the online Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool (EMBL-EBI) and the resulting MSA file was viewed using the free Jalview software (www.jalview.org). The ConSurf server was used to map amino acid conservation to the protein model using SWISS-MODEL (<https://swissmodel.expasy.org/>). The free UCSF Chimera protein model analysis software (<https://www.cgl.ucsf.edu/chimera/download.html>) was used to view and process protein models to emphasize features of interest.

2.3.4 Plasmid Construction

Protein coding sequences for genes of interest (*DUN1*, *DIF1*, and *SML1*) were amplified from DY-1 genomic DNA. Protein coding sequences were cloned into pJG4-6 and pEG202, prey and bait vectors for yeast two-hybrid assays, respectively (Duncker et al., 2002; Ausubel et al., 1988; Gyuris et al., 1993). The bait and prey vectors contain a selectable marker for the biosynthesis of histidine (*HIS3*) and tryptophan (*TRP1*), respectively. The pSH18-34 plasmid contains a selectable marker for the biosynthesis of uracil (*URA3*) (Ausubel et al., 1988; Gyuris et al., 1993). The full-length coding sequence for *DUN1* was amplified using a commercially available High-Fidelity PCR kit (Roche) for the construction of the pJG4-6 *DUN1* and pEG202 *DUN1* plasmids. PCR products were purified using a commercial PCR clean up kit that was also used for the clean-up of restriction enzyme digested PCR products and vectors (Geneaid). All restriction enzymes were purchased from Thermo Fisher Scientific, and their recognition sequences were incorporated into the design of the primers used for PCR amplification. Ligation was carried out using a T4 DNA ligase kit (BioBasic), and the ligated product was transformed into calcium-chloride competent DH5 α Escherichia coli (E. coli) cells grown and plated on LB media (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Colony PCR

was performed to confirm transformation of successfully ligated constructs using a commercial colony PCR kit (New England Biolabs). Primer pairs were designed such that one primer targeted the vector sequence and the other targeted the sequence of the insert. Plasmid DNA was isolated from saturated overnight cultures of DH5 α for positive colonies using a commercial plasmid prep kit (Geneaid; <http://www.geneaid.com/sites/default/files/PD13.pdf>). Plasmid DNA was sent for sequencing of the full length insert at the Sick Kids Sequencing Facility (Toronto, Ontario), to ensure the fidelity of the cloned PCR product.

The protein coding sequence of *DUN1* for the region of the FHA domain, amino acids 21-145, was amplified as described above and cloned into the pJG4-6 vector using forward and reverse primers that incorporated EcoRI and XhoI restriction enzyme sites respectively to generate the pJG4-6 *DUN1-FHA* construct. The following Dun1 FHA domain mutants were generated via site-directed mutagenesis (described below) using the pJG4-6 *DUN1-FHA* prey construct as a template: pJG4-6 *dun1-N121A-fha*, pJG4-6 *dun1-L134A-fha*, pJG4-6 *dun1-K136A-fha*, and pJG4-6 *dun1-R60A-fha*. Site-directed mutagenesis of the full-length pJG4-6 *DUN1* prey construct generated the following Dun1 mutants in the context of full-length *DUN1*: pJG4-6 *dun1- Δ FHA*, pJG4-6 *dun1-HV* (H36A, V38A), pJG4-6 *dun1-EKE* (E45A, K47A, E49A), pJG4-6 *dun1-LK* (L134A, K136A). Mutagenized pJG4-6 *dun1* constructs were digested with EcoRI and XhoI restriction enzymes and purified using a commercial PCR clean up kit (Geneaid). The *DUN1* and *dun1* inserts were extracted and purified from 0.8% agarose gel and ligated into purified EcoRI and XhoI digested pEG202 to generate the following pEG202 constructs: pEG202 *DUN1-FHA*, pEG202 *dun1- Δ FHA*, pEG202 *dun1-N121A*, pEG202 *dun1-L134A*, pEG202 *dun1-K136A*, pEG202 *dun1-R60A*, pEG202 *dun1-HV* (H36A, V38A), pEG202 *dun1-LK* (L134A, K136A), pEG202 *dun1-EKE* (E45A, K47A, E49A), pEG202 *dun1-HVR* (H36A, V38A, R60A), pEG202 *dun1-LKR* (L134A, K136A, R60A), pEG202 *dun1-HVLK* (H36A, V38A, L134A, K136A), pEG202 *dun1-HVLKR* (H36A, V38A, L134A, K136A, R60A). Colony PCR was used to confirm successful transformations of desired ligation products as described above. The full-length coding sequences of *DIF1* and *SML1* were cloned into the pEG202 bait vector as described above using BamHI and XhoI restriction enzymes for *SML1* and EcoRI and NcoI restriction enzymes for *DIF1*. The full-length coding sequence of *SML1* was digested, purified and gel extracted, as described above, from the pEG202 vector using the EcoRI and XhoI restriction enzymes and inserted into EcoRI and XhoI digested pJG4-6 to generate the pJG4-6 *SML1* construct. Colony PCR was used to confirm successful transformation of desired ligation products as described above.

2.3.5 Site-directed Mutagenesis

Mutations within the *DUN1* coding sequence were generated using the QuikChange II XL Site-directed Mutagenesis kit (Agilent Technologies). Mutagenic primers were designed using the QuikChange Site-Directed Mutagenesis online interface (<https://www.genomics.agilent.com/primerDesignProgram.jsp>) and then utilized during PCR to remove or replace the desired nucleotide(s). PCR products were digested using DpnI restriction endonuclease to degrade parental copies of the plasmid containing the gene to be mutagenized and then the product was transformed into XL-10 Gold Ultracompetent cells (Agilent) and plated on Luria-Bertani (LB) agar (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Plasmid DNA isolated from transformants was sent for sequencing of the gene at the Sick Kids Sequencing Facility to confirm successful mutagenesis. A full protocol for site-directed mutagenesis is available online: <http://www.agilent.com/cs/library/usermanuals/public/200523.pdf>.

2.3.6 Yeast Transformation

Two-hybrid bait and prey plasmid DNA was transformed into the DY-1 yeast strain, previously transformed with the pSH18-34 reporter plasmid. A working culture was grown to a concentration of $\sim 1 \times 10^7$ cells/mL in 10 mL of Synthetic Complete (SC) media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, 1X amino acid mix) lacking uracil for the maintenance of the pSH18-34 reporter plasmid. Working cultures were centrifuged for 5 minutes at 4000 rpm (Thermo Scientific centrifuge CL2), resuspended in 1X Tris-EDTA (TE) solution pH 8.0, centrifuged again, resuspended in 200 μ L of lithium acetate/TE solution (100 mM lithium acetate, 2.5 mL 1X TE), and then held at room temperature for 10 minutes. Approximately 0.4 μ g of plasmid DNA was added to 100 μ L of the yeast suspension mix along with 100 μ g of salmon sperm DNA, and then 300 μ L of lithium acetate/TE/PEG4000 solution (100 mM lithium acetate, 2 g polyethylene glycol 4000, 0.5 mL 10X TE) was added. The solution was incubated at 30°C for 30 minutes before 40 μ L of 100% dimethyl sulfoxide (DMSO) was added. Cells were heat shocked at 42°C for 7 minutes, held on ice for 2 minutes and then plated on selective media and grown for 2-3 days.

2.3.7 Yeast two-hybrid Assay

This analysis was performed as described previously (Duncker et al., 2002). For the Dun1-Dif1 analysis, Dun1 was used as the prey protein and Dif1 as the bait. Sml1 in the bait construct resulted in false positives and so bait and prey vectors were switched for the Dun1-Sml1 analysis. All Dun1-Sml1

analyses were conducted with mutants in the context of full-length (FL) Dun1 as the Dun1 FHA domain is not sufficient for the Dun1-Sml1 interaction. Dun1-Dif1 analyses were done using just the Dun1 FHA domain for initial single mutations and then using full-length Dun1 for the combination mutants.

2.3.8 Yeast Whole Cell Extract and Western Blotting

To verify appropriate protein expression, yeast whole cell extracts (WCE) were generated for Western blotting. A 20 mL volume of each yeast culture ($\sim 1.0 \times 10^7$ cells/mL) was centrifuged for 5 minutes at 4000 rpm (Thermo Scientific centrifuge CL2) to pellet cells, which were then resuspended in 300 μ L of lysis buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 100 μ L of Fisher HALT! Protease inhibitor and 1 mM PMSF) and added to a 2 mL tube with 0.3 g of 0.5 mm glass beads before subjecting the samples to lysis at 4°C via the use of a Biospec Mini Bead-Beater for eight cycles of 30 seconds of agitation and 30 seconds of rest on ice. A total of 105 μ L of supernatant (whole cell extract) was collected following the centrifugation of lysed cells for 30 seconds at 13, 200 rpm (Eppendorf 5415D centrifuge) at 4°C: 5 μ L for a Bradford Assay and 100 μ L for SDS-PAGE. A Bradford Assay was used to determine the protein concentrations of each sample (Bio-Rad). Protein extracts were mixed with loading buffer (60% 4X buffer [15% SDS, 40% glycerol, and 166 mM Tris base], 0.26 M DTT, 7% bromophenol blue) at a volume of one-half that of the extract and then boiled for 10 minutes before loading 50 μ g of protein onto SDS polyacrylamide gels (10 % resolving gel and 5% stacking gel). After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane using a wet transfer method (200 mM glycine, 25 mM Tris-base, 20 % methanol, 0.054% SDS for the transfer buffer). Membranes were pre-stained with 0.1% Ponceau S, imaged using a ChemiDoc MP Imaging System (BioRad), and then de-stained with 1X TEN + T (20 mM Tris-HCl, 1 mM EDTA, 0.14 NaCl, 0.05% Tween 20) before detection with antibodies (Table 2). Antibody incubations were performed for 1-2 hours after 1 hour blocking in 1X TEN + T with 5% skim milk powder. Three 10-minute washes were done using 1X TEN + T after each antibody exposure and images were taken with a ChemiDoc MP Imaging System (Bio-Rad).

Table 2.2 List of Antibodies.

Antibody	Dilution	Source
Anti-HA (mouse monoclonal) – pJG4-6	0.75:5000 in 3% BSA	Sigma
Anti-LexA (rabbit polyclonal) – pEG202	1:5000 in 3% BSA	Thermo Fisher
AlexaFluor 488 anti-mouse	1:3000 in 5% skim milk	Thermo Fisher
AlexaFluor 647 anti-rabbit	1:3000 in 5% skim milk	Thermo Fisher

HA, hemagglutinin. BSA, bovine serum albumin.

2.3.9 Spot Plate Assay

A spot-plate assay was used to determine the sensitivity of mutant *dun1* strains to genotoxic stress. Three ten-fold dilutions of each strain were made from an initial concentration of 1×10^7 cells/mL. 5 μ L of each dilution was plated onto yeast peptone dextrose (YPD) plates, containing 0.005%, 0.010%, 0.015% or 0.020% methyl methanesulfonate (Sigma), 50 mM, 100 mM, 150 mM, or 200 mM of hydroxyurea (BioShop), or 1 or 2 μ g/mL of bleomycin or phleomycin (Sigma).

2.3.10 Statistical Analysis

IBM SPSS software was used to ascertain the significance of the differences observed amongst yeast two-hybrid assay samples (<https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software>). The One-way ANOVA test and its associated Tukey post-hoc test was used to determine whether there were significant differences in the mean β -galactosidase activity for each yeast two-hybrid assay sample. The Levene's test and Kolmogorov-Smirnov tests were used to assess the assumptions for homoscedasticity and normality, respectively, to confirm the appropriate use of the One-way ANOVA test. The acceptance threshold was set at 0.05.

2.4 Results and Discussion

2.4.1 The Dun1 FHA domain is necessary but not sufficient for its interactions with Dif1 and Sml1

FHA domains have previously been studied through the lens of their presumed pThr-containing epitope binding specificity. However, in recent years, data from our group and other researchers has suggested more complex and multi-faceted aspects of FHA domain mediated protein-protein interactions involved in signal transduction pathways, dimerization, and the enhancement of ligand specificity (Almawi et al., 2016; Cai et al., 2009; Huo et al., 2012; Li et al., 2002; Luo et al., 2015; Yufeng et al., 2010). Following the discovery that the Rad53 FHA1 domain interacts with Dbf4 through a novel non-canonical lateral surface patch in a manner independent of the pThr-epitope binding site (Matthews et al., 2014), we decided to investigate whether Dun1, a Rad53 paralog, might also use its lateral surface to mediate protein-protein interactions involved in the dNTP regulation pathway in a similar non-canonical fashion.

The RNR enzyme is activated during S phase as well as in response to DNA damage and/or replication stress (Zhou and Elledge, 1993; Zhao and Rothstein, 2002). The Mec1-dependent hyperphosphorylation of Rad53 under checkpoint conditions is recognized by the Dun1 FHA domain leading to Rad53-mediated phosphorylation and activation of Dun1 (Zhou and Elledge, 1993; Zhao and Rothstein, 2002; Lee et al., 2008b). Activated Dun1 in turn phosphorylates downstream factors, including Dif1 and Sml1, leading to the assembly of the RNR holoenzyme and its catalytic activity for the synthesis of dNTPs (Zhou and Elledge, 1993; Zhao and Rothstein, 2002; Lee et al., 2008b; Chen et al., 2007). RNR is also regulated by iron availability due to the iron-dependent nature of the enzyme (Sanvisens et al., 2011). In response to iron deficiency, Dun1 phosphorylates Dif1 and Sml1, targeting them for degradation, leading to the re-distribution of the small subunits of RNR, Rnr2 and Rnr4, from the nucleus to the cytoplasm as well as the loss of Sml1-dependent inhibition of Rnr1 catalytic activity (Sanvisens et al., 2016; Sanvisens et al., 2014). Additionally, it was observed that Dun1 mutants of the FHA domain pThr-epitope binding site exhibited a partial defect in the re-localization of the Rnr2 and Rnr4 subunits in response to iron deficiency, illustrating a partial dependence on the Dun1 FHA domain for Rnr2 and Rnr4 re-localization (Sanvisens et al., 2016).

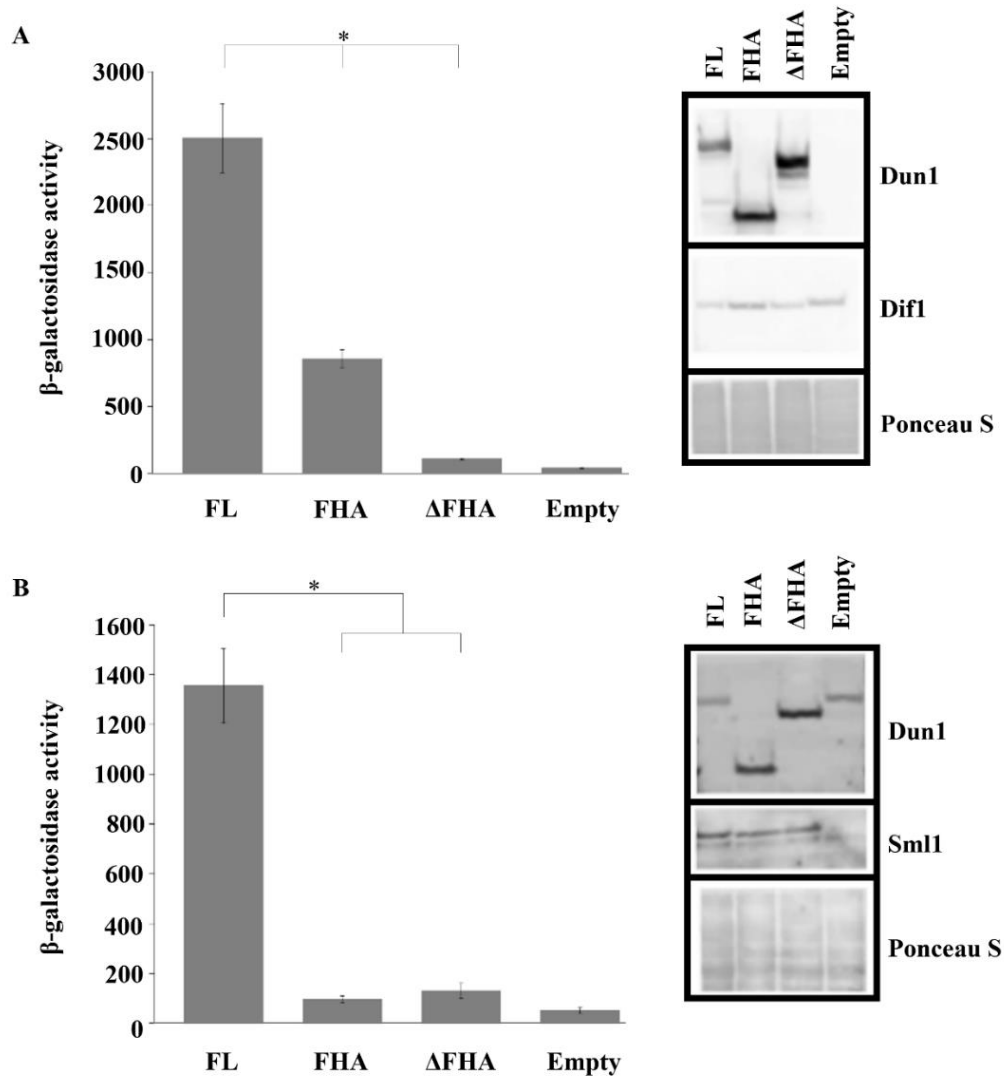


Figure 2.1 The Dun1 FHA domain is necessary but not sufficient for interactions with Dif1 and Sml1.

Yeast two-hybrid assays evaluating the interactions between Dun1 and Dif1 (A) and Dun1 and Sml1 (B). Full-length (FL) Dun1, the Dun1 FHA domain (FHA), full-length Dun1 with a deletion of amino acids 21-145 (ΔFHA), or an empty vector was used as the prey and full-length Dif1 as the bait (A). Full-length (FL) Dun1, the Dun1 FHA domain (FHA), or full-length Dun1 with a deletion of amino acids 21-145 (ΔFHA) was used as the bait and full-length Sml1 or an empty vector as the prey (B). Mean β-galactosidase activity units represent interaction strength, n = 3, and error bars represent standard deviation. *: p < 0.001 (Tukey's HSD), showing significant differences compared to FL excluding statistically significant negative control. Corresponding western blots confirm protein expression of bait and prey constructs. Ponceau S staining indicates relative loading and transfer of protein samples.

To determine whether, and to what extent, the FHA domain is needed for the interaction between Dun1 and its ligands involved in the regulation of RNR for the dNTP pathway, Dif1 and Sml1, yeast two-hybrid assays were conducted (Fig. 2.1). Measures of β -galactosidase activity showed that there was an interaction between Dif1 and the Dun1 FHA domain on its own, but this represented a significant reduction compared to the level of interaction observed with the full-length Dun1 protein (Fig. 2.1A). Without its FHA domain, Dun1 was unable to interact with Dif1, indicating that the Dun1 FHA domain is necessary but not sufficient for the full-strength interaction with Dif1. Analysis of the Dun1-Sml1 interaction revealed that the FHA domain alone was unable to elicit significant β -galactosidase activity compared to empty vector basal levels (Fig. 2.1B). As with Dif1, Sml1 was unable to interact with Dun1 when the FHA domain region was deleted, indicating that the Dun1 FHA domain is also necessary but not sufficient for the interaction between Dun1 and Sml1.

2.4.2 The Dun1 FHA pThr-epitope binding site is required to different extents for interactions with Dif1 and Sml1

Having confirmed the necessity of the Dun1 FHA domain for interactions with Dif1 and Sml1, we next wanted to determine whether Dun1 FHA has another region of conservation, apart from its known phosphothreonine recognition motif (Lee et al., 2008a), analogous to what we have identified for Rad53 FHA1 (Matthews et al., 2014). Sequence conservation mapping amongst Dun1 homologs for the region of the FHA domain was used (Fig. 2.2A) to identify regions that could be important for protein-protein interactions (Fig. 2.2B). As expected, the pThr-epitope binding site located on the apical surface of the FHA domain was highly conserved (Fig. 2.2A; Lee et al., 2008a). Intriguingly, as with the Rad53 FHA1 domain, there was also a second region of conservation on the distal lateral surface of the Dun1 FHA domain, albeit not as extensive as the one found for Rad53 FHA1 (Matthews et al., 2014). Relatively well conserved residues on this lateral surface include N121, L134 and K136 (Fig 2.2). These residues have side chains that appeared to project into the external lateral surface space of the FHA domain model. As such, they were identified as candidates of interest.

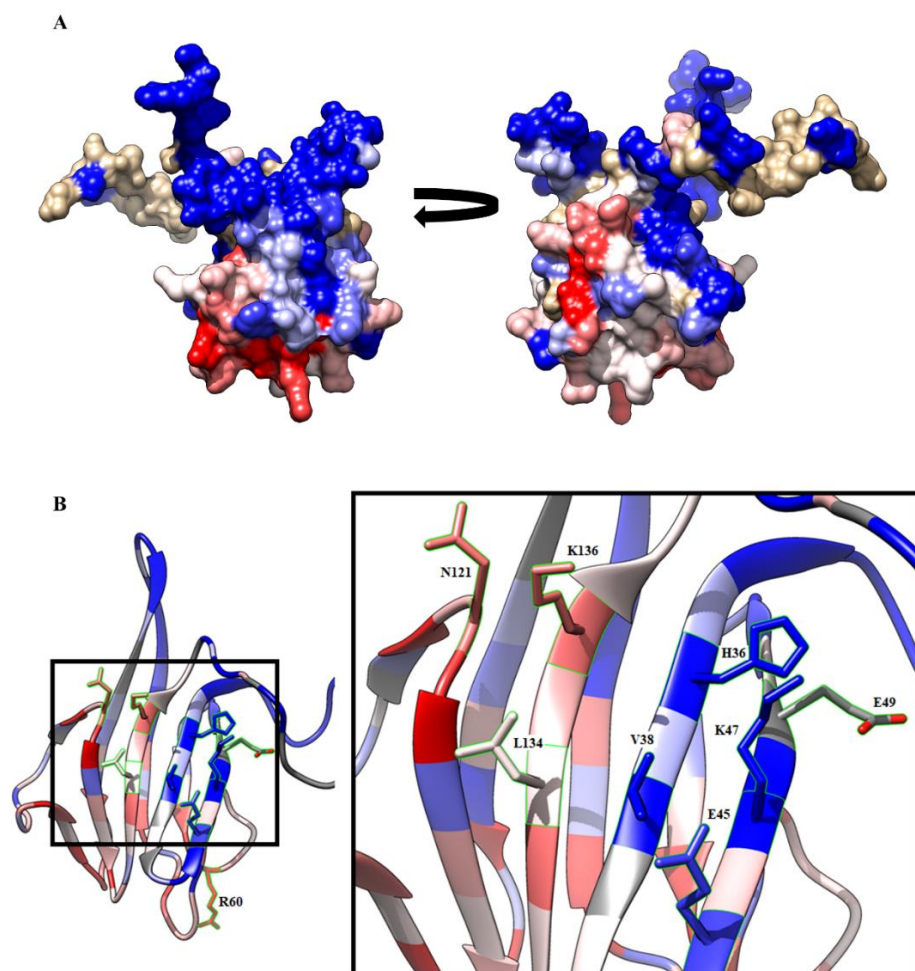


Figure 2.2 The Dun1 FHA domain has a moderately conserved lateral surface patch.

(A) Space filling model of the Dun1 FHA domain showing sequence conservation ranging from red (high conservation) to blue (low conservation), illustrating the apical pThr-epitope binding site and the conservation of the distal lateral surface. (B) Ribbon diagram of the Dun1 FHA domain (PDB template ID: 2JQJ) showing the range of sequence conservation for Dun1 homologs mapped to the 3-D model. Conservation ranges from red (high conservation) to blue (low conservation). Residues exhibiting conservation and external-facing side chains as well as residues that lack sequence conservation but had external-facing side chains were labelled with the single-letter amino acid code and the sequence position. The distal lateral surface was enlarged for the visualization of the residues selected for mutagenesis.

To determine whether these residues could be contributing to the Dun1-Dif1 and Dun1-Sml1 interactions, alanine substitution mutations were created for each individually, as well as for the key canonical pThr-epitope binding site residue, R60, located on the apical surface. Yeast two-hybrid assays

were conducted to measure β -galactosidase activity as an indicator of protein interaction strength (Fig. 2.3). No significant reduction in the Dun1-Dif1 interaction was observed for the Dun1 N121A and L134A mutants of the lateral surface patch (Fig. 2.3A). In marked contrast, the R60A mutation of the pThr-epitope binding site resulted in a complete abrogation of the Dun1-Dif1 interaction, indicating that it relies on the Dun1FHA pThr-epitope binding site, representing a canonical mode of FHA domain ligand binding (Fig. 2.3A). Interestingly, the K136A mutation resulted in a large and significant increase in the interaction between Dun1 and Dif1 (Fig. 2.3A). This unexpected increase in the Dun1-Dif1 interaction is likely due to the removal of the large side chain size and positive charge of K136 through alanine substitution. This suggested that, although the Dun1-Dif1 interaction requires the pThr-epitope binding site, there is also some degree of interaction regulation contributed by the lateral surface of the Dun1 FHA domain. Dun1 K136 may serve to promote a more transient interaction with Dif1 to allow additional and/or subsequent interactions between Dun1 and other ligands, reminiscent of the F146A mutant for Rad53 FHA1 which interacted better with Dbf4 than WT Rad53 FHA1 (Almawi et al., 2016).

A different pattern of effects was observed with the Dun1 single mutants for the interaction with Sml1 (Fig. 2.3B). Whereas the L134A mutation had no significant influence on the Dun1-Dif1 interaction, it significantly reduced the association between Dun1 and Sml1 while K136A, instead of increasing the interaction between Dun1 and Sml1 as was observed for the Dun1-Dif1 association, significantly reduced the level of Dun1-Sml1 binding (Fig. 2.3B). Furthermore, while the R60A mutation significantly reduced the interaction between Dun1 and Sml1, this was in marked contrast to the Dun1-Dif1 interaction where the R60A mutation resulted in the complete abrogation of association between these two proteins. These results illustrate differential contributions of the distinct Dun1 FHA domain interaction surfaces based on the ligand, indicating that, in addition to the Dun1 FHA region that binds to pThr-containing epitopes, there is also a reliance on a non-canonical mechanism of FHA domain-mediated protein-protein interactions present in the Dun1 pathway, like what we and our collaborators previously characterized for the Rad53 FHA1 interaction with Dbf4/Cdc7 (Matthews et al., 2014).

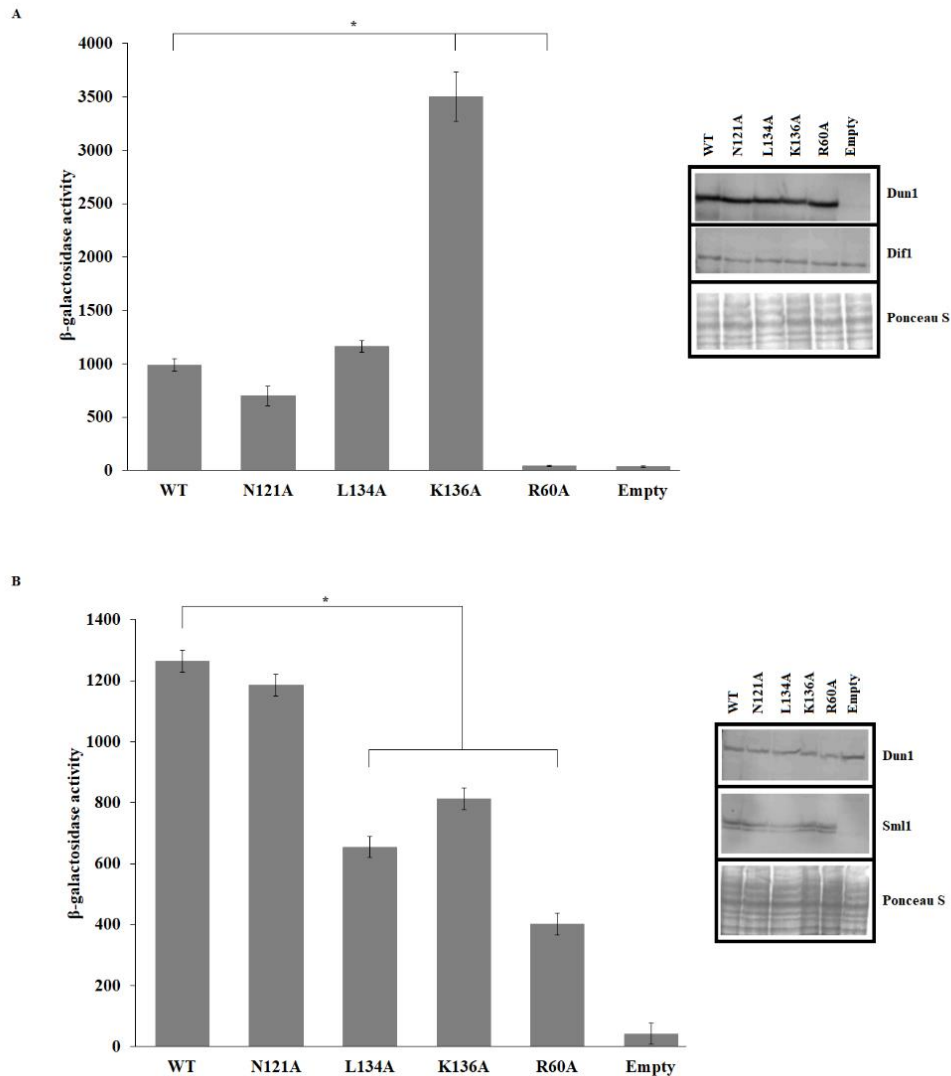


Figure 2.3 Conserved Dun1 lateral surface residues exhibit differential contributions to the Dun1 interactions.

Yeast two-hybrid assays evaluating the interactions between Dun1 and Dif1 (A) and Dun1 and Sml1 (B). Alanine substitutions of conserved residues on the lateral surface of the FHA domain: N121A, L134A, and K136A as well as of the conserved arginine residue of the pThr-binding site, R60A, were used to mutate the Dun1 lateral surface and pThr-binding site. (A) Wild-type (WT) or indicated Dun1 mutants in the context of the FHA domain or an empty vector was used as the prey with full-length Dif1 as the bait. (B) Wild type or indicated Dun1 mutants in the context of full-length Dun1 were used as the bait with full-length Sml1 or an empty vector as the prey. Mean β -galactosidase activity units represent interaction strength, $n = 3$, and error bars represent standard deviation. *: $p < 0.001$ (Tukey's HSD), showing significant differences compared to WT excluding statistically significant negative control. Corresponding western blots confirm protein expression of bait and prey constructs. Ponceau S staining indicates relative loading and transfer of protein samples.

2.4.3 The Dun1-Sml1 interaction utilizes both canonical and non-canonical FHA binding faces.

To further investigate the contribution of the Dun1 lateral surface interface, combination mutants were generated for two or more residues and yeast two-hybrid assays were conducted to evaluate the effects on Dun1 ligand binding to Dif1 and Sml1 (Fig. 2.4). Combinations for mutations were determined based on the proximity of the residues to one another for ease of site-directed mutagenesis, generating multiple mutations with a single primer set. Additionally, residues on the lateral surface that did not exhibit sequence conservation but that had side chains that projected into the external lateral surface space were included as candidate contributors to the lateral patch interface (Fig. 2.2), including H36, V38, E45, K47, and E49.

Due to the observation that the N121A mutation did not affect either the Dun1-Dif1 or Dun1-Sml1 interactions, it was not included in further analyses. The L134 and K136 residues were both mutated to alanine. This LK double mutant did not show a significant decrease in the interaction between Dun1 and Dif1 compared to the wild-type interaction (Fig. 2.4A). Combinations of alanine substitutions were then generated involving additional residues that, despite their lack of conservation, were chosen based on the previously described positioning of their side chains, with groupings determined by the proximity of the residues to one another. The double mutant for H36A and V38A (HV) significantly reduced the interaction between Dun1 and Dif1, whereas the combination of E45A, K47A, and E49A (EKE) did not reduce the Dun1-Dif1 interaction (Fig. 2.4A). For the interaction with Sml1, the Dun1 LK and HV double mutants again resulted in significantly reduced association (Fig. 2.4B). In contrast to what was observed with Dif1, the Dun1 triple mutant EKE showed a significant increase in the interaction between Dun1 and Sml1 (Fig. 2.4), consistent with the notion of the Dun1 FHA lateral surface mediating binding strength such that the absence of the long and charged side chains of E45, K47, and E49 might open a space that facilitates the association of Sml1 with Dun1.

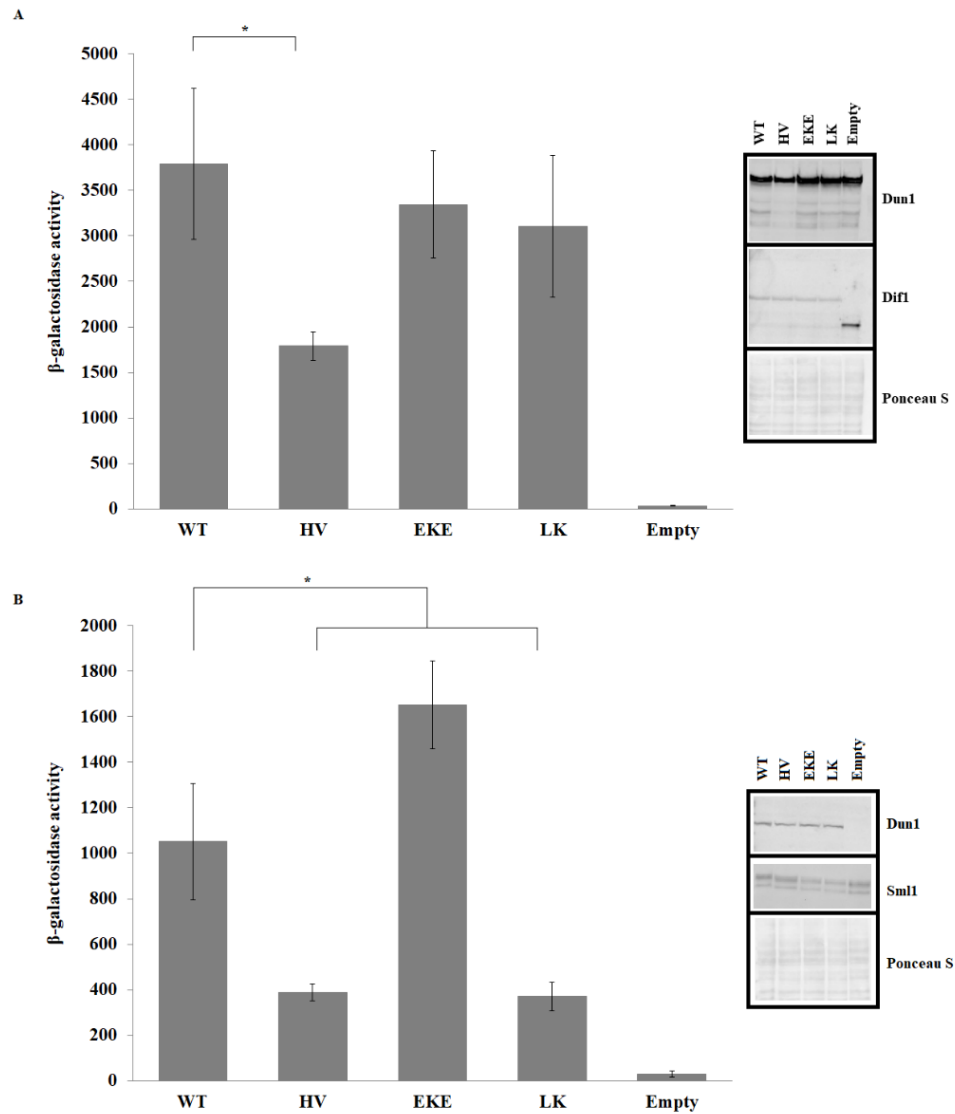


Figure 2.4 Dun1-Sml1 interaction requires the lateral surface and the pThr-epitope binding site.

Yeast two-hybrid assays evaluating Dun1 interactions with Dif1 (A) and Sml1 (B) for combination mutants of the distal lateral surface, in the context of full-length Dun1. Combined alanine substitutions for histidine 36 and valine 38 (HV), aspartic acid 45, lysine 47, and aspartic acid 49 (EKE), and leucine 134 and lysine 136 (LK) were created as double and triple mutants. (A) Dun1 constructs were used as prey with Dif1 as bait. Detection of the empty bait vector LexA tag is seen in the corresponding blot. (B) Sml1 was used as prey with Dun1 constructs as bait. Mean β -galactosidase activity units represent interaction strength, $n = 3$, and error bars represent standard deviation. *: $p < 0.001$ (Tukey's HSD), showing significant differences compared to WT excluding statistically significant negative control. Corresponding western blots confirm protein expression of bait and prey constructs. Ponceau S staining indicates relative loading and transfer of protein samples.

A further series of mutants involving combinations of three or more residues that had exhibited a reduction in the Dun1-Dif1 and/or Dun1-Sml1 interactions was next examined (Fig. 2.5). The combination of the HV double mutation with those for other residues of the lateral surface (HVLK) significantly reduced but did not completely abrogate the interaction between Dun1 and Dif1 (Fig 2.5A). The combination of the HV double mutation with those for other residues of either the lateral surface or the pThr-epitope binding site resulted in the abrogation of the Dun1-Sml1 interaction. In contrast to the other combination mutants for the lateral surface and pThr-epitope binding site, the LKR triple mutant featured a disrupted interaction similar in level to R60A alone (Fig 2.5B). Unsurprisingly, given the HVLK results, complete interaction abrogation with Sml1 was also observed for the penta-mutant Dun1HVLKR which featured the addition of the R60A mutation for the pThr-epitope binding site (Fig. 2.5B).

Taken together, these results show that there is a differential requirement for the lateral surface and/or pThr-binding site of the Dun1 FHA domain that mediates the interaction between Dun1 and two of its ligands, Dif1 and Sml1, and that the Dun1 FHA domain utilizes the two interaction interfaces in a fashion reminiscent of what was observed for Rad53 FHA1 and DDK (Dbf4/Cdc7) (Matthews et al., 2014), with an important distinction. Instead of relying on the lateral surface and pThr-epitope binding sites for simultaneous binding to Dbf4 and a Cdc7 peptide, respectively, Dun1 interactions with Dif1 and Sml1 each utilize both regions of the FHA domain to facilitate ligand binding. In the case of the Dun1-Dif1 interaction, loss of the pThr-epitope binding site prevented Dun1 binding to Dif1 for the kinase cascade involved in dNTP regulation with some reduction in the interaction with mutation of the lateral surface. However, for the Dun1-Sml1 interaction, it was the loss of the lateral surface that prevented the interaction with mutation of the pThr-epitope binding site only resulting in a reduced interaction.

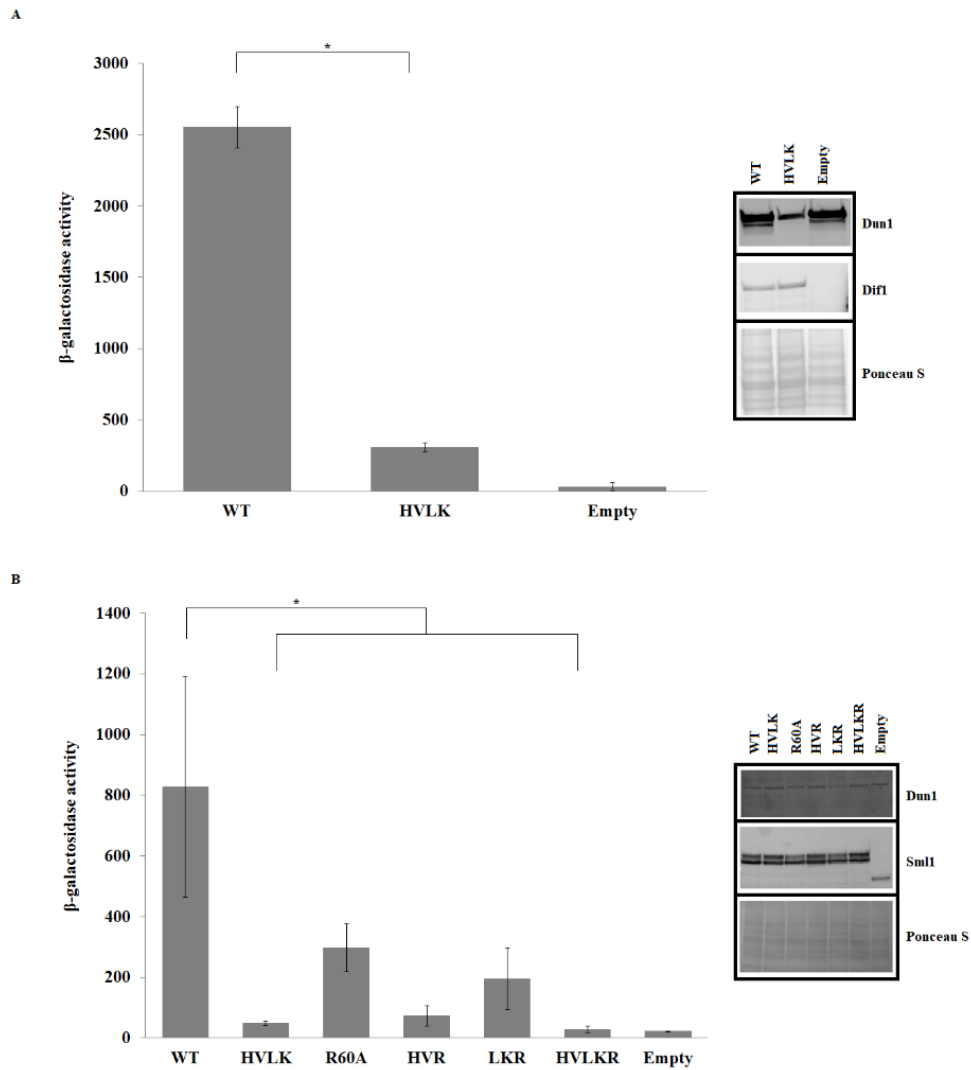


Figure 2.5 Dun1 interactions utilize the lateral surface and pThr-epitope binding site.

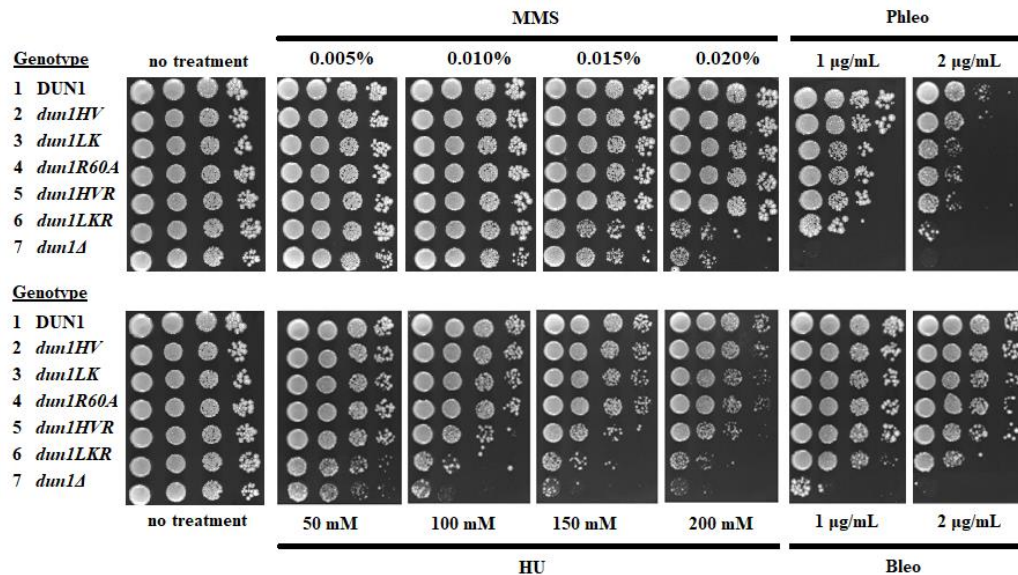
Yeast two-hybrid assays evaluating Dun1 interactions with Dif1 (A) and Sml1 (B) for combination mutants, in the context of full-length Dun1. The quadruple HVLK mutant combined alanine substitutions for histidine 36, valine 38, leucine 134 and lysine 136. (B) Addition of the R60A mutation to the HV and LK double mutants was used to create the HVR and LKR triple mutants for residues of both the lateral surface and the pThr-binding site. Addition of the R60A mutation to the HVLK quadruple mutant was used to create the HVLKR penta-mutant. (A) Dun1 constructs were used as prey with Dif1 as bait. (B) Dun1 constructs were used as bait with Sml1 as the prey. Mean β -galactosidase activity units represent interaction strength, $n = 3$, and error bars represent standard deviation. *: $p < 0.001$ (Tukey's HSD), showing significant differences compared to WT excluding statistically significant negative control. Corresponding western blots confirm protein expression of bait and prey constructs. HA tag for empty prey vector can be seen in blot for Dun1-Sml1 (B). Ponceau S staining indicates relative loading and transfer of protein samples.

2.4.4 The non-canonical Dun1 FHA interaction interface mediates resistance to genotoxic stress.

It has been previously reported that Dif1 abundance significantly decreases in response to methyl methanesulfonate (MMS) and hydroxyurea (HU) (Lee et al., 2008b). Furthermore, mutation of the Dun1 pThr-epitope binding site significantly impedes Rnr2 and Rnr4 re-localization from the cytoplasm to the nucleus in response to MMS (Sanvisens et al., 2016). We were therefore interested in assessing the effects of mutating the Dun1 pThr-epitope binding and/or lateral interaction surface on resistance to MMS and HU, through spot plate assays of *DUN1* genomic mutants (Fig. 2.6). Despite the complete abrogation of the Dun1-Dif1 interaction and reduced Dun1-Sml1 interaction for the Dun1 R60A mutation (Fig. 2.3), *dun1R60A* cells exposed to MMS did not show any difference in growth compared to WT *DUN1* cells (Fig. 2.6A). However, combined mutations of the conserved residues of the lateral surface and the conserved R60 residue of the pThr-epitope binding site (LKR) resulted in MMS hypersensitivity to the level of *dun1Δ* mutant cells (Fig. 2.6A), illustrating the importance of both FHA domain regions. The *dun1HVLK* cells which had mutations for all four residues of the lateral surface patch exhibited hypersensitivity to 0.020% MMS, like *dun1Δ* mutant cells (Fig. 2.6B). Interestingly, the hypersensitivity of the *dun1HVLKR* mutant, which included the full set of mutations of the lateral surface in addition to the pThr-epitope binding site, exceeded that of *dun1Δ* cells (Fig. 2.6B). We speculate that the *dun1HVLKR* combination of mutations might exert its negative phenotypic effect via Dun1 interaction with further ligand(s) mediated by region(s) other than the FHA pThr-interaction region or lateral surface, which could be favored with this mutant. The *dun1R60A* mutant cells did not appear to be hypersensitive to HU, only showing a slight difference in growth for the highest dilution of cells on 200 mM HU (Fig. 2.6A). In combination with alanine substitution mutations for H36 and V38 or L134 and K136, the R60A mutation resulted in hypersensitivity at concentrations of 100 mM HU and above (Fig. 2.6A), as expected given the complete loss of the Dun1-Sml1 interaction observed for combined mutants of the lateral surface patch and the pThr-binding site and the abrogated Dun1-Dif1 interaction with the R60A mutation (Fig. 2.5). The *dun1LKR* strain showed higher sensitivity than the *dun1HVR* mutant, exhibiting signs of HU hypersensitivity at 50 mM HU. (Fig. 2.6A). The quadruple mutant for residues of the Dun1 FHA domain lateral surface showed hypersensitivity at concentrations of 100 mM and 150 mM of HU with no visible growth at 200 mM of HU (Fig. 2.6B). Like what was seen with MMS exposure, the addition of the R60A mutation to the quadruple mutant resulted in enhanced sensitivity beyond the level for even *dun1Δ*

cells for all concentrations of HU, again suggesting that mutations in both the lateral surface and pThr-epitope binding site regions of the Dun1 FHA domain are more severe than just mutation of either region.

A



B

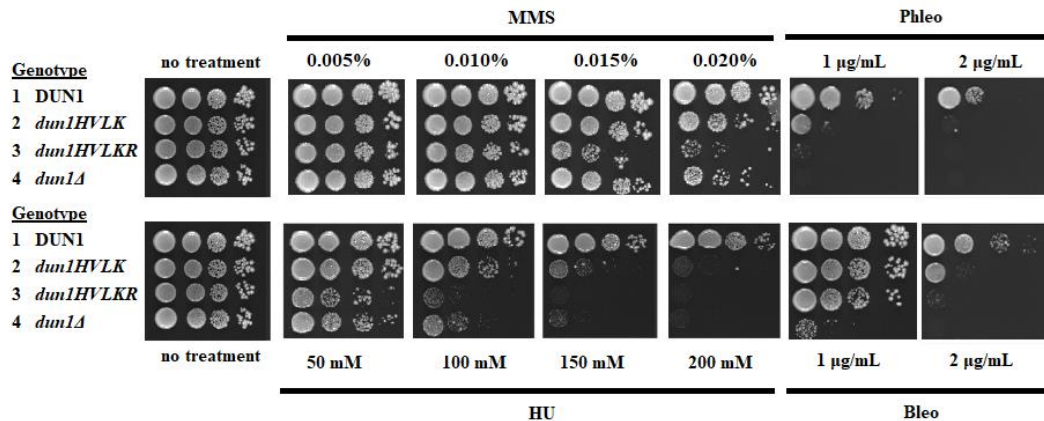


Figure 2.6 Non-canonical FHA domain lateral surface interaction contributes to Dun1-mediated dNTP regulation and cell survival upon exposure to genotoxic stress.

Spot plate assays illustrating genotoxic stress sensitivity of single, double, and triple mutant yeast strains (A) or quadruple and penta-mutant yeast strains (B) to methyl methanesulfonate (MMS), hydroxyurea (HU), bleomycin (bleo), and phleomycin (phleo). YPD plates were incubated for 3 days at 30°C. Four 10-fold serial dilutions from a saturated culture of $\sim 1.0 \times 10^8$ cells/mL were spotted from the highest concentration of cells to the lowest. Figure shows representative image of at least three trials.

Considering evidence that Wtm1 acts as a nuclear anchor for the RNR small subunits in the dNTP regulatory pathway, we were interested in the observation that the interaction between Wtm1 and the RNR small subunits is diminished in response to phleomycin (Lee and Elledge, 2006). This prompted us to examine whether mutations of the Dun1 lateral surface and/or pThr-epitope binding site would result in sensitivity to phleomycin and/or another compound that generates DNA double-strand breaks, bleomycin (Fig. 2.6). Spot plate assays of *dun1* mutants showed that the *dun1LK* and *dun1R60A* mutant cells exhibited some sensitivity relative to wild-type cells, when exposed to phleomycin for the highest dilution of cells but not for bleomycin (Fig. 2.6A), suggesting that the conserved R60 residue of the pThr-epitope binding site and the conserved residues of the lateral surface are each important to the DNA damage response triggered by phleomycin and bleomycin where the disruption of Dun1 ligand recognition via mutation of the lateral surface or pThr-epitope binding site is detrimental to the DNA damage response pathway for exogenous DNA double-strand breaks. The *dun1LKR* mutant cells exhibited hypersensitivity to phleomycin and bleomycin beyond that exhibited by either the *dun1R60A* or *dun1LK* strains, suggesting that combining disruptions of the lateral surface and pThr-epitope binding site for conserved residues of the Dun1 FHA domain is detrimental to DNA damage response pathway involving DNA double-strand breaks (Fig. 2.6). Sensitivity was observed for the *dun1HVLK* mutant at 2 µg/mL bleomycin as well as both concentrations of phleomycin (Fig. 2.6B). Addition of the R60A mutation to the *dun1HVLK* mutant resulted in almost no growth in response to 2 µg/mL of bleomycin or 1 µg/mL of phleomycin (Fig. 2.5B). Interestingly, the enhanced sensitivity of the *dun1HVLKR* mutant cells beyond the level of *dun1Δ* observed with MMS and HU exposure was not seen with phleomycin or bleomycin. Together these results illustrate the importance of the interactions mediated by both the non-canonical lateral surface patch and canonical pThr-epitope binding site of the Dun1 FHA domain, indicating that both regions are important to cell survival during genotoxic stress exposure.

2.5 Conclusions

This study shows that a non-canonical FHA lateral surface protein interaction interface is not unique to the Rad53 FHA1 domain, with Dun1 now identified as a second FHA domain-containing protein that utilizes this mode of ligand binding. Furthermore, our findings point to greater diversity in the use of a non-canonical surface for protein-protein interactions. Previously, we and our collaborators showed that an FHA domain can use its lateral surface as the sole means of mediating a protein-protein interaction with one ligand within a target complex, while simultaneously using the pThr-epitope binding site to engage another, as we observed for Rad53 FHA1 interactions with Dbf4 and Cdc7 respectively. Here, we have demonstrated that an FHA-mediated interaction with a single protein can also use the lateral surface in addition to the pThr-epitope binding site to aid in ligand specificity as well as binding efficiency. Thus, this study adds to the small but growing collection of evidence that FHA domains act through more than their pThr-epitope binding capacity.

The loss of Dun1 interactions with Dif1 and Sml1 and the corresponding hypersensitivities to MMS and HU for mutants within both regions of the FHA domain were expected considering the role of Dun1, during the DNA damage response (DDR), in upregulating genes related to DNA repair (Zhou and Elledge, 1993; Zhao and Rothstein, 2002). Combinations of mutations for both regions of the Dun1 FHA domain, resulting in the loss of both the Dif1 and Sml1 interactions (Fig. 2.5, Table 2.3), exhibited hypersensitivity to DSB-inducing agents phleomycin and bleomycin in agreement with the observation that cells lacking Dun1 have compromised 'G2 phase' arrest in response to DSBs (Yam et al., 2020). The enhanced hypersensitivity of the *dun1HVLKR* mutant cells, relative to *dun1Δ*, was a surprising result (Fig. 2.6). We speculate that the negative effect of the full combination of mutations for both the lateral surface and pThr-epitope binding site may be the consequence of Dun1 dysregulation in DNA repair and/or other pathways (Yam et al., 2020). For example, it has been shown that Dun1 is also involved in 'G2 phase' arrest and the prevention of replication fork collapse in response to DNA DSBs and HU exposure (Yam et al., 2020; Wang et al., 2001; Woolstencroft et al., 2006) where *dun1Δ* cells can recognize DNA damage and have an active checkpoint, seen via Rad53 and Chk1 phosphorylation and activation, but still progress through mitosis (Yam et al., 2020). Not only are Rad53 and Chk1 unable to prevent the onset of mitosis without Dun1, but the anaphase inhibitor Pds1 is also unstable and unphosphorylated in the absence of Dun1, leading to its premature degradation and the progression of chromosome segregation (Yam et al., 2020; Wang et al., 2001; Woolstencroft et al., 2006). It is therefore possible that altered Dun1

activity in one or more of these processes, because of altered ligand interactions, results in a more severe phenotypic outcome than for its complete absence.

Table 2.3 Summary of Dun1 FHA Domain Mutations and Ligand Binding Effects.

Bait	Prey	FHA Mutation(s)	Canonical vs. Non-canonical	Interaction
Dif1	Dun1	R60A	Canonical	Δ
Dif1	Dun1	N121A	Non-canonical	NE
Dif1	Dun1	L134A	Non-canonical	NE
Dif1	Dun1	K136A	Non-canonical	↑
Dif1	Dun1	HV	Non-canonical	↓
Dif1	Dun1	EKE	Non-canonical	NE
Dif1	Dun1	LK	Non-canonical	NE
Dif1	Dun1	HVLK	Non-canonical	↓
Dun1	Sml1	R60A	Canonical	↓
Dun1	Sml1	N121A	Non-canonical	NE
Dun1	Sml1	L134A	Non-canonical	↓
Dun1	Sml1	K136A	Non-canonical	↓
Dun1	Sml1	HV	Canonical	↓
Dun1	Sml1	EKE	Non-canonical	↑
Dun1	Sml1	LK	Non-canonical	↓
Dun1	Sml1	HVLK	Non-canonical	Δ
Dun1	Sml1	HVR	Non-canonical & Canonical	Δ
Dun1	Sml1	LKR	Non-canonical & Canonical	↓
Dun1	Sml1	HVLKR	Non-canonical & Canonical	Δ

NE, no effect. ↑, increased interaction. ↓, decreased interaction. Δ, interaction abrogation.

FHA domain-containing proteins are prevalent in the DNA damage regulation and checkpoint pathways that are central to proper cell division and the maintenance of DNA integrity during DNA replication and repair processes. As such, expanding our view of FHA domains beyond their phospho-dependent interactions and pathways will open new perspectives for understanding the regulatory pathways involved in diseases attributed to errors in DNA damage and checkpoint responses. A thorough understanding of all facets of FHA domains and the interactions that they mediate could broaden the

scope of our understanding of the molecular pathways behind a whole host of diseases such as Li-Fraumeni syndrome for which four tumour-associated mutations have been identified and characterized in Checkpoint kinase 2 (Chk2), the human homolog of Rad53, with two of them being located within the FHA domain (Wu et al., 2001). Our findings emphasize the importance of looking at the full scope of FHA domain interactions, including non-canonical binding modes. In doing so, we will be better able to understand the missing or reduced interactions that compromise DNA damage signaling pathways leading to the faulty repair, and/or compromised cell cycle progression that is characteristic of many human diseases. By understanding the intricacies of these important protein-protein interaction processes, we will be better equipped to design more targeted therapeutic approaches.

Chapter 3

Sequence and Structural Conservation of the FHA and H-BRCT domains in *Saccharomyces cerevisiae* and Humans

3.1 Summary

FHA domains have been historically characterized based on their pThr-epitope binding specificity. However, over the past two decades various research groups have shown isolated incidents of FHA domain-mediated protein-protein interactions that are not just reliant on the pThr-epitope binding site. As such, there exists a need for an in-depth, unbiased analysis of FHA domains beyond the scope of their prominent pThr-epitope binding specificity. The discovery of the H-BRCT domain in budding yeast Dbf4 and the non-canonical interaction between the H-BRCT domain and the FHA1 domain of Rad53 allowed for the characterization of an interaction where an FHA domain was involved in a phospho-independent interaction using the distal lateral surface of the FHA domain to establish the interaction instead of the pThr-epitope binding site. This observation raised the question of whether the non-canonical nature of the Rad53 interaction with Dbf4 in budding yeast was a unique feature of the Rad53 FHA1 domain and/or a consequence of the H-BRCT domain structure, as well as whether other FHA domains could use an alternative surface for mediating protein-protein interactions outside of the apical pThr-epitope binding site. To investigate this question, the list of yeast FHA domain-containing proteins reported in the *Saccharomyces* Genome Database was first analyzed and confirmed using the pHMM available in the PFAM database and the online HMMER analysis tool. All FHA domain-containing protein sequences were subjected to secondary structure prediction and analysis tools, IUPRED and PSIPred, prior to conservation mapping via the Consurf server of SWISS-MODEL and Robetta-derived FHA domain models to visualize any areas of conservation at the apical and lateral FHA domain surfaces. UCSF Chimera, in conjunction with the conservation mapping, was used to identify conserved residues of the pThr-epitope binding site and candidate residues of interest on the proximal and distal lateral surfaces of FHA domain-containing proteins for which a 3-D model of the FHA domain could be visualized. The existence of other H-BRCT domains in budding yeast and humans was investigated using HHPred homology searching and HMMER analysis. The SWISS-MODEL tool was used to model bona fide H-BRCT domains in budding yeast and candidate H-BRCT domains. This data provides an overview and starting point for the characterization of non-canonical FHA domain-based protein-protein interactions.

3.2 Introduction

One of the key signaling mechanisms for mediating protein-protein interactions is phosphorylation. Protein phosphorylation and dephosphorylation events, executed by kinases and phosphatases respectively, are essential to many signal transduction pathways in living cells (reviewed in Seet et al., 2006), with phosphorylation at serine and threonine (Ser/Thr) residues being the most prominent type of phosphorylation in eukaryotes (Yaffe and Elia, 2001). Since the discovery of Ser/Thr phosphorylation, many protein families have been identified based on their ability to recognize those pSer/pThr residues. Distinct from other phosphopeptide-sensing protein families, the Forkhead-associated (FHA) domain family uniquely exhibits pThr-epitope binding specificity (Durocher et al., 2000). In 2002, Durocher and Jackson detailed the structure of the Forkhead-associated (FHA) domain as a module consisting of 100-120 amino acids that fold into an 11-stranded β -sandwich with two apical and two lateral surfaces (Durocher and Jackson, 2002). The phosphopeptide-binding site is located on one of the apical surfaces with both the N- and C-termini localized at the opposite apical surface (reviewed in Almawi et al., 2017). Each of the lateral surfaces can be referred to as either being proximal to the pThr-epitope binding site or distal. FHA domains have a highly conserved structure, although they typically do not exhibit extensive amino acid sequence conservation. The mechanism of the FHA domain pThr-epitope binding specificity relies on two pockets of the apical surface that recognize the pThr residue itself as well as a conserved residue located at the pThr+3 position (Durocher et al., 2002). The β 3- β 4 and β 4- β 5 loops constitute the pThr-epitope binding site where two conserved residues, one of which is an arginine, usually contact the phosphopeptide (Durocher et al., 2002). The β 6- β 7 and β 10- β 11 loops constitute the pThr+3-binding site. However, unlike the pThr pocket, the residues of this second pocket are not conserved. This allows for ligand binding specificity. Additionally, non-conserved residues contribute to hydrogen bonding with the pThr residue to influence interaction stability.

The discovery of FHA domain pThr-epitope binding specificity and the use of peptide libraries to investigate FHA domain binding specificity had propagated the bias that FHA domains were only capable of recognizing pThr-epitopes (reviewed in Almawi et al., 2017). However, discoveries that FHA domains could bind to short peptides that contained pThr residues even if they were not native FHA-binding partners (Chen et al., 2013; Matthews et al., 2012) triggered investigations into FHA domain-mediated interactions with an awareness of the potential for higher complexity interactions. The analysis of human Ki67 and budding yeast Dun1 during the 2000s demonstrated that FHA domains such as these can bind to multi-phosphorylated epitopes (Byeon et al., 2005, Lee et al., 2008, Bashkirov et al., 2003,

Lee et al., 2003). In fact, Dun1 activation requires the recognition and binding of Dun1 to the di-phosphorylated form of the Rad53 checkpoint kinase in budding yeast (Lee et al., 2008). In this case, Rad53 possesses two pThr residues and lacks the conserved residue at the pThr+3 site. The second pThr residue is recognized by the canonical conserved arginine residue of the pThr-epitope binding site instead of being involved in the usual stabilization of the phosphate group of the first threonine (Lee et al., 2008). Differences in binding efficiency and strength have also been observed due to multi-phosphorylation as well as auto-inhibitory interactions mediated by FHA domains (Byeon et al., 2005, Barthe et al., 2009, Nott et al., 2009). FHA domains have also been found to be involved in phosphorylation-dependent oligomerization based on both the apical and/or distal lateral surfaces such as have been observed in Chk2 (Cai et al., 2009) and Mdc1 (Jungmichel et al., 2012, Liu et al., 2012, Almawi et al., 2017).

Of particular interest is the observation that FHA domains can mediate phosphorylation-independent interactions through their apical surfaces as well as their lateral surfaces (reviewed in Almawi et al., 2017). Previous work from our lab, in collaboration with the Guarné lab (McMaster University, currently McGill University) and Dr. Brendan McConkey (University of Waterloo), characterized the phospho-independent interaction between Rad53 and Dbf4 (Matthews et al., 2014, Almawi et al., 2016). Dbf4 is the regulatory subunit of the Dbf4-dependent kinase (DDK), a complex of Dbf4 and Cdc7 kinase (Bruck and Kaplan, 2009). The DDK complex is involved in activating origins of replication, meiosis, mitotic exit, and the intra-S phase checkpoint, which helps to suppress untimely origin firing and to stabilize stalled replication forks to prevent genetic instability (Marston, 2009, Matos et al., 2008, Patterson et al., 1986, Miller et al., 2009, Pessoa-Brandao and Sclafani, 2004, Zegerman and Diffley, 2010). Dbf4 has three short regions of conservation referred to as the N, M, and C motifs based on their location in the polypeptide chain (Masai and Arai, 2000). The N motif is part of a larger structurally conserved unit consisting of amino acid residues 120-250 (Matthews et al., 2009). Matthews et al., discovered that this unit was involved in mediating the interaction between Rad53 and Dbf4 (Matthews et al., 2012). They also found that, unique to Dbf4, residues 105-119 formed an α -helix that was embedded within the core of the BRCT domain (Matthews et al., 2012). This novel structure was termed the Helix-BRCT (H-BRCT) domain. Since the discovery of the Dbf4 H-BRCT domain, only one other H-BRCT domain has been discovered. Sir4, a member of the silent information regulator (SIR) complex involved in transcriptional repression of sub-telomeric regions and the homothallic mating-type loci (Rine and Herskowitz, 1987, Aparicio et al., 1991, Hecht et al., 1995, reviewed in Kueng et al., 2013), has been shown to have an H-BRCT domain closely related to the Dbf4 H-BRCT domain

(Deshpande et al., 2019). Tong et al., also showed that the FHA domain of KIF_{13B} does not possess the typical conserved residues required for pThr recognition and instead utilizes its lateral surfaces to enhance binding at its apical surface (Tong et al., 2010). Human Chk2, as seen with budding yeast Rad53, can also use a conserved hydrophobic patch on the lateral surface to mediate protein-protein interactions (Li et al., 2002, Matthews et al., 2014, Almawi et al., 2016). Mutation of the hydrophobic patch I157 residue on the proximal lateral surface in human Chk2 has been linked to Li-Fraumeni syndrome (Li et al., 2002).

To contribute to the rationalization that FHA domains can in fact mediate and/or contribute to ligand binding specificity and efficiency via the proximal and/or distal lateral surfaces, the prevalence of conserved lateral surface interfaces on all known FHA domain-containing proteins in budding yeast and the existence of other H-BRCT domain-containing proteins was investigated. PSI-BLAST searches to identify homologs, multiple sequence alignments and HMMER analyses, protein domain modeling, and conservation mapping was used to characterize FHA domains in budding yeast, showing lateral surface conservation in near and distant homologs. For the H-BRCT domains, HHPred homology searches, multiple sequence alignment and HMMER analyses, and H-BRCT domain modelling was used to search for other H-BRCT domains in yeast other than Dbf4 and Sir4.

3.3 Methods

3.3.1 Secondary Structure Analysis and Homology Search for FHA Domains

All FHA domain-containing protein amino acid sequences were submitted to the IUPred and PSIPRED online servers for the prediction of the disordered regions and protein secondary structures, respectively, to confirm suggested FHA domain boundaries (<https://iupred2a.elte.hu/>; <http://bioinf.cs.ucl.ac.uk/psipred/>). The peptide sequence corresponding to the FHA domain was entered as the query to search the non-redundant protein sequences (nr) database using the Position-Specific Iterated BLAST (PSI-BLAST) algorithm (NCBI) to establish a collection of homologs to assess amino acid conservation. Returned sequences exhibiting a percent identity of 25% or above to the query sequence were selected as homologs and their sequence data exported for use in generating a multiple sequence alignment (MSA). Homologous sequence data was uploaded to the online Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool (EMBL-EBI) and the resulting MSA file was viewed using the free Jalview software (www.jalview.org) and used as a query in an HMMSearch using the EMBL online tool (<https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch>) to generate a profile Hidden Markov Model (pHMM) for FHA domains.

3.3.2 Homology Searching and Sequence Conservation for H-BRCT Domains

The HHPred search tool (<https://toolkit.tuebingen.mpg.de/tools/hhpred>) was utilized with the Dbf4 H-BRCT domain amino acid sequence as the search query to confirm any distant and/or close homologs to be utilized in the generation of a H-BRCT profile Hidden Markov Model (pHMM). The HHPred online tool's default settings were used for the search. Hit sequences were selected based on E-values that were <0.001. The return alignment was viewed online with the HHPred alignment viewer and a collection of 8 sequences were identified and used to generate a Multiple Sequence Comparison by Log-Expectation (MUSCLE) multiple sequence alignment (MSA; <https://www.ebi.ac.uk/Tools/msa/muscle/>). The MSA was visualized using Jalview software (www.jalview.org) and then used as the query in an HMMSearch using the EMBL online tool (<https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch>) to identify all potential H-BRCT domain-containing proteins across species.

3.3.3 Protein Modelling and Conservation Mapping

Protein models for domains were generated using SWISS-MODEL for proteins with structural data in the protein data bank (PDB, <https://swissmodel.expasy.org/>) or *ab initio* modelling via the Robetta server. UCSF Chimera protein model analysis software (<https://www.cgl.ucsf.edu/chimera/download.html>) was used to view and process protein models to emphasize features of interest. For analysis of the H-BRCT domains, models were generated for the following: Dbf4 (amino acids 100-225), Sir4 (amino acids 960-1090), Chs5 (amino acids 150-260), and TOPBP1 (amino acids 1360-1480). Sequence conservation was mapped to protein models using the Consurf server and visualized in UCSF Chimera (https://consurf.tau.ac.il/consurf_index.php).

3.3.4 Yeast Strains

Table 3.1 Yeast Strains.

Strain	Genotype	Source
DY-1	<i>MATa, ade2-1, can1-100, trp1-1, his3-11, ura3-1, leu2-3, leu2-112, pep4::LEU2</i>	(Duncker et al., 2002)

3.3.5 Genomic DNA Isolation

Genomic DNA was isolated from the DY-1 yeast strain for use as template DNA in PCR reactions contributing to plasmid construction. A working culture for each strain, inoculated from a saturated preculture, was grown to a concentration of $\sim 1 \times 10^7$ cells/mL in 10 mL of YPD (1% yeast extract, 2% peptone, and 2% dextrose) media at 30°C. Cells were centrifuged at 4000 rotations-per-minute (rpm) for 5 minutes (Thermo Scientific centrifuge CL2), resuspended in 500 μ L of sterile water and transferred to a sterile 2 mL screw-cap tube. The cells were then centrifuged for 10 seconds at maximum speed (Eppendorf 5424 R centrifuge), the supernatant decanted, and then vortexed to loosen the pellet. A volume of 200 μ L of 'genomic prep mix' (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8, and 1 mM EDTA) was added to the loosened pellet, followed by 200 μ L of phenol:chloroform:isoamylalcohol (25:24:1) and 0.5 g of 0.5 mm glass beads. All components were mixed for 3-4 minutes using a vortex and then 200 μ L of 1X TE pH 8 was added and mixed. Following a 5-minute centrifugation at maximum speed (Eppendorf 5424 R centrifuge), the top layer was transferred to a new sterile 1.5 mL Eppendorf tube with 1 mL of 100% ethanol and mixed by inversion. After another centrifugation for 2 minutes at maximum speed (Eppendorf 5424 R centrifuge), the pellet was completely re-suspended in 0.4 mL of 1X TE pH 8 to which 10 μ L of 10 mg/mL RNase A (Sigma) was added before incubating at 37°C for 10 minutes. After incubation, 10 μ L of 4 M ammonium acetate and 1 mL of 100% ethanol were added and mixed by inversion. After a 2-minute centrifugation, the supernatant was discarded, and the pellet left to air dry before completely re-suspending the pellet in 50 μ L of 1X TE pH 8. Genomic DNA was stored at -20°C.

3.3.6 Plasmid Construction

Protein coding sequences for Dun1 and Dif1 were amplified from DY-1 genomic DNA and cloned into the pJG4-6 and pEG202 prey and bait vectors for yeast two-hybrid assays respectively (Duncker et al., 2002; Ausubel et al., 1994; Gyuris et al., 1993). The bait and prey vectors contain a selectable marker for the biosynthesis of histidine (*HIS3*) and tryptophan (*TRP1*), respectively. The pSH18-34 plasmid contains a selectable marker for the biosynthesis of uracil (*URA3*) (Ausubel et al., 1994; Gyuris et al., 1993). Coding sequences were amplified using a commercially available High-Fidelity PCR kit (Roche). PCR products were purified using a commercial PCR clean up kit that was also used for the clean-up of restriction enzyme digested PCR products and vectors (Geneaid). All restriction enzymes were purchased from Thermo Fisher Scientific, and their recognition sequences were

incorporated into the design of the primers used for PCR amplification. Ligation was carried out using a T4 DNA ligase kit (BioBasic), and the ligated product was transformed into calcium-chloride competent DH5 α Escherichia coli (E. coli) cells grown and plated on LB media (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Colony PCR was performed to confirm transformation of successfully ligated constructs using a commercial colony PCR kit (New England Biolabs). Primer pairs were designed such that one primer targeted the vector sequence and the other targeted the sequence of the insert. Plasmid DNA was isolated from saturated overnight cultures of DH5 α for positive colonies using a commercial plasmid prep kit (Geneaid; <http://www.geneaid.com/sites/default/files/PD13.pdf>). Plasmid DNA was sent for sequencing of the full length insert at the Sick Kids Sequencing Facility (Toronto, Ontario), to ensure the fidelity of the cloned PCR product. The full-length Dun1 coding sequence was cloned into pJG4-6 using the EcoRI and ApaI restriction enzymes and the full-length Dif1 coding sequence was cloned into pEG202 using the EcoRI and NcoI restriction enzymes. Site-directed mutagenesis was used to generate the pJG4-6 *dun1-H77A* and pJG4-6 *dun1-C64A* constructs.

3.3.7 Site-directed Mutagenesis

Mutations within the Dun1 coding sequence were generated using the QuikChange II XL Site-directed Mutagenesis kit (Agilent Technologies). Mutagenic primers were designed using the QuikChange Site-Directed Mutagenesis online interface (<https://www.genomics.agilent.com/primerDesignProgram.jsp>) and then utilized during PCR to remove or replace the desired nucleotide(s). PCR products were digested using DpnI restriction endonuclease to degrade parental copies of the plasmid containing the gene to be mutagenized and then the product was transformed into XL-10 Gold Ultracompetent cells (Agilent) and plated on Luria-Bertani (LB) agar (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Plasmid DNA isolated from transformants was sent for sequencing of the gene at the Sick Kids Sequencing Facility to confirm successful mutagenesis. A full protocol for site-directed mutagenesis is available online: <http://www.agilent.com/cs/library/usermanuals/public/200523.pdf>.

3.3.8 Yeast Transformation

Two-hybrid bait and prey plasmid DNA was transformed into the DY-1 yeast strain, previously transformed with the pSH18-34 reporter plasmid. A working culture was grown to a concentration of $\sim 1 \times 10^7$ cells/mL in 10 mL of Synthetic Complete (SC) media (0.17% yeast nitrogen base, 0.5% ammonium

sulfate, 2% glucose, 1X amino acid mix) lacking uracil for the maintenance of the pSH18-34 reporter plasmid. Working cultures were centrifuged for 5 minutes at 4000 rpm (Thermo Scientific centrifuge CL2), resuspended in 1X Tris-EDTA (TE) solution pH 8.0, centrifuged again, resuspended in 200 µL of lithium acetate/TE solution (100 mM lithium acetate, 2.5 mL 1X TE), and then held at room temperature for 10 minutes. Approximately 0.4 µg of plasmid DNA was added to 100 µL of the yeast suspension mix along with 100 µg of salmon sperm DNA, and then 300 µL of lithium acetate/TE/PEG4000 solution (100 mM lithium acetate, 2 g polyethylene glycol 4000, 0.5 mL 10X TE) was added. The solution was incubated at 30°C for 30 minutes before 40 µL of 100% dimethyl sulfoxide (DMSO) was added. Cells were heat shocked at 42°C for 7 minutes, held on ice for 2 minutes and then plated on selective media and grown for 2-3 days.

3.3.9 Yeast two-hybrid Assay

This analysis was performed as described previously (Duncker et al., 2002).

3.3.10 Statistical Analysis

GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) was used to analyze yeast two-hybrid data for One-way ANOVA comparison of sample means over several independent replicates for reproducibility. Dunnett multiple comparisons test was used to compare sample means. The acceptance threshold was set at 0.05.

3.4 Results and Discussion

3.4.1 Yeast FHA domain-containing proteins pHMM shows additional conserved motifs and surface residues on lateral surfaces.

To confirm the complete list of FHA domain-containing proteins in budding yeast, as annotated in the Saccharomyces Genome Database (SGD), or to identify novel FHA domain-containing proteins, the multiple sequence alignment (MSA) from the PFAM database profile Hidden Markov Model (profile-HMM) for the FHA domain family was used in an hmmsearch within the online HMMER analysis tool (Fig. 3.1). The alignment profile shows the highly conserved GR motif of the pThr-epitope binding site (Durocher et al., 2002) and a SxxH motif featuring a highly conserved serine and histidine (Fig. 3.1). There also appears to be some conservation of aspartic acid, serine, and asparagine residues downstream of the pThr-epitope binding site in a region likely attributable to a lateral surface (Fig. 3.1). The hit

sequences returned from the hmmsearch did not contain any additional sequences or proteins beyond what has already been identified as FHA domain-containing proteins in the SGD (Table 3.1), leading us to the conclusion that SGD contains a complete list of budding yeast FHA domain-containing proteins.



Figure 3.1 HMMER-derived FHA pHMM shows GR motif of pThr-epitope binding site and SxxH motif.

HMMER alignment profile shows potential FHA domain motifs. The multiple sequence alignment of the profile Hidden Markov Model (profile-HMM) for the FHA domain family available from PFAM (PF00498) was used to search the reference proteomes database in an hmmsearch restricted to *Saccharomyces cerevisiae* for confirmation of the profile-HMM sequence logo for the complete list of FHA domain-containing proteins in budding yeast.

Since the complete list of budding yeast FHA domains was confirmed, a closer analysis of the FHA domain sequence and structure was used to investigate the prevalence of conserved residues on the distal lateral surface that could be involved in mediating protein-protein interactions. To begin the analysis of budding yeast FHA domains, IUPRED and PSIPRED online tools were used to examine the primary amino acid sequence of each protein, their predicted secondary structures, and their ordered and disordered regions. Secondary structure analyses depicted the alpha helices, loops, and beta strands of each protein as well as which regions of the polypeptide sequence should fold into a stable structure. The boundaries or region of the protein to be used in the generation of the FHA domain models were determined based on the secondary structure analysis of each sequence. Boundaries were chosen to encompass the expected 11-stranded β -sandwich FHA domain structure (Table 3.2). A PSI-BLAST search of each FHA domain sequence was used to identify homologs for a MUSCLE multiple sequence alignment (MSA) to assess the amino acid conservation of budding yeast FHA domains and identify the highly conserved GR motifs in FHA domain MSAs, visualized using Jalview, by which the arginine residue(s) of the pThr-epitope binding site were identified (Table 3.2).

Table 3.2 Selected FHA domain region for modelling and conservation mapping and pThr-epitope binding site and lateral surface residues.

Protein Name	Selected region for modelling	Conserved arginine(s) of pThr-epitope binding site	Surface residues of β Strands	
			Proximal lateral surface residues	Distal lateral surface residues
Rad53	1-180	R70 (FHA1)	V65, T67, Q90, L92, L100, N102, N121, L123	R35, I37, D47, W110, K115, T131, E129, S142, V144, F146
	550-750	R605 (FHA2)	F602, F624, F626, K628, D646, W648, K669, L671	T579, K581, E695, V689, W682, K679, E677, Y658
Dun1	1-160	R60, R102	T57, E79, H81, L83, M85, V87, F90, R92, L94, N96, I98, I118	C33, H36, V38, L40, E45, K47, E49, F106, V126, R124, S132, L134, K136
Mek1	1-144	R51	K48, V71, W73, V75, F77, I82, M84, Y86, K88, T107, L109	Y96, E117, V115, M135, V133, R131, N29, E27, H25, P40, T38
Xrs2	1-100	R32	T27, S29, T52, K54, C69, V71, T91	S78, I102, E104, E113, E115, V3, R5
Fkh1	1-175	R80, R132	D100, D102, T77, K112, A115, R117, N119, S124, E126, Q128	L136, I158, D160, Q165, I167, I169, A58, K61, A63, T68, Y70
Fkh2	1-185	R87, R142	N110, D112, S84, K122, I125, K127, E136, H138, P160, R162	L168, D170, Q175, M177, I179, K68, S70, T75, Y77
Vps64	150-280	R189	K186, C229, S231, Y240, R242	F251, T269, D271, D277, K279, I161, I163, F175, V177
Far10	50-205	R103	K100, L148, S150, D152, K157, Y159, R161	R175, F170, V188, D190, E200, R202, I204, I75, V77, T79, H89, V91
Dma1	175-280	R193	-	-
Dma2	270-380	R299	-	-
Tos4	100-182	R122	-	-
Plm2	100-230	R106	-	-
Fhl1	280-390	R304	-	-
Pml1	100-204	R108	-	-
YGL081W (uncharacterized protein)	1-170	R26	-	-

For proteins with FHA domain crystal structures published in the PDB, or that of their homologs (Rad53, Dun1, Mek1 and Xrs2) SWISS-MODEL was used to analyze FHA domain models for the selected boundaries (Appendix B, Figures 1-4, 9). For proteins without published crystal structures or homologs with a published crystal structure in the PDB (Fkh1, Fkh2, Vps64 and Far10), models for selected regions were generated using *ab initio* modelling on the Robetta server (Appendix B, Figures 5-8). Models for the remaining FHA domain-containing proteins listed in Table 3.2 could not be generated using *ab initio* modelling in Robetta, due to a lack of adequate coverage of the FHA domain primary sequence (Data not shown). Surface amino acid conservation for each protein was mapped to generated

models using the Consurf server and models were analyzed using UCSF Chimera. All residues located on β -strands with externally facing side chains were viewed in Chimera and listed as potential residues of interest for mediating interactions with FHA domain ligands (Table 3.2). Residues that have already been investigated are highlighted (Table 3.2, Chapter 2, Chapter 3). Lateral sides, as per the β -sandwich structure of FHA domains, were distinguished based on proximity to the pThr-epitope binding site (reviewed in Almawi et al., 2017). For proteins without successful models, lateral surface residues could not be identified within Chimera. Visualization of FHA domain models illustrated the differential existence of conservation on the proximal and/or distal lateral surfaces in agreement with data showing the involvement of lateral surfaces mediating protein-protein interactions (Li et al., 2002, Tong et al., 2010, Matthews et al., 2014, Almawi et al., 2016). Amongst the Rad53, Dun1, and Mek1 paralogs, there seemed to be a separation of lateral surface conservation that could be attributed to their evolutionary function. The FHA1 domain of Rad53 showed conservation on both the proximal and distal lateral surfaces, whereas the Dun1 FHA domain only featured a few conserved residues on the distal lateral surface and the Mek1 FHA domain only had conserved residues on the proximal lateral surface (Appendix B Figures 1-3). The Rad53 FHA2 domain showed conservation primarily on the proximal lateral surface only (Appendix B Figure 9). Xrs2, Fkh1, Fkh2, Vps64, and Far10 each exhibited conservation on both proximal and distal lateral surfaces (Appendix B Figures 4-8). Preliminary investigation of the Rad53 human homolog, Chk2, showed conservation on both the proximal and distal lateral surfaces. This prompted a further analysis of just Rad53, Dun1, Mek1, and Chk2.

3.4.2 Comparison of human Chk2 with budding yeast paralogs Rad53, Dun1, and Mek1

Given that Mek1 is a meiotic-specific paralog of Rad53 and Dun1 (Chuang et al., 2012), we were interested to see if any distinct characteristics would show up in an analysis of just those FHA domain sequences apart from the other identified yeast FHA domains (Figure 3.2). Additionally, due to the observation that the conservation of residues at the lateral surface can differ amongst paralogs (Appendix B Figures 1-3) as well as a preliminary look at the Rad53 human homolog, Chk2 (Data not shown), which showed high conservation at both lateral surfaces, we decided to investigate the conservation of Rad53, Dun1, and Mek1 in yeast compared with Chk2 in humans with respect to their primary sequence. Using a MSA generated from the FHA domain sequences of Rad53, Dun1, Mek1, and Chk2, a hmmsearch was run to see what the pHMM would look like for those FHA domains compared to that of all budding yeast FHA domains (Fig. 3.2)

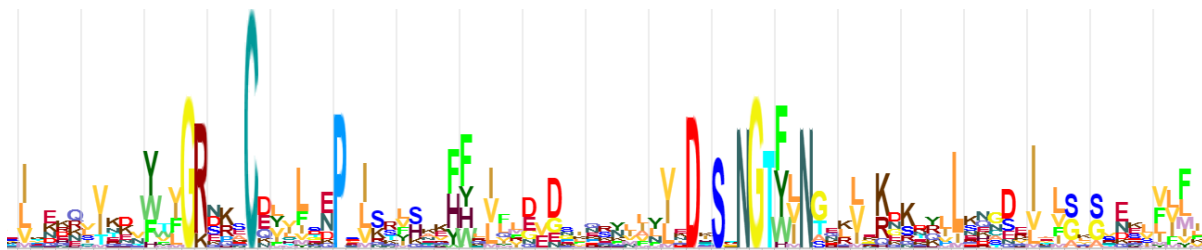


Figure 3.2 HHMER-derived FHA domain pHMM for yeast and human checkpoint proteins shows highly conserved cysteine residue instead of yeast SxxH motif.

HHMER alignment profile shows residue conservation and motifs based on budding yeast Rad53, Dun1, and Mek1 and human Chk2 FHA domain multiple sequence alignment (MSA). The MSA generated using the amino acid sequences of Rad53, Dun1, Mek1 and Chk2 was used to search the reference proteomes database in an hmmsearch to generate the profile-HMM sequence logo across species.

Compared to the pHMM for all budding yeast FHA domains, there appears to be a different secondary “motif” for FHA domains relative to just Rad53, Dun1, Mek1, and hChk2. Instead of the highly conserved serine and histidine residues seen in Figure 3.1, a highly conserved cysteine residue is seen followed by a highly conserved proline residue six “positions” downstream (Fig. 3.2). Like the full budding yeast FHA domain analysis, there are a collection of aspartic acid, serine, asparagine, glycine, and threonine residues downstream of the pThr-epitope binding site that exhibit high conservation which could be conservation within a lateral surface (Fig. 3.2). Yeast two-hybrid analysis conducted by senior Honours Thesis student Renessa Gomes was used to investigate whether the highly conserved histidine and cysteine residues could be involved in mediating FHA domain-based protein-protein interactions. Alanine substitution of Dun1 H77 reduced the interaction of Dun1 with one of its binding partners, Dif1, to about 12%. Alanine substitution of C64 reduced the interaction of Dun1 with Dif1 interaction to about 65% (Fig. 3.3). This suggests that these conserved residues can be integral to mediating protein-protein interactions, binding specificities, and/or FHA domain structural stability and integrity in budding yeast.

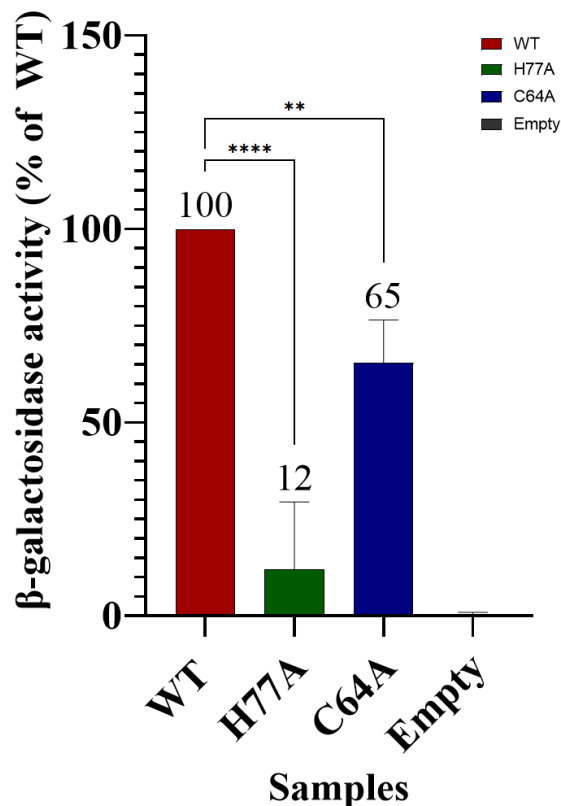


Figure 3.3 Histidine and cysteine residues contribute to the Dun1-Dif1 interaction.

Yeast two-hybrid assay showing average β -galactosidase activity as a percentage of the wild-type (WT) Dun1-Dif1 interaction for alanine substitution mutants of Dun1 at conserved residues H77 and C64 according to FHA domain pHMMs. Dun1 constructs were used as prey with Dif1 or an empty vector as bait. Values indicate average percentage with above error bars for standard deviation. Asterisks represent the degree of statistical significance relative to the WT, <0.01 (**) and <0.001 (****), $n = 3$.

Given the evolutionary relationship between Rad53, Dun1, Mek1, and Chk2 and the consensual pattern of the lateral surfaces, the question of whether key residues of their lateral surfaces would be positionally conserved if their FHA domain models were aligned arose (Fig. 3.4). Previous data has shown that several residues on the distal lateral surface of the Rad53 FHA1 domain (Matthews et al., 2014) and the Dun1 FHA domain (Chapter 2) contribute to protein-protein interactions involved in the DNA damage and cell cycle checkpoint responses in budding yeast. Research done in collaboration with the Hollingsworth lab in Stony Brook, NY has shown that residues on the proximal lateral surface of the Mek1 FHA domain are involved in mediating the interaction between Mek1 and the Ndt80 transcription factor as well as the Rrm3 helicase for the regulation of the meiotic recombination checkpoint in budding

yeast (Chapter 4, unpublished data). Given the homology of human Chk2 with Rad53, Dun1, and Mek1, we decided to consider the location of these conserved residues of Rad53, Dun1 and Mek1 in relation to the I157 residue within the hydrophobic patch of the proximal lateral surface of the Chk2 FHA domain (Li et al., 2002). Looking at the structural alignment of the Rad53 FHA1, Dun1, Mek1 and Chk2 FHA domains, the alignment of the pThr-epitope binding sites is clear on the apical surface (Fig. 3.4A). The positional conservation of the identified residues contributing to the Rad53-Dbf4 interaction (Matthews et al., 2014, Almawi et al., 2016) and those for the Dun1-Sml1 interaction (Chapter 2) can be seen on the distal lateral surface (Fig. 3.4B).

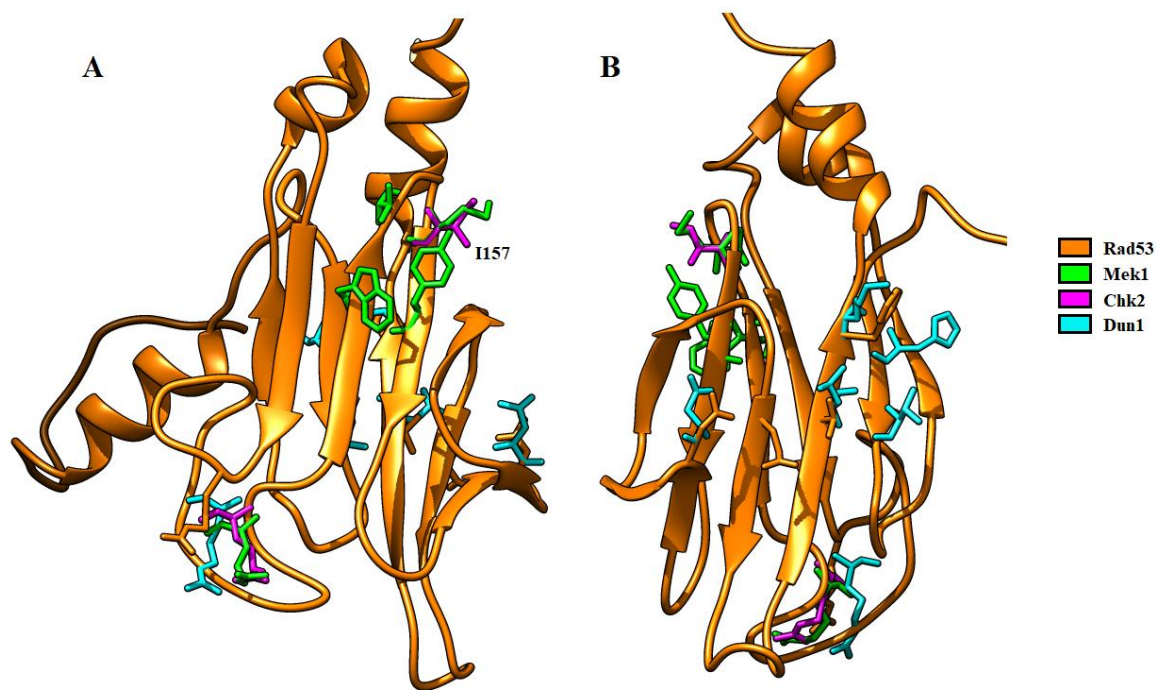


Figure 3.4 Structural alignment of yeast Rad53, Dun1, and Mek1 FHA domains and human Chk2 FHA domain show positional conservation of key residues.

Protein models of the Rad53 FHA1, Dun1, Mek1 and human Chk2 FHA domains were aligned using UCSF Chimera to illustrate the spatial conservation of physiologically relevant residues located at the pThr-epitope binding site and lateral surfaces of each domain. Amino acid side chains for residues that have been identified as contributing to important protein-protein or protein-ligand interactions in yeast are shown for the pThr-epitope binding site and proximal lateral surface (A) and the distal lateral surface (B). (A) The I157 residue implicated in Li-Fraumeni syndrome in humans is shown on the proximal lateral surface.

Interestingly, the residues on the proximal lateral surface of the Mek1 FHA domain, which when mutated to alanine resulted in the reduction or complete abrogation of the Mek1-Ndt80 interaction (Chapter 4), are found in the same region as the I157 residue of the Chk2 FHA domain. Taken together, these results demonstrate the relevancy of non-canonical FHA domain-based interactions to other interactions besides the Rad53-Dbf4 interaction, showing conservation in a human homolog which has already been implicated in human pathology (Li et al., 2002).

3.4.3 Linker region between TopBP1 BRCT7 and BRCT8 could be part of an H-BRCT domain as seen in yeast Dbf4 and Sir4

The discovery of the Dbf4 H-BRCT domain structure in conjunction with the observation that it was interacting with the distal lateral surface of the Rad53 FHA1 domain in a phospho-independent manner (Matthews et al., 2014) led to the question of whether the uniqueness of this non-canonical interaction was a consequence of the H-BRCT domain structure and whether other H-BRCT domains could be identified based on sequential or structural features.



Figure 3.5 HMMER-derived H-BRCT pHMM shows highly conserved tryptophan.

HMMER alignment profile shows positional residue conservation amongst Dbf4 H-BRCT homologs. The multiple sequence alignment generated using the amino acid sequences of selected sequences was used to search the reference proteomes database in an hmmsearch to generate the profile-HMM sequence logo.

The sequence of the Dbf4 H-BRCT domain was used in an HHPred search to identify homologous protein sequences. The resulting sequences (Appendix B Table 1) were used to generate a MSA that was then input into the hmmsearch tool to create a pHMM sequence logo (Fig. 3.5). One of the sequences identified in the search was that of Sir4, which is known to have an H-BRCT domain (Deshpande et al.,

2019). The identification of the Sir4 H-BRCT domain sequence confirmed the success of the bioinformatics tools used to search for additional H-BRCT domains.

In total, eight proteins in budding yeast and humans were identified to have candidate H-BRCT domain sequences according to the pHMM (Appendix B Table 1). Alignments for each hit sequence compared to the Dbf4 H-BRCT domain were visualized using the online alignment viewer to look for any features that could distinguish between BRCT and H-BRCT-like domains. There appeared to be a segment of the peptide sequence upstream of what appeared to be the corresponding peptide sequence for the core BRCT domain. Using the Dbf4 H-BRCT domain as a template, BRCT domain models were generated for hit peptide sequences with an alignment that resembled the Dbf4 and Sir4 H-BRCT domains in terms of the potential α -helical segment. Those BRCT domain models were aligned using USCF chimera to look for the positioning of the α -helix relative to that of the Dbf4 and Sir4 H-BRCT domains (Fig. 3.6).

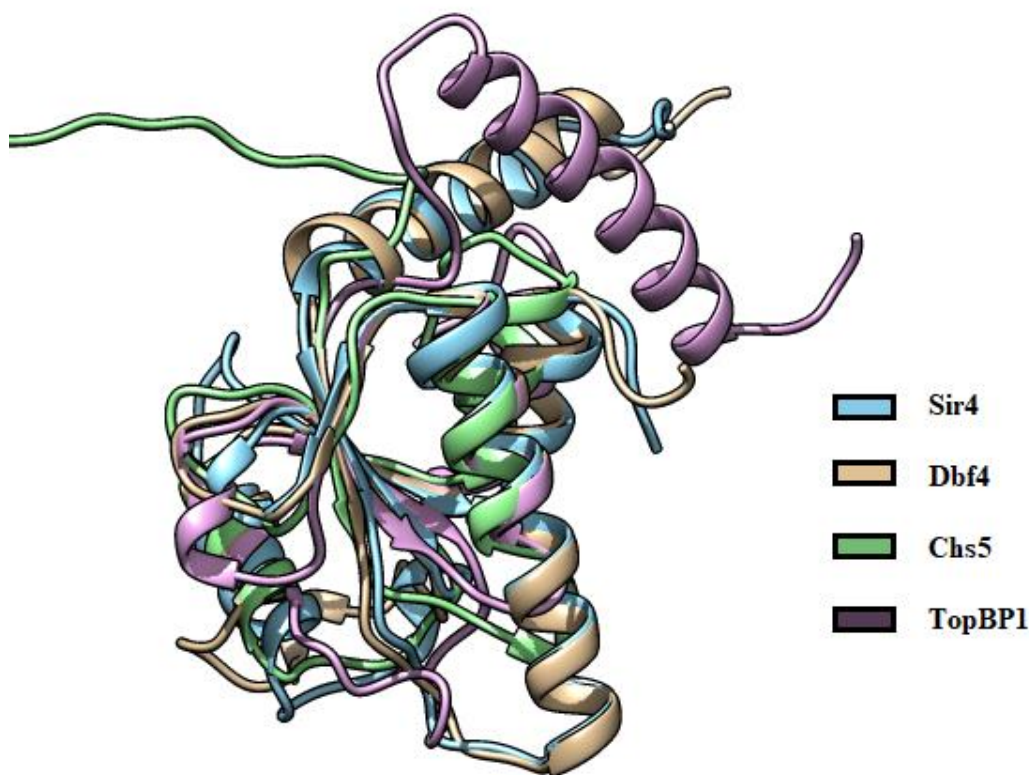


Figure 3.6 Structural alignment of H-BRCT domains with yeast Chs5 and human TopBP1 show possible α -helix on TopBP1 BRCT domain.

Domain models for the H-BRCT domains of Dbf4 and Sir4 were aligned with models generated for yeast Chs5 and human TopBP1 candidate H-BRCT domain sequences. Structural alignment shows the positioning of the α -helical segment of the Dbf4 and Sir4 H-BRCT domains to distinguish between which, if any, of the candidate sequences could possess an H-BRCT domain structure.

According to the structural alignment of the Dbf4, Sir4, Chs5 and TopBP1 domains, only TopBP1 appeared to have a segment that could be an α -helix for an H-BRCT domain as the Chs5 BRCT domain model did not show a clear α -helical structure in the region of the α -helix seen for Dbf4 and Sir4 (Fig. 3.6). Unfortunately, the alignment of the α -helix in the TopBP1 model does not align with those of Dbf4 and Sir4. As is common of BRCT domain-containing proteins, TopBP1 features tandem BRCT domains (reviewed in Mesquita et al., 2010). As a result of the tandem BRCT7/8 domains in the region of TopBP1 modelled, the additional α -helix seen in the structural alignment could merely represent the linker region between BRCT7 and BRCT8 in TopBP1 (Leung et al., 2011). However, this linker region in

TopBP1 BRCT7/8 is unusually longer than any other linker region seen in other tandem BRCT domains involved in phospho-peptide binding (Leung et al., 2011). This suggests that Dbf4 and Sir4 might be the only two isolated cases of single H-BRCT domain-containing proteins, however, other proteins that could be identified as possessing an H-BRCT domain structure might have an extended α -helix linker region between the tandem BRCT domains (Leung et al., 2011). Whether there is any evolutionary significance to the single H-BRCT domain structure in budding yeast Dbf4 and Sir4 and tandem BRCT domains and extended α -helix linker regions has yet to be determined.

3.5 Conclusions

The investigation and characterization of the lateral surface regions of all budding yeast FHA domains is necessary for a full understanding of the protein-protein interactions mediated by FHA domain lateral surfaces. The collection of FHA domains in budding yeast produces a distinct pHMM that emphasizes the highly conserved glycine and arginine residues of the pThr-epitope binding site and a highly conserved SxxH motif (Fig. 3.1). However, when looking at just the FHA domain proteins of budding yeast Rad53, Dun1 and Mek1 paralogs and the human homolog Chk2, the profile instead showed highly conserved cysteine and proline residues (Fig. 3.2). When the histidine or cysteine residues of the Dun1 FHA domain were mutated to alanine, the interaction between Dun1 and Dif1 was reduced by more than 50 % of the wild-type interaction and about 50% of the wild-type interaction, respectively (Fig. 3.3).

Looking at the conservation mapping of budding yeast FHA domain models, most FHA domains possess a collection of conserved residues on both proximal and distal lateral surfaces. However, the conservation of the Dun1 FHA domain was localized to the distal lateral surface whereas conservation of the Mek1 FHA domain was localized at the proximal lateral surface (Appendix B Figures 2 and 3). We found it interesting that the Rad53, Dun1, and Mek1 paralogs in budding yeast had overlapping conservation in regions that align with conservation on the distal and proximal lateral surfaces of the human Chk2 FHA domain (Fig. 3.4). The residues of the Rad53 FHA1 domain which were involved in interaction with Dbf4 were in the same region of the distal lateral surface as the residues of the Dun1 FHA domain that interact with Sml1 (Fig. 3.4). The residues of Mek1 on the proximal lateral surface aligned with those on the proximal lateral surface of the Chk2 FHA domain, suggesting a possible relational conservation of those regions for checkpoint responses in mitotically active cells versus meiotically active cells in budding yeast.

HHPred and HMMER analyses of H-BRCT domains, looking for the potential existence of more H-BRCT domains in budding yeast and humans, did not identify other H-BRCT domains like that of Dbf4 and Sir4. A close candidate was found in the human TopBP1 BRCT7/BRCT8 region where the extended linker region between the two BRCT domains appears to be positioned in the region of the additional α -helix which distinguishes the Dbf4 and Sir4 H-BRCT domains from typical BRCT domains (Leung et al., 2011). However, the linker region is common amongst tandem BRCT domain, so it is unclear whether the structure can be classified as an H-BRCT domain versus just two tandem BRCT domains with an unusually long linker region. We did not have adequate evidence to prove that there are no other H-BRCT domains in existence. However, we were unable to identify any others.

Together, the analysis of the FHA and H-BRCT domain structures adds to the growing collection of evidence that the non-canonical nature of the Rad53 FHA1 and Dbf4 H-BRCT domain interaction was not a consequence of the H-BRCT structure or unique to the Rad53-Dbf4 interaction. FHA domains themselves can interact with binding partners in a variety of ways beyond just recognition at the pThr-epitope binding site (reviewed in Almawi et al., 2017). Our analysis of FHA domains shows that regions of conservation can exist on the distal and/or proximal lateral surfaces and can be involved in mediating protein-protein and protein-ligand interactions in budding yeast as well as humans. The summary of the bioinformatics analysis allows forms a starting point for further in-depth analyses of the interactions mediated by FHA domains by providing candidate residues for mutagenesis of the pThr-epitope binding site and lateral surfaces for the characterization of interactions and the effects of interaction disruption or abrogation, aiding in the expansion of research perspectives concerning FHA domain-mediated interactions.

Chapter 4

Mek1 FHA domain utilizes non-canonical ligand binding of Ndt80 to regulate the meiotic recombination checkpoint.

4.1 Summary

Mec1/Tel1 phosphorylation of Hop1-T318 leads to the recruitment and activation of the meiotic recombination checkpoint kinase, Mek1. The Ndt80 transcriptional activator is a downstream target of Mek1 kinase and promotes the expression of genes necessary for the dissolution of the synaptonemal complex and release of cells from meiotic recombination checkpoint arrest. Mek1 is known to interact with and phosphorylate Ndt80, inhibiting Ndt80 activity such that cells are held in meiotic arrest whilst Mek1 function promotes the repair of DNA double strand breaks (DSBs) using the homologous chromosome instead of the neighbouring sister chromatid. In contrast to the Mek1-Hop1 interaction that requires R51 of the Mek1 FHA domain, the Mek1-Ndt80 interaction does not require this pThr-binding site. Here we show that the RPSKR motif of the Ndt80 “bypass checkpoint” or “bc” domain required for Ndt80 to interact with Mek1 associates with a region of the proximal lateral surface of the Mek1 FHA domain. Several aspartic and glutamic acid residues: D78, E79, and D80, on an alpha helical loop connecting beta strands of the proximal lateral surface specifically mediate the Mek1-Ndt80 interaction. Mutation of highly conserved beta strand residues W73, V75, M84, and Y86 disrupts the Mek1-Ndt80 interaction, but leads to a mutant version of Mek1 that appears to lack kinase activity. We propose that the Mek1 FHA domain, like the Rad53 FHA1 and Dun1 FHA domains, utilizes its lateral surface to mediate protein-protein interactions independent of the pThr-epitope binding site, in this case involved in the meiotic recombination checkpoint.

4.2 Introduction

The timely and efficient repair of DNA double strand breaks (DSBs) is essential to maintaining genomic integrity. Whether DSBs are generated due to endogenous or exogenous causes, cells have a host of DNA damage repair and checkpoint response pathways to resolve programmed or spontaneous DSBs. Spo11-induced DSBs at recombination hotspots initiate recombination events (reviewed in Keeney et al., 2014), with the mitotic recombinase, Rad51, and the meiosis-specific recombinase, Dmc1, binding to the 3' single stranded ends of the 5' end-resected DSBs (Hunter, 2007; Brown et al., 2015). The “strand invasion” of a homologous sequence on a non-sister chromatid leads to the resolution of the double Holliday junction as either a crossover or non-crossover event (Hollingsworth and Gaglione, 2019). Every pair of homologs requires at least one crossover for species survival. To ensure adequate crossover amongst homologous chromosomes, a surplus of DSBs is generated throughout the genome. In budding yeast, approximately 160 DSBs are triggered throughout the genome (reviewed in Keeney et al., 2014) with the meiotic recombination checkpoint pathway promoting inter-homolog repair instead of sister chromatid-based repair.

The 3' end of a resected DSB will either use its sister chromatid or one of the two chromatids of the homologous chromosome as a repair template. The meiosis specific Rad53 paralog, Mek1 functions to encourage the 3' end to invade the homologous chromosome as only crossover events between homologous chromosomes can connect chromosomes (Lao and Hunter, 2010). Without Mek1 kinase activity, DSBs are repaired using the sister chromatid instead of the homologous chromosome (Niu et al., 2005; Kim et al., 2010; Goldfarb and Lichten, 2010). Since the homologs are not connected during DSB repair with the sister chromatid, chromosomal segregation is impacted leading to aneuploidy and the formation of inviable spores following completion of meiosis (Rockmill and Roeder, 1991; Leem and Ogawa, 1992). Mek1 kinase activity prevents Rad51-mediated strand invasion at the sister chromatid via the phosphorylation of T132 on the Rad51 accessory factor protein, Rad54. Rad54 phosphorylation reduces the affinity of Rad54 towards Rad51, preventing the Rad54-Rad51 association (Petukhova et al., 1999; Niu et al., 2009). Mek1 also phosphorylates and stabilizes the meiosis-specific Hed1 protein such that it binds to Rad51 also preventing the Rad51-Rad54 association needed for strand invasion at the sister chromatid (Tsubouchi and Roeder, 2006; Busygina et al., 2008; Callendar et al., 2016). The blockage of Rad51 recombinase activity allows for interhomolog bias at DSBs such that they are then predominantly repaired via the Dmc1 meiosis-specific recombinase (Hong et al., 2013; Lao et al., 2013; Lu et al., 2014).

A downstream target of Mek1 is the Ndt80 transcription factor. Ndt80 binds to the promoters of more than 300 target genes to activate their transcription during the meiotic recombination checkpoint as well as its own promoter region (Hepworth et al., 1995; Ozsarac et al., 1997; Chu and Herskowitz, 1998; Hollingsworth and Gaglione, 2019). One such gene is that of the Cdc5 cyclin which, alongside DDK, is responsible for triggering the resolution of the Holliday junction for DSB repair and the dissolution of the synaptonemal complex (SC) via the degradation of a member of the axial element of the SC, Red1 (Sourirajan and Lichten, 2008; Okaz et al., 2012; Argunhan et al., 2017; Prugar et al., 2017; reviewed in Tsubouchi et al., 2018). Within the middle region of Ndt80, a 57 amino acid domain was discovered to be required for meiotic recombination checkpoint delay/arrest in mutant cells that were unable to progress through the meiotic recombination checkpoint due to unrepaired DSBs (Wang et al., 2011). The deletion of this 57-amino acid region, referred to as the “bc” or “bypass checkpoint” domain, resulted in a version of the Ndt80 transcriptional activator that no longer responded to the meiotic recombination checkpoint (Wang et al., 2011). The ability of Ndt80 without the region of the “bc” domain to allow cells to bypass the meiotic recombination checkpoint was found to be the result of the absence of the Mek1-Ndt80 interaction (Chen et al., 2018). Mek1 binds to a conserved RPSKR amino acid sequence in the “bc” domain of Ndt80 and phosphorylates multiple sites on the transcription factor, impeding Ndt80 activity and function (Chen et al., 2018). As a result, deletion of the RPSKR motif of Ndt80 prevents Mek1 from binding to, phosphorylating, and inactivating Ndt80 (Chen et al., 2018). Interestingly, mutation of the key Mek1 FHA domain pThr-epitope binding residue R51 does not affect the Mek1-Ndt80 interaction (Chen et al., 2018).

Here we show that the Mek1 interaction with the RPSKR motif of the Ndt80 “bc” domain requires the D78, E79, and D80 residues located on an alpha helical loop connecting two beta strands of the proximal lateral surface of the Mek1 FHA domain. Conserved residues of the proximal lateral surface beta strands are required for Mek1 kinase activity as well as the Mek1-Ndt80 interaction, whereas the D78, E79, and D80 residues selectively mediate the Mek1-Ndt80 interaction without impacting Mek1 kinase activity. Loss of the Mek1-Ndt80 interaction but not Mek1 kinase activity still permits cells to bypass the meiotic recombination checkpoint while maintaining a small degree of interhomolog-based DSB repair. Mutation of the Mek1 proximal lateral surface that disrupts the Mek1-Ndt80 interaction and Mek1 kinase activity results in cells that can bypass the meiotic recombination checkpoint, but their repair of DSBs mimics the *mek1* null mutant.

4.3 Methods

4.3.1 Yeast Strains

All strains used for tetrad dissection and sporulation assays were derived from the *SK1* background and are listed below (Table 4.1). The yeast integrating plasmid pLP37 (pRS316) containing the coding sequence of Mek1 was provided by Nancy Hollingsworth (Stony Brook University) and used to generate diploid yeast strains for sporulation assays and tetrad dissections to assess spore viability for genomic *mek1* mutants as described in Chen et al. (2018) (Table 4.1). Site-directed mutagenesis (described below) was used to generate *mek1* mutant sequences in the pLP37 plasmid. Lithium acetate yeast transformation (described below) was used to introduce pLP37 plasmid constructs for integration into the genome of haploid yeast cells derived from the DY-405 diploid yeast strain to create *mek1* mutant strains in the *dmc1Δ* background. Haploid strains successfully carrying the integrated plasmids were then mated to generate diploid strains carrying mutations for the proximal lateral surface of the Mek1 FHA domain (DY-409 – DY-411) or the pThr-epitope binding site R51 residue (DY-412). The wild-type sequence of MEK1 was also integrated into DY-405 haploids for functional complementation of the *mek1Δ* deletion mutation. For analysis of *mek1* mutations in the context of the *DMC1* wild-type background, Lihong Wan (Stony Brook University) introduced pLP37 constructs into the NH2740 yeast strain for genomic integration.

Table 4.1 List of Yeast Strains

Strain	Genotype	Source
DY-1	<i>MATa, ade2-1, can1-100, trp1-1, his3-11, ura3-1, leu2-3, leu2-112, pep4::LEU2</i>	(Duncker et al., 2002)
DY-403	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) ho::hisG ura3(deltaSma-Pst)/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) ho::hisG ura3(deltaSma-Pst)</i>	N. Hollingsworth
DY-404	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	N. Hollingsworth
DY-405	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) mek1delta::natMX4 ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) mek1delta::natMX4 ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	N. Hollingsworth
DY-408	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) mek1delta::natMX4::MEK1 ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) mek1delta::natMX4::MEK1 ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	This study.
DY-409	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) mek1delta::natMX4::mek1MY ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) mek1delta::natMX4::mek1MY ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	This study.
DY-410	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) mek1delta::natMX4::mek1WV ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) mek1delta::natMX4::mek1WV ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	This study.
DY-411	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) mek1delta::natMX4::mek1L109A ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) mek1delta::natMX4::mek1L109A ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	This study.
DY-412	<i>MATa leu2::hisG HIS4::LEU2-(Bam+ ori) mek1delta::natMX4::mek1R51A ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6/MATalpha leu2::hisG his4-x::LEU2-(NgoMIV + ori) mek1delta::natMX4::mek1R51A ho::hisG ura3(deltaSma-Pst) dmc1delta-2E::kanMX6</i>	This study.
NH2740	<i>mek1Δ CEN8::CFP::LEU2/CEN8::CFP::TRP1</i>	N. Hollingsworth

4.3.2 Genomic DNA Isolation

Genomic DNA was isolated from the DY-1 yeast strain for use as template DNA in PCR reactions contributing to plasmid construction as well as from yeast strains for use as template DNA in PCR reactions to confirm the successful integration of *MEK1* or *mek1* mutant gene sequences. A working culture for each strain, inoculated from a saturated preculture, was grown to a concentration of $\sim 1 \times 10^7$ cells/mL in 10 mL of YPD (1% yeast extract, 2% peptone, and 2% dextrose) medium at 30°C. Cells were centrifuged at 4000 rotations-per-minute (rpm) for 5 minutes (Thermo Scientific centrifuge CL2), resuspended in 500 μ L of sterile water and transferred to a sterile 2 mL screw-cap tube. The cells were then centrifuged for 10 seconds at maximum speed (Eppendorf 5424 R centrifuge), the supernatant decanted, and then vortexed to loosen the pellet. 200 μ L of ‘genomic prep mix’ (2% Triton X-100, 1%

SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8, and 1 mM EDTA) was added to the loosened pellet, followed by 200 μ L of phenol:chloroform:isoamylalcohol (25:24:1) and 0.5 g of 0.5 mm glass beads. All components were mixed for 3-4 minutes using a vortex and then 200 μ L of 1X TE pH 8 was added and mixed. Following a 5-minute centrifugation at maximum speed (Eppendorf 5424 R centrifuge), the top layer was transferred to a new sterile 1.5 mL Eppendorf tube with 1 mL of 100% ethanol and mixed by inversion. After another centrifugation for 2 minutes at maximum speed (Eppendorf 5424 R centrifuge), the pellet was completely re-suspended in 0.4 mL of 1X TE pH 8 to which 10 μ L of 10 mg/mL RNase A (Sigma) was added before incubating at 37°C for 10 minutes. After incubation, 10 μ L of 4 M ammonium acetate and 1 mL of 100% ethanol were added and mixed by inversion. After a 2-minute centrifugation, the supernatant was discarded, and the pellet left to air dry before completely re-suspending the pellet in 50 μ L of 1X TE pH 8. Genomic DNA was stored at -20°C.

4.3.3 Bioinformatics

The Mek1 amino acid sequence was submitted to the IUPred and PSIPRED online servers for the prediction of disordered regions and protein secondary structure, respectively, to confirm the complete FHA domain boundaries (<https://iupred2a.elte.hu/>; <http://bioinf.cs.ucl.ac.uk/psipred/>). The peptide sequence corresponding to the FHA domain was entered as the query to search the non-redundant protein sequences (nr) database using the Position-Specific Iterated BLAST (PSI-BLAST) algorithm (NCBI) to establish a collection of homologs to assess amino acid conservation. Returned sequences exhibiting a percent identity of 25% or above to the query sequence were selected as homologs (Appendix C Table 1) and their sequence data exported for use in generating a multiple sequence alignment (MSA). Homolog sequence data was uploaded to the online Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool (EMBL-EBI) and the resulting MSA file was viewed using the free Jalview software (www.jalview.org). The ConSurf server was used to map amino acid conservation to the protein model using SWISS-MODEL (<https://swissmodel.expasy.org/>). The free UCSF Chimera protein model analysis software (<https://www.cgl.ucsf.edu/chimera/download.html>) was used to view and process protein models to emphasize features of interest.

4.3.4 Plasmid Construction

Table 4.2 lists plasmids constructed and used in this study. The protein coding sequences for *NDT80* and *MEK1* were amplified from DY-1 genomic DNA and cloned into pJG4-6 and pEG202, prey and bait vectors for yeast two-hybrid assays completed in the Duncker Lab, respectively (Duncker et al.,

2002; Ausubel et al., 1994; Gyuris et al., 1993). Coding sequences were amplified using a commercially available High-Fidelity PCR kit (Roche). PCR products were purified using a commercial PCR clean up kit that was also used for the clean-up of restriction enzyme digested PCR products and vectors (Geneaid). All restriction enzymes were purchased from Thermo Fisher Scientific, and their recognition sequences were incorporated into the design of the primers used for PCR amplification. The coding sequence of Ndt80 without the DNA-binding domain (amino acids 287-627), according to Chen et al., 2018, was PCR amplified, purified, and the products were then digested using *ApaI* and *XhoI* restriction enzymes for the construction of the pJG4-6 *Ndt80-DBD* plasmid (Table 4.2). Ligation was carried out using a T4 DNA ligase kit (BioBasic), introducing the Ndt80 sequence into the pJG4-6 vector, and the ligated product was transformed into calcium-chloride competent DH5 α *Escherichia coli* (*E. coli*) cells grown and plated on LB media (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Colony PCR was performed to confirm transformation of successfully ligated constructs using a commercial colony PCR kit (New England Biolabs). Primer pairs were designed such that one primer targeted the vector sequence and the other targeted the sequence of the insert. Plasmid DNA was isolated from saturated overnight cultures of DH5 α for positive colonies using a commercial plasmid prep kit (Geneaid; <http://www.geneaid.com/sites/default/files/PD13.pdf>). Plasmid DNA was sent for sequencing of the full length insert at the Sick Kids Sequencing Facility (Toronto, Ontario), to ensure the fidelity of the cloned PCR product. The Mek1 coding sequence was cloned into pJG4-6 for the construction of the pJG4-6 *MEK1* plasmid using the *EcoRI* and *BglII* restriction enzymes for co-immunoprecipitation (described below). The full-length coding sequence of Red1 was PCR amplified, digested with *ApaI* and *BglIII* restriction enzymes and ligated into the pJG4-6 vector for the construction of the pJG4-6 *RED1* plasmid. Alanine substitution mutations were generated within pJG4-6 Mek1 constructs via site-directed mutagenesis (described below) for the construction of the pJG4-6 *mek1 MY*, pJG4-6 *mek1 WV*, pJG4-6 *mek1 L109A*, and pJG4-6 *mek1 R51A* plasmids. *EcoRI* and *XhoI* digestion of pJG4-6 *MEK1* and all pJG4-6 *mek1* constructs were done and ligation of the Mek1 inserts into pEG202 allowed for the generation of the pEG202 *MEK1*, pEG202 *mek1 MY*, pEG202 *mek1 WV*, pEG202 *mek1 L109A*, and pEG202 *mek1 R51A* constructs for yeast two-hybrid analyses. Site-directed mutagenesis of the pLP37 construct provided by Nancy Hollingsworth (Stony Brook) was used to generate the pLP37-*MY*, pLP37-*WV*, pLP37-*L109A*, and pLP37-*R51A* constructs. *LexA-MEK1* and GAD-fusions used for yeast two-hybrid screens and spotting assays were generated by Hollingsworth lab members as described in (Chen et al., 2018; Hollingsworth and Gaglione, 2019). The pTS3-AAA, pTS3-D78A E79A, pTS3-

D78A D80A, pTS3-E79A D80A, pLP37-AAA, pLP37-D78A E79A, pLP37-E79A D80A, pLP37-D78A D80A, pLP37-D78A, pLP37-E79A, and pLP37-D80A plasmids were made by site-directed mutagenesis of the pTS3 and pLP37 plasmids using the QuikChange II kit (Agilent) in the Hollingsworth Lab (Stony Brook). The entire gene from each plasmid was sequenced at the Stony Brook University DNA Sequencing facility to confirm the presence of the desired mutations. For the pCM190myc13 vector, 13 copies of the Myc coding sequence were cloned into pCM190 between the NotI (5') and PstI (3') sites by Aaron Donahue in the Duncker Lab introducing additional restriction enzyme recognition sequences for NheI and BglII within the multiple cloning site (MCS) directly after NotI.

Table 4.2 List of Plasmids

Plasmid	Features	Source
pTS3	2 μ , TRP1, ADH1p <i>lexA-MEK1</i> , ADE2, <i>ampR</i>	(Chen et al., 2018)
pTS3-AAA	2 μ , TRP1, ADH1p <i>lexA-mek1-D78A E79A D80A</i> , ADE2, <i>ampR</i>	Hollingsworth Lab
pACTII	2 μ <i>GAD LEU2</i>	(Durfee et al., 1993)
pXC13	2 μ , <i>GAD-NDT80²⁸⁴⁻⁶²⁷</i> , <i>LEU2 ampR</i>	(Chen et al., 2018)
pTS3-D78A E79A	2 μ , ADH1p <i>lexA-mek1-D78A E79A</i> , TRP1 ADE2, <i>ampR</i>	Hollingsworth Lab
pTS3-E79A D80A	2 μ , ADH1p <i>lexA-mek1-E79A D80A</i> , TRP1, ADE2, <i>ampR</i>	Hollingsworth Lab
pTS3-D78A D80A	2 μ , ADH1p <i>lexA-mek1-D78A D80A</i> , TRP1, ADE2, <i>ampR</i>	Hollingsworth Lab
pLP37	<i>CEN, MEK1, URA3, amp^R</i>	(de los Santos and Hollingsworth, 1999)
pLP37-AAA	<i>CEN, mek1-D78A E79A D80A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-D78A E79A	<i>CEN, mek1-D78A E79A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-D78A D80A	<i>CEN, mek1-D78A D80A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-D78A	<i>CEN, mek1-D78A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-D80A	<i>CEN, mek1-D80A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-E79A D80A	<i>CEN, mek1-E79A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-E79A	<i>CEN, mek1-E79A D80A, URA3, amp^R</i>	Hollingsworth Lab
pLP37-MY	<i>CEN, mek1-M84A Y86A, URA3, amp^R</i>	This study
pLP37-WV	<i>CEN, mek1-W73A V75A, URA3, amp^R</i>	This study
pLP37-L109A	<i>CEN, mek1-L109A, URA3, amp^R</i>	This study
pLP37-R51A	<i>CEN, mek1-R51A, URA3, amp^R</i>	This study
pEG202	2 μ , ADH1p <i>lexA, RAF_{part}</i> , <i>HIS3, ampR</i>	(Ausubel et al., 1994)
pEG202 MEK1	2 μ , ADH1p <i>lexA-MEK1, RAF_{part}</i> , <i>HIS3, ampR</i>	This study
pEG202 mek1 MY	2 μ , ADH1p <i>lexA-mek1-M84A Y86A, RAF_{part}</i> , <i>HIS3, ampR</i>	This study
pEG202 mek1 WV	2 μ , ADH1p <i>lexA-mek1-W73A V75A, RAF_{part}</i> , <i>HIS3, ampR</i>	This study
pEG202 mek1 L109A	2 μ , ADH1p <i>lexA-mek1-L109A, RAF_{part}</i> , <i>HIS3, ampR</i>	This study
pEG202 mek1 R51A	2 μ , ADH1p <i>lexA-mek1-R51A, RAF_{part}</i> , <i>HIS3, ampR</i>	This study
pJG4-6	2 μ , <i>GAL1p B42 AD-HA, TRP1, ampR</i>	(Longtine et al. 1998)
pJG4-6 Ndt80-DBD	2 μ , <i>GAL1p B42 AD-HA-Ndt80ΔDBD, TRP1, ampR</i>	This study
pJG4-6 MEK1	2 μ , <i>GAL1p B42 AD-HA-MEK1, TRP1, ampR</i>	This study
pJG4-6 mek1 MY	2 μ , <i>GAL1p B42 AD-HA-mek1-M84A Y86A, TRP1, ampR</i>	This study
pJG4-6 mek1 WV	2 μ , <i>GAL1p B42 AD-HA-mek1-W73A V75A, TRP1 ampR</i>	This study
pJG4-6 mek1 L109A	2 μ , <i>GAL1p B42 AD-HA-mek1-L109A, TRP1, ampR</i>	This study
pJG4-6 mek1 R51A	2 μ , <i>GAL1p B42 AD-HA-mek1-R51A, TRP1, ampR</i>	This study
pJG4-6 Red1	2 μ , <i>GAL1p B42 AD-HA-Red1, TRP1, ampR</i>	This study
pCM190	2 μ , <i>tTA, CMVp, TetO, CYC1p, URA3, ampR</i>	(Gari et al. 1997)
pCM190myc13	2 μ , <i>tTA, CMVp, TetO, CYC1p-myc13, URA3, ampR</i>	This study.
pCM190Dbf4myc13	2 μ , <i>tTA, CMVp, TetO, CYC1p-Dbf4-myc13, URA3, ampR</i>	(Ramer et al., 2013)
pCM190Cdc7myc13	2 μ , <i>tTA, CMVp, TetO, CYC1p-Cdc7-myc13, URA3, ampR</i>	(Ramer et al., 2013)

4.3.5 Site-directed Mutagenesis

Mutations within the *MEK1* coding sequence were generated using the QuikChange II XL Site-directed Mutagenesis kit (Agilent Technologies). Mutagenic primers were designed using the QuikChange Site-Directed Mutagenesis online interface (<https://www.genomics.agilent.com/primerDesignProgram.jsp>) and then utilized during PCR to remove or replace the desired nucleotide(s). PCR products were digested using DpnI restriction endonuclease to degrade parental copies of the plasmid containing the gene to be mutagenized and then the product was transformed into XL-10 Gold Ultracompetent cells (Agilent) and plated on Luria-Bertani (LB) agar (1% NaCl, 1% tryptone, 0.5% yeast extract, and 2.4% agar) containing the antibiotic ampicillin (BioShop). Plasmid DNA isolated from transformants was sent for sequencing of the gene at the Sick Kids Sequencing Facility to confirm successful mutagenesis. A full protocol for site-directed mutagenesis is available online: <http://www.agilent.com/cs/library/usermanuals/public/200523.pdf>.

4.3.6 Yeast Transformation

Two-hybrid bait and prey plasmid DNA was transformed into the DY-1 yeast strain, previously transformed with the pSH18-34 reporter plasmid, for assays completed in the Duncker Lab. A working culture was grown to a concentration of $\sim 1 \times 10^7$ cells/mL in 10 mL of Synthetic Complete (SC) media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, 1X amino acid mix) lacking uracil for the maintenance of the pSH18-34 reporter plasmid. Working cultures were centrifuged for 5 minutes at 4000 rpm (Thermo Scientific centrifuge CL2), resuspended in 1X Tris-EDTA (TE) solution pH 8.0, centrifuged again, resuspended in 200 μ L of lithium acetate/TE solution (100 mM lithium acetate, 2.5 mL 1X TE), and then held at room temperature for 10 minutes. Approximately 0.4 μ g of plasmid DNA was added to 100 μ L of the yeast suspension mix along with 100 μ g of salmon sperm DNA, and then 300 μ L of lithium acetate/TE/PEG4000 solution (100 mM lithium acetate, 2 g polyethylene glycol 4000, 0.5 mL 10X TE) was added. The solution was incubated at 30°C for 30 minutes before 40 μ L of 100% dimethyl sulfoxide (DMSO) was added. Cells were heat shocked at 42°C for 7 minutes, held on ice for 2 minutes and then plated on selective media and grown for 2-3 days.

4.3.7 Yeast two-hybrid Assay

β -galactosidase activity assays were performed as described previously for liquid yeast two-hybrid assays with Ndt80 as the prey construct and Mek1 as the bait (Duncker et al., 2002; Ausubel et al., 1994; Gyuris et al., 1993). The bait and prey vectors for yeast two-hybrid assays contain a selectable

marker for the biosynthesis of histidine (*HIS3*) and tryptophan (*TRP1*), respectively. The pSH18-34 reporter plasmid contains a selectable marker for the biosynthesis of uracil (*URA3*) (Ausubel et al., 1994; Gyuris et al., 1993). The yeast two-hybrid liquid and spotting assays shown in Figure 4.6 were conducted by the Hollingsworth lab as described previously (Chen et al., 2018).

4.3.8 Yeast Whole Cell Extract and Western Blotting

To verify appropriate protein expression for samples processed in the Duncker Lab, yeast whole cell extracts (WCE) were generated for Western blotting. A 20 mL of each yeast culture ($\sim 1.0 \times 10^7$ cells/mL) was centrifuged for 5 minutes at 4000 rpm to pellet cells (Thermo Scientific centrifuge CL2), which were then resuspended in 300 μ L of lysis buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 100 μ L of Fisher HALT! Protease inhibitor and 1 mM PMSF) and added to a 2 mL tube with 0.3 g of 0.5 mm glass beads before subjecting the samples to lysis at 4°C via the use of a Biospec Mini Bead-Beater for eight cycles of 30 seconds of agitation and 30 seconds of rest on ice. A total volume of 105 μ L of supernatant (whole cell extract) was collected following the centrifugation of lysed cells for 30 seconds at 13, 200 rpm at 4°C (Eppendorf 5415D centrifuge): 5 μ L for a Bradford Assay and 100 μ L for SDS-PAGE. A Bradford Assay was used to determine the protein concentrations of each sample (Bio-Rad). Protein extracts were mixed with loading buffer (60% 4X buffer [15% SDS, 40% glycerol, and 166 mM Tris base], 0.26 M DTT, 7% bromophenol blue) at a volume of one-half that of the extract and then boiled for 10 minutes before loading 50 μ g of protein onto SDS polyacrylamide gels (10 % resolving gel and 5% stacking gel). After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane using a wet transfer method (200 mM glycine, 25 mM Tris-base, 20 % methanol, 0.054% SDS for the transfer buffer). Membranes were pre-stained with 0.1% Ponceau S, imaged using a ChemiDoc MP Imaging System (BioRad), and then de-stained with 1X TEN + T (20 mM Tris-HCl, 1 mM EDTA, 0.14 NaCl, 0.05% Tween 20) before detection with antibodies (Table 2). Antibody incubations were performed for 1-2 hours after 1 hour blocking in 1X TEN + T with 5% skim milk powder. Three 10-minute washes were done using 1X TEN + T after each antibody exposure and images were taken with a ChemiDoc MP Imaging System (Bio-Rad). Immunoblots for Arp7, Mek1, Hed1 and pHed1 detection were conducted by Lihong Wan (Hollingsworth Lab) using the tri-chloroacetic acid method (TCA) (Falk et al., 2010) as described previously (Chen et al., 2018).

Table 2. List of Antibodies.

Primary	Dilution	Source	Secondary	Dilution	Source
Anti-HA (mouse monoclonal) – pJG4-6	0.75:5000	Sigma H3663	AlexaFluor 488 anti-mouse	1:3000	Thermo Fisher A32723
Anti-LexA (rabbit polyclonal) – pEG202	1:5000	Thermo Fisher PA1-4966	AlexaFluor 647 anti-rabbit	1:3000	Thermo Fisher A32733
Anti-myc13	1:5000	Sigma M5546	AlexaFluor 488 anti-mouse	1:3000	Thermo Fisher A32723
α -Arp7	1:50000	Santa Cruz sc-8961	α -Goat	1:15000	Santa Cruz sc-2354
α -GST-Mek1	1:20000	N. M. Hollingsworth	α -Guinea Pig	1:10000	Santa Cruz sc-2903
α -Hed1	1:50000	Patrick Sung	α -Rabbit	1:10000	Santa Cruz sc-2357
α -phospho Hed1-T40	1:20000	N. M. Hollingsworth	α -Rabbit	1:20000	Santa Cruz sc-2357

4.3.9 Sporulation Assay and Tetrad Dissection

To assess spore formation and viability in the Duncker Lab, sporulation assays and tetrad dissections were conducted for yeast strains harbouring mutations in *MEK1*. Saturated cultures of diploid strains were incubated overnight at 30°C in 10 mL of YPD (1% yeast extract, 2% peptone, and 2% dextrose). After incubation, 1 mL of saturated culture was transferred to 10 mL of pre-warmed pre-sporulation media (0.5% yeast extract, 1% peptone, 1% potassium acetate, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.05 M potassium hydrogen phthalate, 20 μ L/L polypropylene glycol 2000, pH 5.5) and incubated for 24 hrs at 30°C. A 1.5 mL aliquot of “pre-sporulated” culture was centrifuged at maximum speed for 1 minute (Eppendorf 5424 R centrifuge), the supernatant was discarded and the pellet washed with 1 mL sterile water, then resuspended in 2.5 mL of pre-warmed sporulation media (1% potassium acetate, 0.02% raffinose, 20 μ L/L polypropylene glycol 2000, pH 7) and incubated for 24 hrs at 30°C. After 24 hours, 1 mL of sporulated culture was centrifuged at maximum speed for 1 minute (Eppendorf 5424 R centrifuge), the supernatant discarded, and the cells fixed in 70% ethanol. For counting sporulated cells, 10 μ L of fixed cells were mounted on microscope slides and imaged for counting. Five fields of view with an average of 200 cells were counted for each strain. Sporulation

assays conducted by the Hollingsworth lab utilized solid sporulation media as described in (Chen et al., 2018; Hollingsworth and Gaglione, 2019).

For spore viability, 500 μ L of sporulated culture was spun down at maximum speed for 1 minute (Eppendorf 5424 R centrifuge) in a 1.5 mL centrifuge tube and the supernatant discarded. Cells were washed in 1 mL of sterile water and then resuspended in 50 μ L of zymolyase solution (0.5 mg/mL in 1M sorbitol) and incubated for 8-10 minutes at 30°C. After incubation, 800 μ L of sterile water was slowly added to the tube and held on ice for 5 minutes until tetrads settled on the bottom of the tube. The water was then carefully removed with a pipette and discarded. Tetrads were streaked onto the end of a dry YPD plate with a flamed loop and separated using a MSM micromanipulator (Singer Instruments).

4.3.10 Co-immunoprecipitation

Co-immunoprecipitation was used to investigate whether Mek1 or the synaptonemal complex protein, Red1, interact with components of Dbf4-dependent kinase (DDK). DY-1 cells were transformed with pJG4-6 constructs, containing the gene sequences of Mek1 or Red1, and pCM190, containing the gene sequences of Dbf4 or Cdc7 and saturated cultures were grown in 10 mL of Synthetic Complete (SC) media (0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 2 % glucose, 1X amino acid mix lacking uracil and tryptophan) supplemented with tetracycline (5 μ g/mL) for two days at 30°C and 250 rpm (Varrin et al. 2005). Working cultures were grown to a density of 1×10^7 cells/mL in the same synthetic complete media for 17 hours and then centrifuged at 4000 rpm for 3 minutes (Thermo Scientific centrifuge CL2) and washed with sterile water, resuspended in Gal/Raff media (2% galactose, 1% raffinose, 1X amino acid mix lacking uracil and tryptophan), and then incubated for 6 hours at 30°C. The day before induction, 40 μ L of sheep anti-mouse IgG Dynabeads (Thermo Fisher) were added to 1.5 mL centrifuge tubes, washed 5 times with 200 μ L of 1X PBS + BSA (0.05%), resuspended in 40 μ L of 1X PBS + BSA (0.05%) with 1.2 μ L of mouse anti-Myc IgG (Sigma) per tube. Tubes were incubated overnight on a rotating wheel at 4°C.

During Gal/Raf induction the prepped beads were washed twice with 500 μ L of 1X PBS + BSA (0.05%) and then resuspended in 40 μ L 1X PBS for the immunoprecipitated samples. Once the induction period was over, cultures were centrifuged for 5 minutes at 4000 rpm (Thermo Scientific centrifuge CL2), washed with 40 mL sterile water, and then resuspended in 320 μ L of ice-cold lysis buffer (50 mM HEPES-KOH, pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 3% protease inhibitor, 1% PMSF). Cells were lysed at 4°C via the use of a Biospec Mini Bead-Beater for

eight cycles of 30 seconds of agitation and 30 seconds of rest on ice and then 200 μ L of lysate was transferred to the tubes with the magnetic beads for the immunoprecipitated samples. The remaining lysate was used for protein quantification via Bradford assay and as the protein samples for the input or whole cell extract. IP samples were incubated on the rotating platform for 45 minutes at 4°C. Beads were then washed twice with 400 μ L of lysis buffer and then three times with wash buffer (100 mM Tris-HCl, pH 8, 250 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 3% protease inhibitor). Whole cell extract for the input protein samples were mixed with loading buffer (60% 4X buffer [15% SDS, 40% glycerol, and 166 mM Tris base], 0.26 M DTT, 7% bromophenol blue) at a volume of one-half that of the extract while the immunoprecipitated (IP) samples were resuspended in 40 μ L of loading buffer and then all samples were boiled for 10 minutes before loading 50 μ g of protein onto SDS polyacrylamide gels (10 % resolving gel and 5% stacking gel). After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane using a wet transfer method (200 mM glycine, 25 mM Tris-base, 20 % methanol, 0.054% SDS for the transfer buffer). Membranes were pre-stained with 0.1% Ponceau S, imaged using a ChemiDoc MP Imaging System (BioRad), and then de-stained with 1X TEN + T (20 mM Tris-HCl, 1 mM EDTA, 0.14 NaCl, 0.05% Tween 20) before detection with antibodies (Table 2). Antibody incubations were performed for 1-2 hours after 1 hour blocking in 1X TEN + T with 5% skim milk powder. Three 10-minute washes were done using 1X TEN + T after each antibody exposure and images were taken with a ChemiDoc MP Imaging System (Bio-Rad).

4.4 Results

4.4.1 The Mek1 FHA domain proximal lateral surface contributes to the Mek1-Ndt80 interaction in budding yeast meiotic recombination.

Alanine substitution of the pThr-epitope binding site residue (R51) did not disrupt the Mek1-Ndt80 interaction (Chen et al., 2018). This led to the conclusion that the Mek1 FHA must not mediate Ndt80 binding (Wan et al., 2004; Durocher et al., 2000). We questioned whether the lack of disruption of the Mek1-Ndt80 interaction with the mutation of the pThr-epitope binding site was sufficient evidence to rule out a role for the FHA domain in mediating the Mek1-Ndt80 interaction, given the accounts of FHA domain-mediated interactions that utilize alternative interfaces beyond the pThr-binding site (Matthews et al., 2014; reviewed in Almawi et al., 2017). To investigate whether the Mek1 FHA domain lateral surface(s) could be involved in mediating the Mek1-Ndt80 interaction such as was observed with Rad53 (Matthews et al., 2014) and Dun1 (Chapter 2), we utilized a collection of bioinformatics tools to identify any conserved lateral surface residues on the Mek1 FHA domain.

Starting with an analysis of Mek1 homologs using PSI-BLAST, we looked at the sequence conservation of Mek1 and mapped it to the model of the Mek1 FHA domain using the Consurf server (Figure 4.1). Sequence conservation mapping of Mek1 homologs identified a region of highly conserved residues on the proximal lateral surface of the Mek1 FHA domain (Figure 4.1A). As a result, we questioned whether this conserved lateral surface could be involved in mediating a non-canonical interaction between the Mek1 FHA and Ndt80 “bc” domains. Looking at the residues of the proximal lateral surface, five of the highly conserved residues appeared to project into the external lateral surface space (Figure 4.1B). Structural alignment of the Mek1 FHA domain and the human Chk2 FHA domain placed the identified residues of the Mek1 FHA domain lateral surface in the same region as the Chk2 I157 residue which when mutated contributes to Li-Fraumeni syndrome (Chapter 3; Li et al., 2002). These residues were then selected as candidates for mediating the Mek1-Ndt80 interaction.

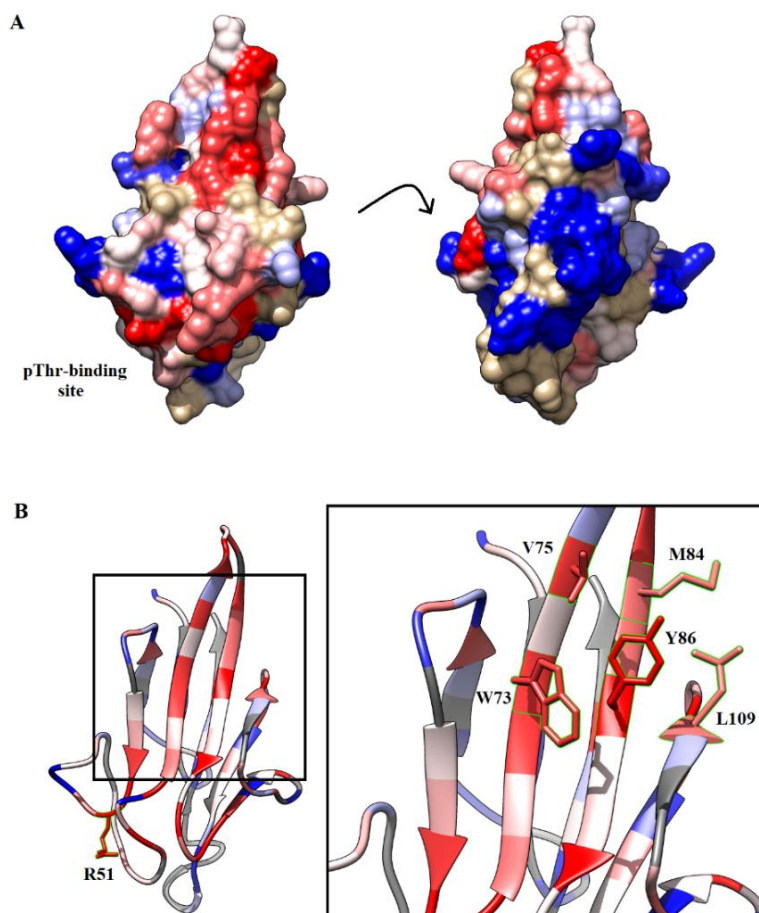


Figure 4.1 Mek1 structure shows highly conserved residues on proximal lateral surface of FHA domain.

(A) Space filling model of the Mek1 FHA domain (PBD template ID: 5YYX) showing sequence conservation ranging from red (high conservation) to blue (low conservation), illustrating the apical pThr-epitope binding site and the conservation of the proximal lateral surface. (B) Ribbon diagram of the Mek1 FHA domain showing the range of sequence conservation for Mek1 homologs mapped to the 3-D model. Conservation ranges from red (high conservation) to blue (low conservation). Residues exhibiting conservation and external-facing side chains were labelled with the single-letter amino acid code and the sequence position. The region of the lateral surface was enlarged for the visualization of the residues selected for mutagenesis.

Considering the sequential proximity of the methionine and tyrosine residues as well as the tryptophan and valine residues to each other (Fig. 4.1), we generated two double and one single Mek1 FHA domain lateral surface mutants to analyze whether mutation of the lateral surface could disrupt the

Mek1-Ndt80 interaction. Site-directed mutagenesis was used to change the codons for methionine 84 and tyrosine 86 such that they both encoded for alanine for the *mek1-MY* mutant, as was also done for tryptophan 73 and valine 75 for the *mek1-WV* mutant. Alanine substitution of leucine 109 generated the *mek1-L109A* single mutant. LexA fusions of wild-type (WT) *MEK1* or *mek1* mutants were overexpressed in cells along with B42 transcriptional activation domain (AD) fusions of *Ndt80* Δ *DBD-HA* lacking the region of the Ndt80 DNA-binding domain (Chen et al., 2018) for the β -galactosidase yeast-two hybrid assay (Ausubel et al., 1994; Gyuris et al., 1993; Figure 4.2). As was seen previously, the R51A mutation of the pThr-epitope binding site of Mek1 was not sufficient to disrupt the Mek1-Ndt80 interaction (Chen et al., 2018; Figure 4.2A). We observed that the Mek1-MY and Mek1-WV mutant proteins were unable to interact with Ndt80 whereas alanine substitution of L109 had no effect (Figure 4.2A). Protein expression of plasmid-expressed Mek1 and Ndt80 constructs was detected via western blotting from whole cell extracts of yeast two-hybrid samples, confirming that the loss of interaction with mutant Mek1 was not due to the absence of Mek1 protein (Figure 4.2B).

To examine the phenotypic effects of the loss of the Mek1-Ndt80 interaction for the Mek1-MY and Mek1-WV mutant proteins and the persistence of the Ndt80 interaction with the Mek1-L109A and Mek1-R51A mutants, we generated diploid mutant strains in the SK1 background to evaluate sporulation efficiency (Figure 4.2C). The absence of the Dmc1 recombinase in sporulation deficient *dmc1 Δ* cells prevents the interhomolog repair of Spo11-induced DNA DSBs due to the presence of active Mek1 inhibiting Rad51 recombinase. As a result, *dmc1 Δ* cells arrest in meiotic prophase (Callender et al., 2016; Bishop et al., 1992; Hunter and Kleckner, 2001; Wan et al., 2004). Loss of Mek1 permits cells to use inter-sister chromatid repair mediated by the Rad51 recombinase to relieve meiotic arrest in response to DNA DSBs, at the expense of spore viability (Callender et al., 2016; Bishop et al., 1992; Hunter and Kleckner, 2001; Wan et al., 2004; Xu et al., 1997; Niu et al., 2005; Kim et al., 2010; Goldfarb and Lichten, 2010). Given the persistence of the interaction between Mek1 and Ndt80 when the R51 residue is mutated to alanine, one might anticipate that *mek1-R51A* mutant cells would have poor sporulation efficiency. However, considering the importance of the pThr-epitope binding site of the Mek1 FHA domain to the establishment of the Mek1-pHop1 interaction for Mek1 recruitment to DSB hotspots, high sporulation efficiency should be expected as Mek1 function in meiotic arrest would likely be impacted by the disruption of the Mek1-pHop1 interaction (Niu et al., 2007; Carballo et al., 2008). This seemed to be the case as *mek1-R51A* mutant cells had a high sporulation efficiency in the *dmc1 Δ* background (Figure 4.2C).

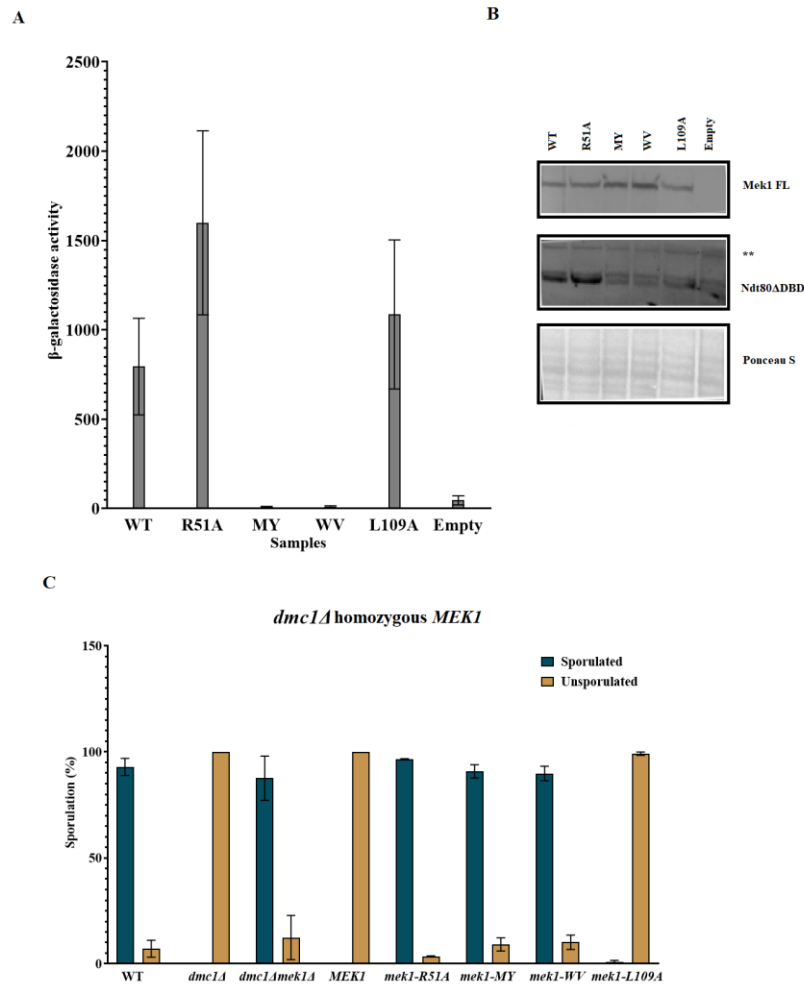


Figure 4.2 Mek1 FHA lateral surface mutants are required for interaction with Ndt80 independent of the pThr-epitope binding site.

(A) Yeast two-hybrid assay evaluating Mek1 mutant interactions with Ndt80, in the context of full-length Mek1, of the pThr-binding site or lateral surface of the Mek1 FHA domain. Wild-type (WT) Mek1 compared to alanine substitution mutants of the pThr-epitope binding site arginine 51 (R51A), or conserved residues of the proximal lateral surface (methionine 84 and tyrosine 86, tryptophan 73 and valine 75, and leucine 109) were tested for interaction with an Ndt80 construct lacking the N-terminal DNA-binding domain. Mean β -galactosidase activity units represent interaction strength. Corresponding western blots confirm protein expression of Mek1 and Ndt80 constructs (B). Ponceau S staining indicates relative loading and transfer of protein samples. Asterisks indicate non-specific binding. (C) Percent sporulation for diploid mutant cells in the *dmc1Δ* background. Sporulated and un-sporulated cells were counted following 24-hour incubation in liquid sporulation media for an average of 200 cells, $n = 3$, and error bars represent standard deviation.

As expected, *mek1-MY* and *mek1-WV* mutant cells exhibited high sporulation efficiency as those mutant proteins were not able to bind to and inhibit Ndt80 (Figure 4.2A and C). The L109A substitution mutation of Mek1 did not affect the Mek1-Ndt80 interaction, so consequently and as expected, *mek1-L109A* mutant cells were unable to sporulate as Mek1 is still able to bind, phosphorylate, and inhibit Ndt80 transcriptional activity for meiotic arrest exit (Figure 4.2C; Chen et al., 2018).

For strains that were able to generate spores, tetrad dissection was used to examine spore viability (Table 4.3). Given the repair of DSBs using Rad51 in the *dmc1Δmek1Δ* strain, inviable spores were expected since Spo11-induced DSBs would be repaired using the sister chromatid, instead of a homologous chromosome, resulting in improper chromosomal segregation during meiosis due to the lack of proper synapsis formation (Xu et al., 1997; Niu et al., 2005; Kim et al, 2010; Goldfarb and Lichten, 2010). We observed a similar lack of viable spore production with the *mek1-R51A* mutant cells, where the disruption of the Mek1-pHop1 interaction and downstream recruitment and activation of Mek1 likely affects Mek1 activity in the meiotic recombination checkpoint (Niu et al., 2007; Carballo et al., 2008; Table 4.3). The *mek1-MY* and *mek1-WV* mutant cells, which had high sporulation efficiencies in the absence of the Mek1-Ndt80 interaction, still had a spore viability defect. However, spore viability was not completely absent (Table 4.3). The presence of some viable spores suggests that, to some extent, mutation of the proximal lateral surface and the ensuing disruption of the Ndt80 interaction prevents full Mek1 function in mediating interhomolog repair for Spo11-induced DNA DSBs while possibly still allowing some degree of DSB repair using homologous chromosomes instead of just inter-sister chromatid repair (Table 4.3).

Table 4.3 Spore Viability by Tetrad Dissection

Genotype	Strain	Viability (%)	Asci dissected	Colonies formed
WT	SK1	91.7	30	110
<i>dmc1Δmek1Δ</i>	SK1	1.7	30	2
<i>dmc1Δmek1Δ::mek1MY</i>	SK1	65.8	30	79
<i>dmc1Δmek1Δ::mek1WV</i>	SK1	60.0	30	72
<i>dmc1Δmek1Δ::mek1R51A</i>	SK1	6.7	30	8

4.4.2 The D78, E79, and D80 residues of the Mek1 FHA domain are necessary for the Mek1-Ndt80 interaction and Mek1 activation.

Despite the initial conclusion that the Mek1 FHA domain was not contributing to the Mek1-Ndt80 interaction (Chen et al., 2018; Wan et al., 2004; Durocher et al., 2000), Aaron Neiman (Stony Brook) also questioned whether an interface of the Mek1 FHA domain other than the pThr-epitope binding site was involved in the interaction. He pursued the development of an interaction model between Mek1 and the “bc” domain of Ndt80 using AlphaFold (Figure 4.3). This model also predicted that a region of the Mek1 FHA domain apart from the pThr-epitope binding site was responsible for mediating the interaction as indicated by yeast two-hybrid analysis (Figure 4.2A). However, according to the model an alpha-helical loop connecting beta strands of the proximal lateral surface, specifically the aspartic and glutamic acid residues: D78, E79, and D80, were directly interacting with the “KR” residues of the RPSKR sequence within the “bc” domain of Ndt80 (Figure 4.3). To further elucidate the phenotypic effect of mutating the residues in the proximal lateral surface of Mek1 implicated in mediating the Mek1-Ndt80 interaction, we used a collection of Mek1 mutants including those for residues on the beta strands examined above, as well as the residues identified in the AlphaFold model and assessed sporulation efficiency and spore viability (Figure 4.4).

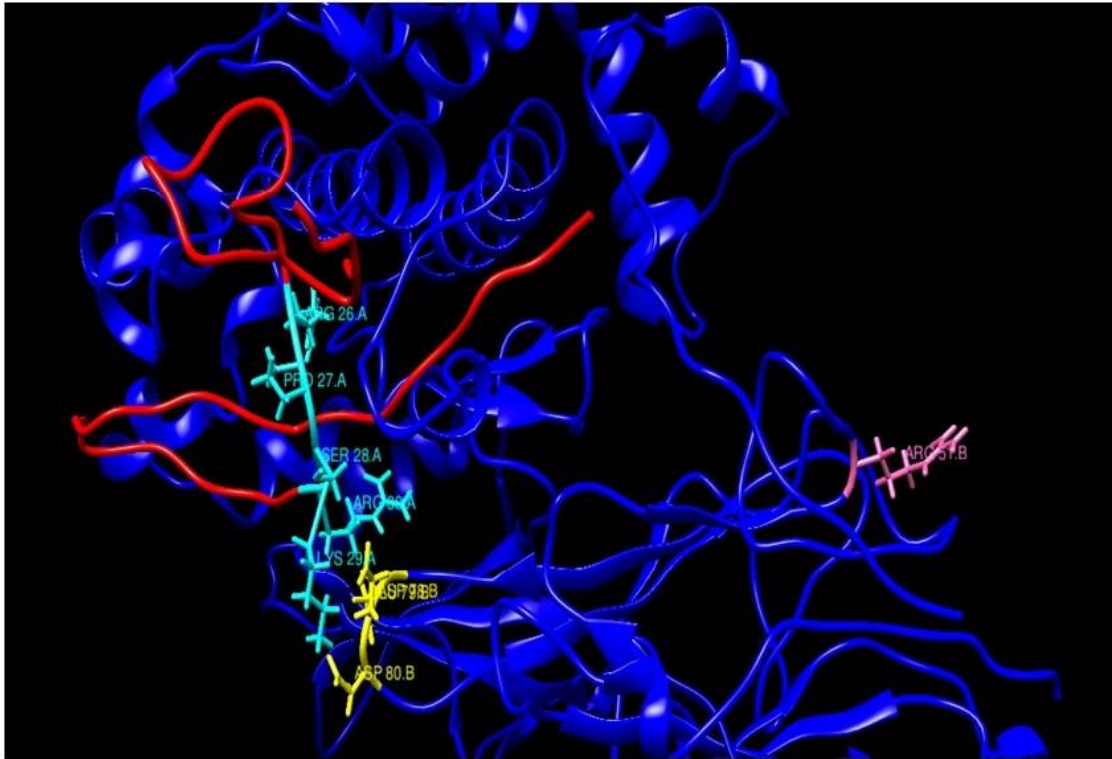


Figure 4.3 Ndt80 bc domain interacts with Mek1 FHA apart from the canonical pThr-epitope binding site.

Alpha Fold representation of Mek1 protein (shown in dark blue) binding to the Ndt80 “bypass-checkpoint” domain peptide (shown in dark red with cyan coloured residues indicating the RPSKR motif). The pink residue illustrates the pThr-epitope binding site arginine (R51) on the apical surface. Residues of the Mek1 FHA domain that are proposed via the model to interact with the RPSKR motif of the Ndt80 “bc” domain are shown in yellow: aspartic acid 78, glutamic acid 79, and aspartic acid 80. Aaron Neiman (Stony Brook) completed AlphaFold modelling.

Lihong Wan (Stony Brook) re-introduced the beta strand mutations to the *dmc1Δmek1Δ* strain to re-generate diploid mutants for the beta strand residues and initially compared them to a triple mutant diploid strain for the alanine substitutions of the three loop residues, referred to as *mek1-DED>AAA*, and a double mutant strain for the two aspartic acid residues of the loop, *mek1-D78A D80A* (Figure 4.4A). Using solid sporulation media, cells were incubated for several days before a colony was selected, mounted in water on a microscope slide, and scored for sporulation. In agreement with previous results, the *mek1-R51A*, *mek1-MY*, and *mek1-WV* mutants exhibited high sporulation efficiency in the *dmc1Δ* background (Figure 4.2C, Figure 4.4A). The *mek1-DED>AAA* triple mutant cells also exhibited high

sporulation efficiency, however, the double mutant *mek1-D78A D80A* cells showed a mild sporulation defect in the *dmc1Δ* background (Figure 4.4A). To determine which of the loop residues had a more profound phenotypic effect on sporulation, loop residue single and double mutants were generated for all possible combinations of mutations and scored for sporulation (Figure 4.4B). Looking at just the single mutants, only the *mek1-D78A* mutant cells showed any sporulation with a sporulation efficiency percentage of approximately 20% (Figure 4.4B). The *mek1-E79A* and *mek1-D80A* single mutants lacked any sporulation, like the phenotype of *dmc1Δ* cells. With the double mutants, *mek1-E79A D80A* mutant cells had an average of about 10% sporulation with the *mek1-D78A E79A* and *mek1-D78A D80A* mutants cells exhibiting a milder sporulation defect with about 50-60% sporulation (Figure 4.4B). The difference in phenotypic effects between the beta strand mutants and the loop mutants suggested that the functional importance of the mutations could also be different. One concern was that the beta strand mutations were disrupting Mek1 structure and function whilst the loop residues might be more specifically affecting the Mek1-Ndt80 interaction. To elucidate the mutation-specific effects on Mek1 function, spore viability was assessed through tetrad dissection in the *mek1Δ* background (Figure 4.4C and D).

Although the *mek1-MY* and *mek1-WV* mutant proteins were unable to interact with Ndt80, they should still be able to recognize pHop1 via the pThr-binding site. However, the absence of a sporulation defect suggests that mutation of the lateral surface residues might disrupt Mek1 activity in addition to disrupting the Mek1-Ndt80 interaction resulting in a *mek1Δ* phenotype. The *mek1Δ* null phenotype features wild-type sporulation. Although, without Mek1 kinase activity to drive interhomolog bias, all generated spores are inviable (Xu et al., 1997; Rockmill and Roeder, 1990, 1991; Leem and Ogawa, 1992). As a result, we chose to evaluate the spore viability of *mek1* mutants in a wild-type *DMC1* background to further investigate mutant Mek1 function (Figure 4.4C and D). The *R51A* mutation resulted in the *mek1Δ* phenotype of inviable spores and the phenotypes for the *mek1-MY* and *mek1-WV* mutant cells were essentially null as well (Figure 4.4C). In contrast, approximately 10% of the *mek1-DED>AAA* triple mutant spores were viable (Figure 4.4C). For the single loop residue mutants and the *mek1-D78A E79A* and *mek1-E79A D80A* double mutants the percentage spore viability was very high (Figure 4.4D). However, the *mek1-D78A D80A* mutants had a more profound spore viability defect with only about 60% spore viability (Figure 4.4D). This suggested that the mutations on the loop might be more specific to the Mek1-Ndt80 interaction, as seen with the AlphaFold model (Figure 4.3), and that perhaps mutations of the beta strand residues were affecting Mek1 activity.

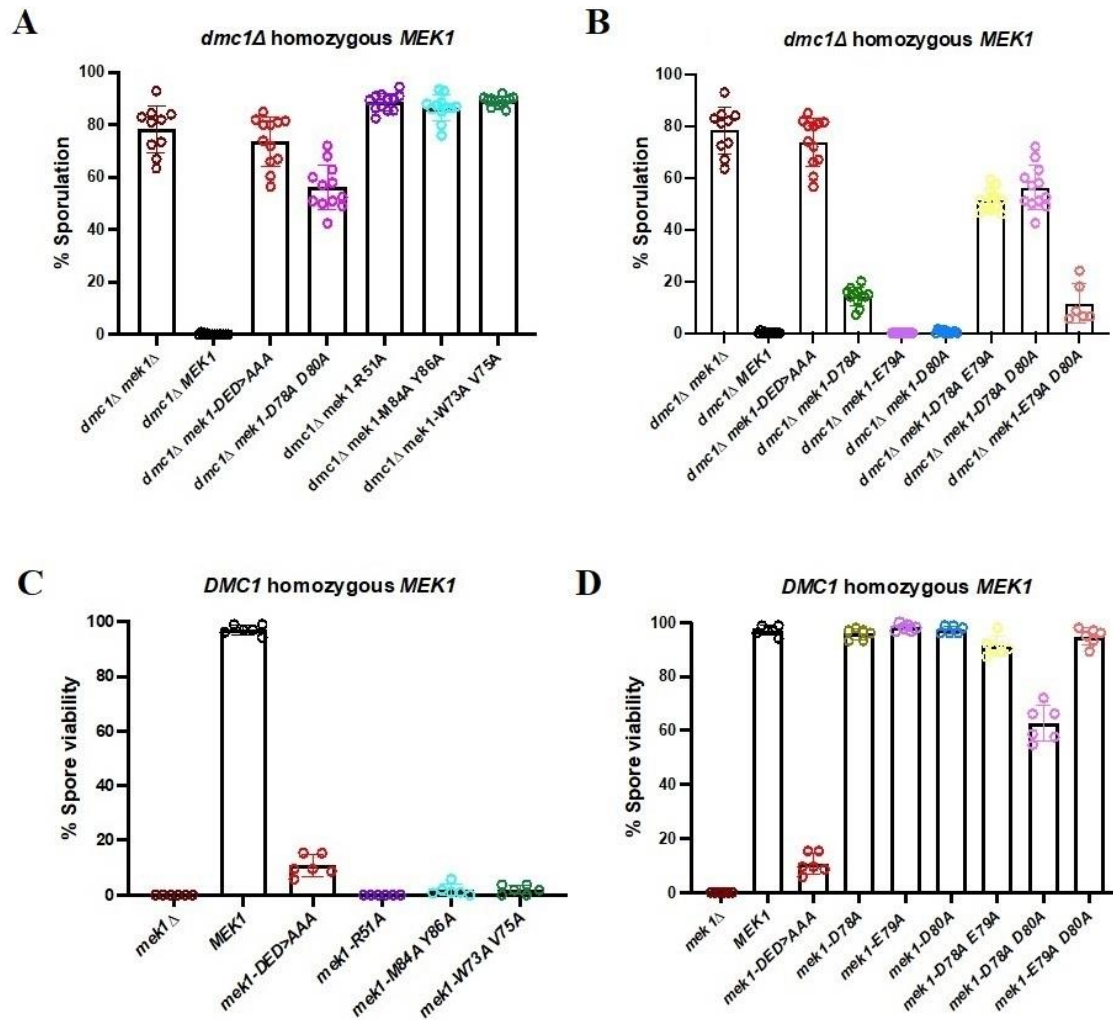


Figure 4.4 Mek1 FHA D78, E79, and D80 are key residues for Mek1-Ndt80 interaction and meiotic recombination checkpoint bypass.

Percent sporulation for *mek1* mutant diploid strains for residues on the beta strands of the proximal lateral surface (A) or for residues identified via the Alpha Fold model (B) were used to evaluate the ability of mutant cells to bypass the meiotic recombination checkpoint arrest in the *dmc1Δ* background. Sporulation was observed following 3 days of incubation at 30°C on solid sporulation medium. Sporulated cells were dissected to evaluate spore viability in the *DMC1* background for *mek1* mutant diploid strains for residues on the beta strands of the proximal lateral surface (C) or for residues identified via the Alpha Fold model (D). Circles represent individual replicates and error bars indicated standard deviations. Analysis was conducted by Lihong Wan in the Hollingsworth Lab.

To determine whether the various mutations were affecting Mek1 stability or activity, immunoblots from diploid strains were used to visualize Mek1 protein expression as well as Mek1 kinase activity via the detection of phosphorylated Hed1 (Callendar et al., 2016; Figure 4.5). Mek1 protein appeared to be stably expressed throughout all mutant samples, however, the alanine substitution mutations of the M84 and Y86 residues on the beta strand of Mek1 impaired Mek1 kinase activity shown by the lack of detectable phosphorylated Hed1 (Figure 4.5). The Mek1-WV mutant was not analyzed based on the assumption that the results would be the same. Noticeably, the mutants for the loop residues were all able to phosphorylate Hed1, indicating that the proposed loss of the Mek1-Ndt80 interaction through mutation of the loop residues of the proximal lateral surface of the Mek1 FHA domain is likely directly responsible for the phenotypic effects and that mutation of the beta-strand residues not only interrupts the Mek1-Ndt80 interaction but also the ability of Mek1 kinase, possibly through the disruption of the Mek1 FHA domain structure, to be active .

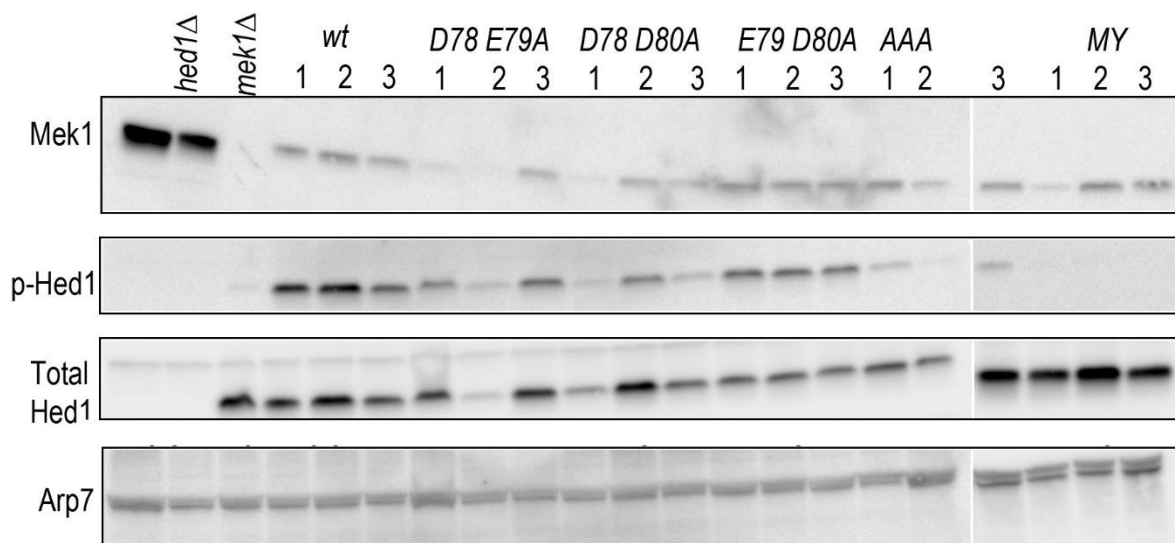


Figure 4.5 Mek1 MY mutant protein does not phosphorylate Hed.

Immunoblot analysis of 3 replicates from sporulated diploid strains for *MEK1*, *mek1-D78A E79A*, *mek1-D78A D80A*, *mek1-E79A D80A*, *mek1-DED>AAA*, and *mek1-M84A Y86A (mek1-MY)* with *mek1Δ* and *hed1Δ* as controls. Hed1 phosphorylation at T40 (p-Hed1) was used as an indicator of Mek1 kinase activity. Arp7 was used as a loading control. Western blots were performed by Lihong Wan.

Tying together the phenotypic effects of the loop residue mutations and the predictions of the AlphaFold model, spotting assays were used to confirm the loss of the Mek1-Ndt80 interaction when the

DED loop residues were mutated to alanine (Fig. 4.6A). Spotting assays confirmed the observation that Mek1 interacts with the fragment of Ndt80 corresponding to amino acid residues 284-627 (Chen et al., 2018) and showed that when all three loop residues are mutated to alanine, the Mek1-Ndt80 interaction is lost (Fig. 4.6A). B-galactosidase activity was measured using liquid yeast two-hybrid assays to illustrate and emphasize the differences in interaction between Mek1 and Ndt80 for the Mek1 loop residue double mutants to isolate the key residues involved in mediating the interaction (Fig. 4.6B).

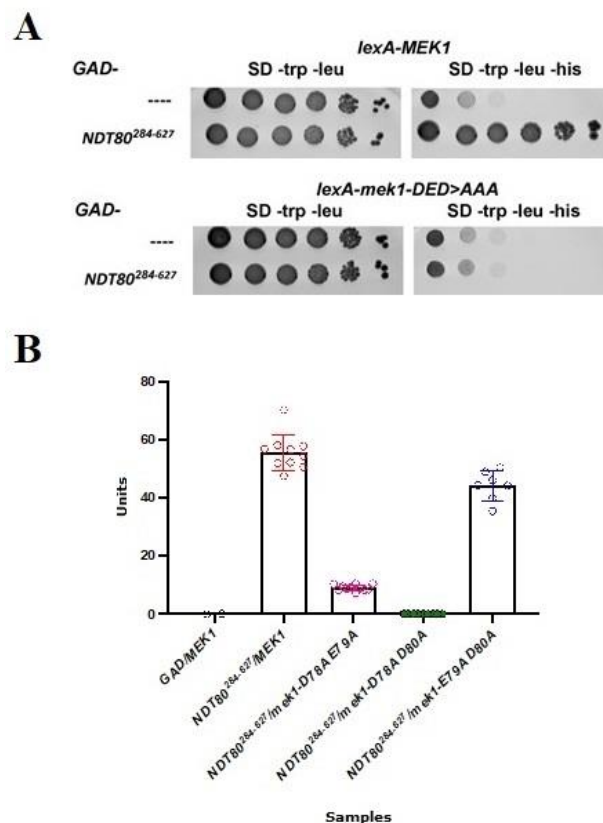


Figure 4.6 *LexA-mek1-D78 E79* weakens the interaction with *GAD-NDT80*.

(A) Auxotrophic growth-based yeast two-hybrid spotting assays using synthetic minimal media (SD) for the analysis of the interaction between a fragment of Ndt80 (amino acids 284-627) and GAD fusions of *lexA-MEK1* or *lexA-mek1-DED>AAA*. (B) Liquid-based yeast two-hybrid assays were used to depict changes in interaction strength between various *mek1* double mutants and Ndt80. Each circle represents an independent transformant with average β -galactosidase activity units shown in the graph. Errors bars depict the standard deviations.

The observation that the *mek1-D78A D80* double mutant resulted in the complete abrogation of the Mek1-Ndt80 interaction suggests that these two negatively charged residues are highly important for the Mek1-Ndt80 interaction. Specifically, the D78 residue appears to have a larger contribution as the *mek1-D78A E79A* mutant interaction with Ndt80 is more affected by the loss of the D78 residue since the *mek1-E79A D80A* mutant since exhibits near wild-type levels of interaction with Ndt80 (Fig. 4.6B).

Considering the likelihood of more Mek1 binding partners, we utilized co-immunoprecipitation to test whether Mek1 and one of its associating proteins Red1 could be interacting with components of the DDK complex (Figure 4.7). Given that the discovery of non-canonical lateral surface interactions for FHA domains originated with the study of the interaction between the Rad53 FHA1 domain and the H-BRCT domain of Dbf4 (Matthews et al., 2014) and the relation of Mek1 to Rad53 as its meiosis-specific paralog, we wanted to investigate whether Mek1 might also be associating with Dbf4 in a non-canonical manner. Mek1-dependent DDK phosphorylation of Zip1 for recombination resolution (Chen et al., 2015; Kim et al., 2010) and the knowledge that the synaptonemal complex protein Red1 is phosphorylated by a kinase other than Mek1 even though it functions in close association with Mek1 (Wan et al., 2004) led us to hypothesize that Mek1 and/or Red1 might associate with Dbf4 and/or Cdc7. Co-immunoprecipitation results indicate that Mek1 and Red1, either individually or due to their complex formation with each other and other associating proteins, can bind to Dbf4 and possibly, to a lesser extent, Cdc7 (Figure 4.7A and B). Testing the theory that Mek1 might also be associated with Dbf4 through its lateral surface, co-IPs with the Mek1 beta strand mutants and Dbf4 were conducted. However, looking at the immunoprecipitated Mek1 mutants, preliminary data was inconclusive as all mutant protein bands can be detected on the blot (Figure 4.7C). Possible recruitment of DDK to local DSBs via the Mek1 lateral surface could contribute to DDK activities during meiotic recombination such as Zip1 phosphorylation and could be another example of a Mek1 interaction that uses the proximal lateral surface (Chen et al., 2015; Kim et al., 2010), but further analysis is required to investigate the possibility.

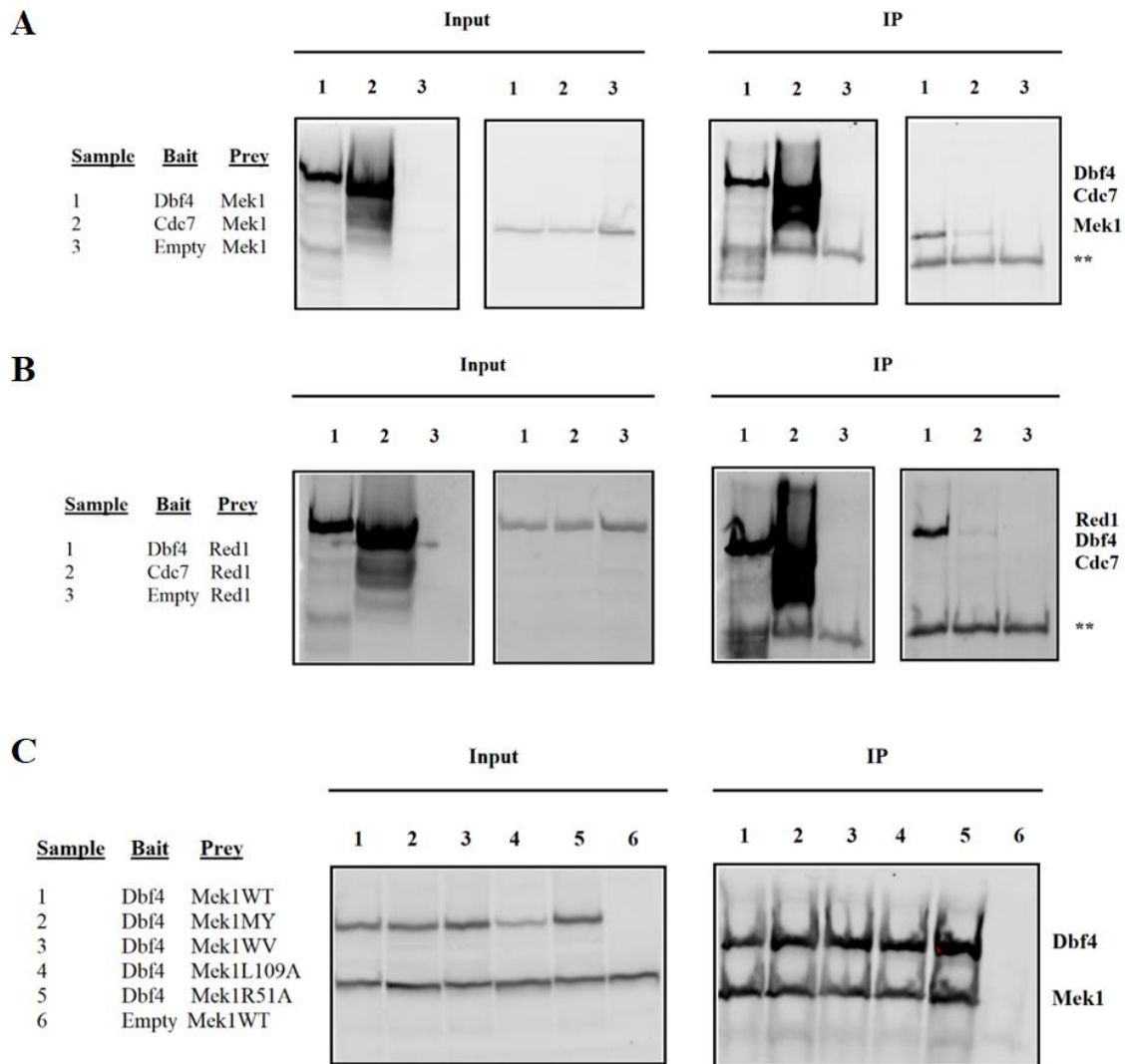


Figure 4.7 Mek1 and Red1 interact with the DDK complex in yeast.

Co-immunoprecipitation of Mek1 (A) and Red1 (B) with components of the DDK complex conducted to investigate the possible interaction between Mek1/Red1 and Dbf4/Cdc7. Alanine substitution mutants for the Mek1 FHA domain on the proximal lateral surface: *mek1-M84A Y86A (MY)*, *mek1-W73A V75A (WV)*, or *mek1-L109A*, the pThr-epitope binding site (*mek1-R51A*), or Mek1 wild-type (WT) were assessed for an interaction with Dbf4 (C). ** indicates non-specific binding. Figures (A) and (B) show a representative image of three trials. Figure (C) shows the image for one preliminary trial. IP refers to the immunoprecipitated samples.

4.5 Conclusions

Taken together, this data shows that the Mek1 FHA domain does in fact mediate the Mek1-Ndt80 interaction albeit not through the pThr-epitope binding site. These results confirm the accuracy and precision of the AlphaFold prediction model and, although in this case residues of an alpha helical loop instead of the beta strands were directly responsible for mediating the FHA domain-based interaction, show that the Mek1 FHA domain represents another model of non-canonical lateral surface interactions that can function without the use of the pThr-epitope binding site. The region of interest for the Mek1 interactions also coincides with a similar region on the human Chk2 FHA domain that when mutated contributes to Li-Fraumeni syndrome (Li et al., 2002; Chapter 3). Irregularities in the pathways involved in meiotic recombination can lead to the occurrence of genetic abnormalities and aneuploidies like Down Syndrome. With each discovery of protein-protein interactions and the mechanisms that establish and/or regulate them, it becomes increasingly clear that no two interactions are the same. To understand the abnormalities and diseases that arise from biological mishaps, we must understand the mechanisms behind the biological processes that govern life. To understand those mechanisms, we need to have a clear view of how the relevant biological pathways work. By having a clear view of how the biological pathways should progress naturally, we can better anticipate and/or correct the mistakes that often occur so that we can move beyond the predominant symptom management-based healthcare. Further analysis of Mek1 interactions and the meiotic recombination checkpoint in budding yeast can lead to a more advanced understanding of similar processes in humans, potentially leading to preventative measures and/or therapeutics that can benefit individuals affected by such genetic abnormalities and aneuploidies.

Chapter 5

General Conclusions and Future Directions

5.1 Dun1

The analysis of the Dun1 FHA domain interactions with binding partners Dif1 and Sml1 demonstrated a similar, yet different, mechanism of non-canonical FHA domain lateral surface interactions compared with its paralog, Rad53. Whereas the distal lateral surface of the Rad53 FHA1 domain mediates its interaction with the Dbf4 H-BRCT domain (Matthews et al., 2014), the Dun1 FHA domain uses both the pThr-epitope binding site and the distal lateral surface to establish and regulate ligand binding and specificity (Chapter 2). For the Dun1-Dif1 interaction, mutation of the pThr-epitope binding site was individually capable of complete interaction abrogation. However, in the case of the Dun1-Sml1 interaction, mutation of the pThr-epitope binding site only reduced the interaction. (Fig. 2.3, Table 2.3, Chapter 2). Complete loss of the Dun1-Sml1 interaction was only achieved upon combining mutations for the pThr-epitope binding site with mutations of residues within the distal lateral surface. Mutants for just the distal lateral surface were still able to interact with Dif1 and Sml1 (Fig. 2.4 and 2.5, Table 2.3, Chapter 2).

Phenotypic effects of combined pThr-epitope binding site and distal lateral surface mutations were observed as hypersensitivity to genotoxic agents MMS and HU beyond the level of the *dun1Δ* null mutant. This enhanced hypersensitivity is speculated to be a consequence of Dun1 dysregulation in DNA repair pathways, G2 phase arrest, and prevention of replication fork collapse in response to DNA DSBs and HU exposure (Fig. 2.6, Chapter 2; Yam et al., 2020; Wang et al., 2001; Woolstencroft et al., 2006). Combination mutations also led to sensitivity to DSB-inducing agents bleomycin and phleomycin but did not mirror the enhanced hypersensitivity beyond the *dun1Δ* null mutant as was seen with MMS and HU treatment (Fig. 2.6, Chapter 2). This chapter demonstrates the importance of the identified distal lateral surface residues to the establishment of the Dun1 interactions in budding yeast. The data presented here contributes to the list of isolated non-canonical FHA domain interactions that have been characterized, supporting evidence that the non-canonical nature of the Rad53-Dbf4 interaction was not a unique occurrence and that there are likely many uncharacterized non-canonical interactions mediated by interfaces other than the pThr-epitope binding site of FHA domains (reviewed in Almawi et al., 2017).

5.2 Bioinformatics

This chapter presents a culmination of bioinformatic analyses of the main structures involved in the non-canonical FHA domain lateral surface interaction characterized between the Rad53 FHA1 and Dbf4 H-BRCT domains (Matthews et al., 2014; Almawi et al., 2016). The primary goal of this chapter was to investigate the prevalence of conserved residues on the lateral surfaces of budding yeast FHA domains and to explore the possibility of identifying novel FHA and H-BRCT domains. Out of this analysis, several key features/characteristics of FHA domains were documented and an intriguing possibility of identifying conserved regions of FHA domain lateral surfaces that can even be found in human homologs was explored.

The comparison of known budding yeast FHA domains was used to probe the *Saccharomyces cerevisiae* proteome for novel FHA domains. Although no novel proteins were identified, the resulting profile Hidden Markov Model (pHMM) depicted two motifs of FHA domains in budding yeast where the highly conserved GR motif can be used to identify the arginine residue of the pThr-epitope binding site. A secondary motif also seemed to be drawn out through analysis of the known budding yeast FHA domains. Downstream of the GR motif, a highly conserved SxxH motif can be seen (Fig. 3.1, Chapter 3). Taking multiple sequence alignments for homologs of each of the FHA domain-containing proteins, sequence conservation was mapped to FHA domain models and residues on the lateral surfaces were visualized. From those models, residues on the proximal and distal lateral surfaces were identified based on conservation and/or positioning of their side chains. Residues with side chains that projected into the external FHA domain lateral space were identified and listed as residues of interest (Table 3.2, Chapter 3).

Given the evolutionary relationship between Rad53, Dun1, and Mek1 (Chuang et al., 2012) and the human Rad53 homolog Chk2, a similar FHA domain analysis was used to see if any unique features could be drawn out of a pHMM based on their MSA alignment. There was a difference in the motif following the conserved GR motif where instead of the SxxH motif, highly conserved cysteine and proline residues were observed (Fig. 3.2, Chapter 3). The alignment of those related FHA domains showed the positional conservation of key residues involved in mediating protein-protein interactions in budding yeast and, in the case of Chk2, a pathological condition (Matthews et al., 2014; Chapter 2 and 4; Li et al., 2002). Although novel H-BRCT domains were not found using a bioinformatics approach, the human TopBP1 extended linker between BRCT7 and BRCT8 could be seen in the vicinity of the

additional α -helix of the H-BRCT domains of Dbf4 and Sir4. Whether single BRCT domains with an additional α -helix preceded the more common tandem BRCT domain structure evolutionarily remains to be determined. In any case, the data presented in this chapter contributes to the growing body of data on FHA domain lateral surfaces and their relevance to human homologs as well, where researchers can be directed as to potential residues of interest on lateral surfaces to investigate for interactions that do not rely on the canonical pThr-epitope binding site. This data also lists the conserved arginine residues of the pThr-epitope binding site and can be used to initiate protein interaction analyses using FHA domains.

5.3 Mek1

The meiotic recombination checkpoint kinase Mek1 presents as another FHA domain-containing protein that does not rely on its pThr-epitope binding site for the establishment of all protein-protein interactions. Upon mutation of the conserved arginine residue of the pThr-epitope binding site, the interaction between Mek1 and the Ndt80 transcription factor persists (Chen et al., 2018). The conclusion that the lack of interaction abrogation upon mutation of the Mek1 FHA domain pThr-epitope binding site indicates that the Mek1 FHA domain does not mediate Ndt80 binding (Chen et al., 2018; Wan et al., 2004; Durocher et al., 2000) was challenged in this study. Data presented shows that the Mek1 FHA domain utilizes a region of the proximal lateral surface to establish the interaction with Ndt80. Residues located on the β -strands of the proximal lateral surface are required for the Mek1-Ndt80 interaction (Fig. 4.2A, Chapter 4). However, mutation of these residues also compromised Mek1 kinase activity (Fig. 4.5, Chapter 4). Although mutants of the proximal lateral surface β -strand residues have compromised Mek1 function, there appears to be a fraction of DSB repair that allows for the generation of some viable spores in the *dmc1 Δ* background, suggesting possible homologous recombination-based repair of Spo11-induced DSBs (Table 4.4, Chapter 4). Several residues located on an α -helical loop connecting proximal lateral surface β -strands: D78, E79, and D80, predicted via an AlphaFold model, were shown to be directly involved in mediating the association of Mek1 with the Ndt80 “bc” domain. Mutation of these residues resulted in the abrogation of the interaction with the persistence of wild-type Mek1 kinase function (Fig. 4.4 - 4.6, Chapter 4). Unpublished data from the Hollingsworth Lab has shown that Mek1 is also able to utilize the exact same surface to mediate the interaction of the Mek1 FHA domain with another ligand, the Rrm3 helicase, and co-immunoprecipitation data showed a potential interaction between Mek1 and the DDK kinase complex.

The observation that Mek1 shares homology with human Chk2 and that the same β -strand residues investigated in this chapter align with the I157 residue of Chk2 involved in the pathology of Li-Fraumeni syndrome (Li et al., 2002; Chapter 3) presents a prime example of the significance of the characterization of FHA domain lateral surfaces. A complete understanding of the full extent of FHA domain-based interactions is only possible by including the characterization of the many interactions that have likely been missed over the past two decades of FHA domain research where studies have been primarily focused on the pThr-binding specificity feature of FHA domains.

5.4 Future Directions

Since the discovery of the non-canonical interaction between the Rad53 FHA1 domain and the H-BRCT domain of Dbp4, the collection of non-canonical FHA domain-based interactions reported has increased. However, given the complexities of protein-protein interactions and the molecular pathways that they govern, there is more than likely many interactions that have yet to be characterized that could also be using an alternative interface for the establishment of FHA domain-based interactions that do not rely on the pThr-epitope binding site.

Data gathered from the characterization of the Dun1-Dif1 and Dun1-Sml1 interactions support the evidence showing that non-canonical interactions using the lateral surface of an FHA domain are a lot more prevalent than initially believed. Even though we were able to show that Dun1 utilizes the FHA domain distal lateral surface to mediate interactions with Dif1 and Sml1 and that disruptions of those interactions can lead to genotoxic sensitivity, further studies should be done to characterize the impact of mutations to the lateral surface residue on the re-localization of the ribonucleotide reductase subunits. Analyses like FRET might also be able to elucidate other Dun1 interactions that could not be detected using yeast two-hybrid assays such as the interaction between Dun1 and Rad53. A co-crystallization structure for the Dun1-Dif1 and Dun1-Sml1 interactions would also be extremely valuable in detailing the specific contacts points between Dun1 and its binding partners.

Considering the Mek1 analysis, we were able to show that non-canonical FHA domain interactions exist beyond mitosis. FHA domain interactions using the lateral surface can also drive meiotic recombination checkpoint regulation in budding yeast. We have even found that the region of the lateral surface of Mek1 aligns with the same region in human Chk2 according to the analysis of their FHA domain models. The relevance of that discovery is supported by data showing that mutation of the I157 residue on the Chk2 FHA domain lateral surface can abrogate the interaction between Chk2 and several

other proteins: Cdc25, p53, and BRCA1 without disrupting Chk2 interactions with phosphorylated epitopes or impairing kinase activity (Li et al., 2001; Falck et al., 2001b; Falck et al., 2001a). Further exploration into other Mek1 interactions and the requirements of the pThr-epitope binding site and/or proximal lateral surface will be key to a more complete characterization of the Mek1 FHA domain and the involvement of its lateral surface in mediating protein-protein interactions. One such exploration would be that of the mechanism of Mek1-mediated DDK phosphorylation of Zip1 and the phosphorylation and degradation of the Red1 synaptonemal complex protein. Additionally, future research looking into the structural effects of the mutations of the proximal lateral surface β -strand residues would be key to understanding the importance of those residues to Mek1 kinase activity. We assume that the Mek1 β -strand lateral surface mutants should still be able to interact with pHop1, but that should be confirmed given the observation that the kinase activity of those mutants is compromised. With regards to the spore viability observed in the *dmc1 Δ* background for the lateral surface β -strand mutants, it would be valuable to develop and use a microscopy-based system to visualize the meiotic recombination process and/or the progression of meiotic cellular divisions.

Overall, FHA domains require more analysis. A focus on the pThr-epitope binding site is too narrow to fully grasp the complexity of the interactions that are mediated by FHA domains. As such, the collection of bioinformatics analysis presents a great demonstration of using such tools to assist in the beginning stages of FHA domain characterization. We have only begun to scratch the surface of bioinformatics tools and their applications to *in silico* analysis of FHA domain-based interactions. A continuation of exploring the uses of tools like AlphaFold and higher-level *ab initio* domain modelling could shed more light on FHA domain surfaces and better predict alternative interaction interfaces.

5.5 Impact and Relevance

FHA domains are prevalent in a lot of the molecular pathways that govern the ability of cells to replicate and divide efficiently. FHA domains are enriched in many transcription factors, checkpoint proteins, and DNA damage and repair factors. As such, studying these processes requires an understanding of FHA domains and how they work to regulate the interactions in which they are involved. Observations showing that FHA domain lateral surface mutations can disrupt specific protein-protein interactions without eliminating all interactions and/or protein function, as well as the positional conservation of examined lateral surface residues in yeast and human paralogs, speaks to the impact and relevance of characterizing FHA domain alternative interfaces. Understanding the interfaces of FHA

domains and how they participate in their various interactions and molecular pathways could potentially lead to the development of small molecules that could be used to activate or inhibit the regions of FHA domain-containing proteins, providing extensive therapeutic benefit.

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Appendix A

Chapter 2 Supplementary Table

Appendix A Table 1 Protein BLAST Query Selected Dun1 Homologs

Accession #	Organism	Product
KMQ85521.1	<i>Lasius niger</i>	Kanadaptin
XP_020282762.1	<i>Pseudomyrmex gracilis</i>	kanadaptin
WP_095723188.1	<i>Calothrix elsteri</i>	FHA domain-containing protein
WP_017300371.1	<i>Nodosilinea nodulosa</i>	FHA domain-containing protein
WP_073606707.1	<i>Phormidium tenue</i>	FHA domain-containing protein
CAH04269.1	<i>Schizosaccharomyces pombe</i>	Cds1 kinase
XP_013025187.1	<i>Schizosaccharomyces cryophilus</i> OY26	CAMK/RAD53 protein kinase Cds1
TNJ27817.1	<i>Giardia muris</i>	FHA domain-containing protein
EJD42329.1	<i>Auricularia subglabra</i> TFB-10046 SS5	SMAD/FHA domain-containing protein
WP_073255868.1	<i>Caldanaerovirga acetigignens</i>	FHA domain-containing protein
WP_066352592.1	<i>Fervidicola ferrireducens</i>	FHA domain-containing protein
WP_073344202.1	<i>Caldanaerobius fijiensis</i>	FHA domain-containing protein
WP_026486109.1	<i>Caldanaerobius polysaccharolyticus</i>	FHA domain-containing protein
WP_131115722.1	<i>Westiellopsis prolifica</i>	FHA domain-containing protein
XP_013933722.1	<i>Ogataea parapolymorpha</i> DL-1	Cell-cycle checkpoint serine-threonine kinase
XP_011275575.1	<i>Wickerhamomyces ciferrii</i>	DNA damage response protein kinase DUN1
XP_002492175.1	<i>Komagataella phaffii</i> GS115	Cell-cycle checkpoint serine-threonine kinase
XP_003867435.1	<i>Candida orthopsilosis</i> Co 90-125	Dun1 protein
KGU25514.1	<i>Candida albicans</i> P75063	serine/threonine-protein kinase Chk2
XP_026595130.1	<i>Candida viswanathii</i>	DNA damage response protein kinase DUN1
XP_002546640.1	<i>Candida tropicalis</i> MYA-3404]	DNA damage response protein kinase DUN1
OEJ88444.1	<i>Hanseniaspora osmophila</i>	DNA damage response protein kinase DUN1
XP_022674963.1	<i>Kluyveromyces marxianus</i> DMKU3-1042	DNA damage response protein kinase DUN1
AQZ11783.1	<i>Zygosaccharomyces parabailii</i>	DUN1 (YDL101C)
NP_010182.1	<i>Saccharomyces cerevisiae</i> S288C	serine/threonine protein kinase DUN1
KTB25543.1	<i>Candida glabrata</i>	DNA damage response protein kinase DUN1
WP_109763196.1	<i>Pleionea mediterranea</i>	FHA domain-containing protein
WP_102043286.1	<i>Thauera hydrothermalis</i>	FHA domain-containing protein
RMH47081.1	<i>Gammaproteobacteria</i> bacterium	FHA domain-containing protein

Appendix B

Chapter 3 Supplementary Tables and Figures

Appendix B Table 1 Dbf4 H-BRCT Query and Selected HHPred Hit Sequences

Accession #	Organism	Product
3QBZ_A	<i>Saccharomyces cerevisiae</i>	DDK kinase regulatory subunit DBF4
6RRV_A	<i>Saccharomyces cerevisiae</i>	Regulatory protein SIR4
3AL2_A	<i>Homo sapiens</i>	DNA topoisomerase 2-binding protein 1
2HTF_A	<i>Homo sapiens</i>	DNA polymerase mu
1L0B_A	<i>Rattus norvegicus</i>	BRCA1
4BMD_A	<i>Schizosaccharomyces pombe</i>	S-M checkpoint control protein RAD4
4Q66_A	<i>Saccharomyces cerevisiae</i>	Chitin biosynthesis protein
4N40_A	<i>Homo sapiens</i>	Protein ECT2

Appendix B Table 2 Protein BLAST Query Selected Rad53 Homologs

Accession #	Organism	Product
XP_023576427.1	<i>Octodon degus</i>	E3 ubiquitin-protein ligase CHFR
XP_023459675.1	<i>Cercospora beticola</i>	Serine/threonine-protein kinase cds1
GEM08343.1	<i>Rhodotorula toruloide</i>	ser/thr/tyr protein kinase RAD53
XP_014152720.1	<i>Sphaeroforma arctica JP610</i>	CAMK/RAD53 protein kinase
CAH04269.1	<i>Schizosaccharomyces pombe</i>	Cds1 kinase
CCG80711.1	<i>Taphrina deformans PYCC 5710</i>	Serine/threonine-protein kinase cds1
XP_024665939.1	<i>Wickerhamiella sorbophila</i>	Serine/threonine-protein kinase RAD53
ONH72964.1	<i>Pichia kudriavzevii</i>	Serine/threonine-protein kinase RAD53
XP_013935328.1	<i>Ogataea parapolymorpha DL-1</i>	ser/thr/tyr protein kinase RAD53
EIF45440.1	<i>Brettanomyces bruxellensis AWRI1499</i>	rad53p
KGU10814.1	<i>Candida albicans 19F</i>	Ser/Thr/Tyr protein kinase RAD53
XP_026597083.1	<i>Candida viswanathii</i>	Serine/threonine-protein kinase RAD53
OEJ83107.1	<i>Hanseniaspora osmophila</i>	Serine/threonine-protein kinase RAD53
XP_022674542.1	<i>Kluyveromyces marxianus DMKU3-1042</i>	serine/threonine-protein kinase RAD53
KTB15434.1	<i>Candida glabrata</i>	Serine/threonine-protein kinase RAD53
5XZV_A	<i>Saccharomyces cerevisiae S288C</i>	Serine/threonine-protein Kinase Rad53
AQZ10671.1	<i>Zygosaccharomyces parabailii</i>	RAD53 (YPL153C)

Appendix B Table 3 Protein BLAST Query Selected Dun1 Homologs

Accession #	Organism	Product
KMQ85521.1	<i>Lasius niger</i>	Kanadaptin
XP_020282762.1	<i>Pseudomyrmex gracilis</i>	kanadaptin
WP_095723188.1	<i>Calothrix elsteri</i>	FHA domain-containing protein
WP_017300371.1	<i>Nodosilinea nodulosa</i>	FHA domain-containing protein
WP_073606707.1	<i>Phormidium tenue</i>	FHA domain-containing protein
CAH04269.1	<i>Schizosaccharomyces pombe</i>	Cds1 kinase
XP_013025187.1	<i>Schizosaccharomyces cryophilus</i> OY26	CAMK/RAD53 protein kinase Cds1
TNJ27817.1	<i>Giardia muris</i>	FHA domain-containing protein
EJD42329.1	<i>Auricularia subglabra</i> TFB-10046 SS5	SMAD/FHA domain-containing protein
WP_073255868.1	<i>Caldanaerovirga acetigignens</i>	FHA domain-containing protein
WP_066352592.1	<i>Fervidicola ferrireducens</i>	FHA domain-containing protein
WP_073344202.1	<i>Caldanaerobius fijiensis</i>	FHA domain-containing protein
WP_026486109.1	<i>Caldanaerobius polysaccharolyticus</i>	FHA domain-containing protein
WP_131115722.1	<i>Westiellopsis prolifica</i>	FHA domain-containing protein
XP_013933722.1	<i>Ogataea parapolymorpha</i> DL-1	Cell-cycle checkpoint serine-threonine kinase
XP_011275575.1	<i>Wickerhamomyces ciferrii</i>	DNA damage response protein kinase DUN1
XP_002492175.1	<i>Komagataella phaffii</i> GS115	Cell-cycle checkpoint serine-threonine kinase
XP_003867435.1	<i>Candida orthopsilosis</i> Co 90-125	Dun1 protein
KGU25514.1	<i>Candida albicans</i> P75063	serine/threonine-protein kinase Chk2
XP_026595130.1	<i>Candida viswanathii</i>	DNA damage response protein kinase DUN1
XP_002546640.1	<i>Candida tropicalis</i> MYA-3404]	DNA damage response protein kinase DUN1
OEJ88444.1	<i>Hanseniaspora osmophila</i>	DNA damage response protein kinase DUN1
XP_022674963.1	<i>Kluyveromyces marxianus</i> DMKU3-1042	DNA damage response protein kinase DUN1
AQZ11783.1	<i>Zygosaccharomyces parabailii</i>	DUN1 (YDL101C)
NP_010182.1	<i>Saccharomyces cerevisiae</i> S288C	serine/threonine protein kinase DUN1
KTB25543.1	<i>Candida glabrata</i>	DNA damage response protein kinase DUN1
WP_109763196.1	<i>Pleionea mediterranea</i>	FHA domain-containing protein
WP_102043286.1	<i>Thauera hydrothermalis</i>	FHA domain-containing protein
RMH47081.1	<i>Gammaproteobacteria bacterium</i>	FHA domain-containing protein

Appendix B Table 4 Protein BLAST Query Selected Mek1 Homologs

Accession #	Organism	Product
ONH69372.1	<i>Cyberlindnera fabianii</i>	Meiosis-specific serine/threonine-protein kinase MEK1
XP_026597502.1	<i>Candida viswanathii</i>	Meiosis-specific serine/threonine-protein kinase MEK1
RLV93349.1	<i>Spathaspora sp. JA1</i>	Meiosis-specific serine/threonine-protein kinase MEK1
OEJ92352.1	<i>Hanseniaspora osmophila</i>	Meiosis-specific serine/threonine-protein kinase MEK1
BAP72958.1	<i>Kluyveromyces marxianus</i>	meiosis-specific serine/threonine-protein kinase MEK1
SSD60567.1	<i>Saccharomyces ludwigii</i>	related to Meiosis-specific serine/threonine-protein kinase MEK1
KTB01294.1	<i>Candida glabrata</i>	Meiosis-specific serine/threonine-protein kinase MEK1
SMN20722.1	<i>Kazachstania saulgeensis</i>	similar to <i>Saccharomyces cerevisiae</i> YOR351C MEK1 Meiosis-specific serine/threonine protein kinase
EJT41423.1	<i>Saccharomyces kudriavzevii</i> IFO 1802	MEK1-like protein
EJS41534.1	<i>Saccharomyces arboricola</i> H-6	mek1p
NP_014996.3	<i>Saccharomyces cerevisiae</i> S288C	serine/threonine protein kinase MEK1
AQZ09139.1	<i>Zygosaccharomyces parabailii</i>	MEK1 (YOR351C)
GCE97546.1	<i>Zygosaccharomyces mellis</i>	meiosis-specific serine/threonine-protein kinase mek1
OLL26992.1	<i>Neoelecta irregularis</i> DAH-3	Meiosis-specific serine/threonine-protein kinase mek1
XP_002174585.1	<i>Schizosaccharomyces japonicus</i> yFS275	CAMK/RAD53 protein kinase Mek1
XP_017996945.1	<i>Phialophora attae</i>	Meiosis-specific serine/threonine-protein kinase mek1
GBF67102.1	<i>Trichophyton mentagrophytes</i>	meiosis-specific serine/threonine-protein kinase
XP_002845045.1	<i>Microsporum canis</i> CBS 113480	mek1
PIG87779.1	<i>Aspergillus arachidicola</i>	serine/threonine protein kinase
GCB24254.1	<i>Aspergillus awamori</i>	meiosis-specific serine/threonine-protein kinase MEK1

Appendix B Table 5 Protein BLAST Query Selected Xrs2 Homologs

Accession #	Organism	Product
XP_024259580.1	<i>Oncorhynchus tshawytscha</i>	nibrin-like
XP_021476940.1	<i>Oncorhynchus mykiss</i>	nibrin
XP_029928446.1	<i>Myripristis murdjan</i>	nibrin
XP_030014179.1	<i>Sphaeramia orbicularis</i>	nibrin
XP_020462857.1	<i>Monopterus albus</i>	nibrin
XP_028435477.1	<i>Perca flavescens</i>	nibrin
XP_022621874.1	<i>Seriola dumerili</i>	nibrin
BAP70595.1	<i>Kluyveromyces marxianus</i>	DNA repair protein XRS2
KTB14357.1	<i>Candida glabrata</i>	DNA repair protein XRS2
AAP22944.1	<i>Vanderwaltozyma yarrowii</i>	recombinational repair protein Xrs2
SMN19671.1	<i>Kazachstania saulgeensis</i>	similar to <i>Saccharomyces cerevisiae</i> YDR369C XRS2 Protein required for DNA repair
AQZ10787.1	<i>Zygosaccharomyces parabailii</i>	XRS2 (YDR369C)
NP_010657.3	<i>Saccharomyces cerevisiae</i> S288C	Xrs2p
ABI48903.1	<i>Saccharomyces cariocanus</i>	XRS2
EJT43390.1	<i>Saccharomyces kudriavzevii</i> IFO 1802	XRS2-like protein
ABI48904.1	<i>Saccharomyces pastorianus</i>	XRS2
XP_018223905.1	<i>Saccharomyces eubayanus</i>	XRS2-like protein

Appendix B Table 6 Protein BLAST Query Selected Dma1 Homologs

Accession #	Organism	Product
BAP72975.1	<i>Kluyveromyces marxianus</i>	E3 ubiquitin-protein ligase DMA2
CDH11121.1	<i>Zygosaccharomyces bailii</i> ISA1307	related to E3 ubiquitin-protein ligase DMA2
NP_014283.3	<i>Saccharomyces cerevisiae</i> S288C	ubiquitin-conjugating protein DMA2
NP_011983.1	<i>Saccharomyces cerevisiae</i> S288C	ubiquitin-conjugating protein DMA1
EJT44049.1	<i>Saccharomyces kudriavzevii</i> IFO 1802	DMA1-like protein
OEJ89155.1	<i>Hanseniaspora uvarum</i>	E3 ubiquitin-protein ligase DMA2
ONH68342.1	<i>Cyberlindnera fabianii</i>	E3 ubiquitin-protein ligase DMA2
XP_026589568.1	<i>Candida viswanathii</i>	E3 ubiquitin-protein ligase DMA2
XP_712303.2	<i>Candida albicans</i> SC5314	ubiquitin-conjugating protein
OVF08415.1	<i>Clavispota lusitaniae</i>	putative ubiquitin-conjugating protein
XP_020063987.1	<i>Suomyces tanzawaensis</i> NRRL Y-17324	SMAD/FHA domain-containing protein
XP_006687842.1	<i>Yamadazyma tenuis</i> ATCC 10573	SMAD/FHA domain-containing protein
ONH74380.1	<i>Pichia kudriavzevii</i>	E3 ubiquitin-protein ligase DMA2
OMP83762.1	<i>Diplodia seriata</i>	E3 ubiquitin-protein ligase DMA1
TKX20975.1	<i>Elsinoe australis</i>	E3 ubiquitin-protein ligase DMA2
PNS19365.1	<i>Sphaceloma murrayae</i>	E3 ubiquitin-protein ligase DMA2
KKF94563.1	<i>Ceratocystis platani</i>	E3 ubiquitin-protein ligase DMA2
PHH49269.1	<i>Ceratocystis fimbriata</i> CBS 114723	E3 ubiquitin-protein ligase DMA2
XP_017995465.1	<i>Phialophora attae</i>	E3 ubiquitin-protein ligase DMA2
TVY44060.1	<i>Lachnellula subtilissima</i>	E3 ubiquitin-protein ligase
TVY34760.1	<i>Lachnellula occidentalis</i>	E3 ubiquitin-protein ligase
TVY19895.1	<i>Lachnellula arida</i>	E3 ubiquitin-protein ligase DMA1
TVY57914.1	<i>Lachnellula cervina</i>	E3 ubiquitin-protein ligase DMA1
CZR57194.1	<i>Phialocephala subalpina</i>	related to dma1, component of spindle assembly checkpoint
CZT41004.1	<i>Rhynchosporium secalis</i>	related to dma1, component of spindle assembly checkpoint
KUI65069.1	<i>Valsa mali</i>	E3 ubiquitin-protein ligase DMA2
KXX78950.1	<i>Madurella mycetomatis</i>	E3 ubiquitin-protein ligase DMA2
OLN85651.1	<i>Colletotrichum chlorophyti</i>	E3 ubiquitin-protein ligase DMA2
TDZ35230.1	<i>Colletotrichum trifolii</i>	E3 ubiquitin-protein ligase DMA2
TDZ18847.1	<i>Colletotrichum orbiculare</i> MAFF 240422	E3 ubiquitin-protein ligase DMA2
CCE32481.1	<i>Claviceps purpurea</i> 20.1]	related to dma1, component of spindle assembly checkpoint
KGQ07648.1	<i>Beauveria bassiana</i> D1-5	E3 ubiquitin-protein ligase DMA2
KFH41647.1	<i>Acremonium chrysogenum</i> ATCC 11550	E3 ubiquitin-protein ligase-like protein
PTD11087.1	<i>Fusarium culmorum</i>	E3 ubiquitin-protein ligase DMA2
SCV30046.1	<i>Fusarium fujikuroi</i>	related to dma1, component of spindle assembly checkpoint
CVL01843.1	<i>Fusarium mangiferae</i>	related to dma1, component of spindle assembly checkpoint

Appendix B Table 7 Protein BLAST Query Selected Dma2 Homologs

Accession #	Organism	Product
ONH68342.1	<i>Cyberlindnera fabianii</i>	E3 ubiquitin-protein ligase DMA2
XP_003870781.1	<i>Candida orthopsilosis</i> Co 90-125	Dma1 protein
XP_712303.2	<i>Candida albicans</i> SC5314	ubiquitin-conjugating protein
XP_026589568.1	<i>Candida viswanathii</i>	E3 ubiquitin-protein ligase DMA2
ONH74380.1	<i>Pichia kudriavzevii</i>	E3 ubiquitin-protein ligase DMA2
TKX20975.1	<i>Elsinoe australis</i>	E3 ubiquitin-protein ligase DMA2
PNS19365.1	<i>Sphaceloma murrayae</i>	E3 ubiquitin-protein ligase DMA2
CZR57194.1	<i>Phialocephala subalpina</i>	related to dma1, component of spindle assembly checkpoint
TVY34760.1	<i>Lachnellula occidentalis</i>	E3 ubiquitin-protein ligase
TVY58157.1	<i>Lachnellula suecica</i>	E3 ubiquitin-protein ligase DMA2
KKF94563.1	<i>Ceratocystis platani</i>	E3 ubiquitin-protein ligase DMA2
PHH49269.1	<i>Ceratocystis fimbriata</i> CBS 114723	E3 ubiquitin-protein ligase DMA2
KUI65069.1	<i>Valsa mali</i>	E3 ubiquitin-protein ligase DMA2
KXX78950.1	<i>Madurella mycetomatis</i>	E3 ubiquitin-protein ligase DMA2
TDZ35230.1	<i>Colletotrichum trifolii</i>	E3 ubiquitin-protein ligase DMA2
OLN85651.1	<i>Colletotrichum chlorophyti</i>	E3 ubiquitin-protein ligase DMA2
CCE32481.1	<i>Claviceps purpurea</i> 20.1	related to dma1, component of spindle assembly checkpoint
KFH41647.1	<i>Acremonium chrysogenum</i> ATCC 11550	E3 ubiquitin-protein ligase-like protein
KGQ07648.1	<i>Beauveria bassiana</i> D1-5	E3 ubiquitin-protein ligase DMA2
PTD11087.1	<i>Fusarium culmorum</i>	E3 ubiquitin-protein ligase DMA2
KLP11871.1	<i>Fusarium fujikuroi</i>	dma1, component of spindle assembly checkpoint
CVL01843.1	<i>Fusarium mangiferae</i>	related to dma1, component of spindle assembly checkpoint
OEJ88286.1	<i>Hanseniaspora osmophila</i>	E3 ubiquitin-protein ligase DMA2
OEJ89155.1	<i>Hanseniaspora uvarum</i>	E3 ubiquitin-protein ligase DMA2
BAP72975.1	<i>Kluyveromyces marxianus</i>	E3 ubiquitin-protein ligase DMA2
SCV13248.1	<i>Candida glabrata</i>	related to E3 ubiquitin-protein ligase DMA2
NP_014283.3	<i>Saccharomyces cerevisiae</i> S288C	ubiquitin-conjugating protein DMA2
CDH17377.1	<i>Zygosaccharomyces bailii</i> ISA1307	related to E3 ubiquitin-protein ligase DMA2
NP_011983.1	<i>Saccharomyces cerevisiae</i> S288C	ubiquitin-conjugating protein DMA1
XP_018221897.1	<i>Saccharomyces eubayanus</i>	DMA1-like protein

Appendix B Table 8 Protein BLAST Query Selected Fhl1 Homologs

Accession #	Organism	Product
OEJ87605.1	<i>Hanseniaspora opuntiae</i>	Pre-rRNA-processing protein FHL1
RDB23270.1	<i>Hypsizygus marmoreus</i>	Pre-rRNA-processing protein FHL1
TCD71369.1	<i>Steccherinum ochraceum</i>	Pre-rRNA-processing protein fhl1
OBZ76889.1	<i>Grifola frondosa</i>	Fork-head transcriptional regulator 2
OJT10535.1	<i>Trametes pubescens</i>	Fork-head transcriptional regulator 2
OMJ27350.1	<i>Smittium culicis</i>	Fork head transcription factor 1
XP_009265692.1	<i>Wallemia ichthyophaga</i> EXF-994	Pre-rRNA-processing protein FHL1
OEJ85051.1	<i>Hanseniaspora osmophila</i>	Pre-rRNA-processing protein FHL1
AYO44122.1	<i>Malassezia restricta</i> CBS 7877	Pre-rRNA-processing protein FHL1
XP_714827.2	<i>Candida albicans</i> SC5314	Fhl1p
XP_003867708.1	<i>Candida orthopsilosis</i> Co 90-125	Fhl1 transcription factor
RCH84511.1	<i>Rhizopus azygosporus</i>	Pre-rRNA-processing protein fhl1
OBZ81142.1	<i>hoanephora cucurbitarum</i>	Fork-head transcriptional regulator 2
CDO55671.1	<i>Geotrichum candidum</i>	similar to <i>Saccharomyces cerevisiae</i> YPR104C FHL1 Regulator of ribosomal protein (RP) transcription
ONH74933.1	<i>Pichia kudriavzevii</i>	Pre-rRNA-processing protein FHL1
XP_024664099.1	<i>Wickerhamiella sorbophila</i>	Fork-head transcriptional regulator FHL1
NP_015429.1	<i>Saccharomyces cerevisiae</i> S288C	Fhl1p
KTB19613.1	<i>Candida glabrata</i>	Pre-rRNA-processing protein FHL1
BAP70339.1	<i>Kluyveromyces marxianus</i>	pre-rRNA-processing protein FHL1
GCE99203.1	<i>Zygosaccharomyces mellis</i>	pre-rRNA-processing protein fhl1
EXX58090.1	<i>Rhizophagus irregularis</i> DAOM 197198w	Fhl1p
NP_594272.1	<i>Schizosaccharomyces pombe</i>	fork head transcription factor Fhl1
XP_013934614.1	<i>Ogataea parapolymorpha</i> DL-1	Pre-rRNA-processing protein FHL1
XP_011276948.1	<i>Wickerhamomyces ciferrii</i>	Pre-rRNA-processing protein
ONH68329.1	<i>Cyberlindnera fabianii</i>	Pre-rRNA-processing protein FHL1
CEP22730.1	<i>Cyberlindnera jadinii</i>	FHL1

Appendix B Table 9 Protein BLAST Query Selected Fkh1 Homologs

Accession #	Organism	Product
OEJ89402.1	<i>Hanseniaspora uvarum</i>	Fork head 2
CRL17746.1	<i>Penicillium camemberti</i>	SMAD/FHA domain
GAQ36547.1	<i>Aspergillus niger</i>	forkhead transcription factor Fkh1/2
XP_957006.1	<i>Neurospora crassa</i> OR74A	FKH1
XP_014174987.1	<i>Grosmannia clavigera</i> kw1407	forkhead transcription factor fkh1
EPE10174.1	<i>Ophiostoma piceae</i> UAMH 11346	forkhead transcription factor fkh1
XP_003002403.1	<i>Verticillium alfalfae</i> VaMs.102	FKH1
OLN81816.1	<i>Colletotrichum chlorophyti</i>	Fork-head transcriptional regulator 2
TDZ29250.1	<i>Colletotrichum spinosum</i>	Fork head protein-like protein 1
XP_016642752.1	<i>Scedosporium apiospermum</i>	FKH1-like protein
KOS19255.1	<i>Escovopsis weberi</i>	Fork-head transcriptional regulator 2
RFU74167.1	<i>Trichoderma arundinaceum</i>	fork head transcription factor
OPB45948.1	<i>Trichoderma guizhouense</i>	Forkhead
TQV92378.1	<i>Cordyceps javanica</i>	forkhead transcription factor Fkh1/2
KGQ08078.1	<i>Beauveria bassiana</i> D1-5	Fork head protein 1
KDB17429.1	<i>Ustilaginoidea virens</i>	Ustilaginoidea virens
KPA40119.1	<i>Fusarium langsethiae</i>	fkh1
PTD04904.1	<i>Fusarium culmorum</i>	Fork-head transcriptional regulator 2
XP_018145094.1	<i>Pochonia chlamydosporia</i> 170	forkhead transcription factor Fkh1/2
XP_007812818.1	<i>Metarhizium acridum</i> CQMa 102	FKH1-like protein
OAQ80443.1	<i>Purpureocillium lilacinum</i>	forkhead transcription factor Fkh1/2
PNY26601.1	<i>Tolypocladium capitatum</i>	Fork-head transcriptional regulator 2
KND92644.1	<i>Tolypocladium ophioglossoides</i> CBS 100239	Fork-head transcriptional regulator 2
POR37114.1	<i>Tolypocladium paradoxum</i>	Fork-head transcriptional regulator 2
OEJ88296.1	<i>Hanseniaspora osmophila</i>	Fork-head transcriptional regulator 2
ONH77267.1	<i>Pichia kudriavzevii</i>	Fork-head transcriptional regulator 2
XP_026597550.1	<i>Candida viswanathii</i>	Fork-head transcriptional regulator 2
CEP20962.1	<i>Cyberlindnera jadinii</i>	Fork head protein homolog 2
ONH68535.1	<i>Cyberlindnera fabianii</i>	Fork-head transcriptional regulator 2
XP_011275688.1	<i>Wickerhamomyces ciferrii</i>	Fork head protein
BAP73260.1	<i>Kluyveromyces marxianus</i>	fork head protein homolog 2
NP_012135.1	<i>Saccharomyces cerevisiae</i> S288C	forkhead family transcription factor FKH1
NP_014331.3	<i>Saccharomyces cerevisiae</i> S288C	forkhead family transcription factor FKH2
SCV12779.1	<i>Candida glabrata</i>	related to Fork head protein homolog 2

Appendix B Table 10 Protein BLAST Query Selected Fkh2 Homologs

Accession #	Organism	Product
XP_018735449.1	<i>Sugiyamaella lignohabitans</i>	forkhead family transcription factor FKH2
ORY82022.1	<i>Protomyces lactucaedebilis</i>	fork head domain-domain-containing protein
XP_024663841.1	<i>Wickerhamiella sorbophila</i>	Fork-head transcriptional regulator 2
OEJ88106.1	<i>Hanseniaspora opuntiae</i>	Fork head 2
OEJ89402.1	<i>Hanseniaspora uvarum</i>	Fork head 2
KMU85147.1	<i>Coccidioides immitis H538.4</i>	fork head transcription factor Fkh2
OKO99307.1	<i>Penicillium subrubescens</i>	Fork-head transcriptional regulator 2
KKK14209.1	<i>Aspergillus ochraceoroseus</i>	forkhead transcription factor Fkh1/2
XP_028483776.1	<i>Byssoschlamys spectabilis</i>	forkhead transcription factor Fkh1/2
XP_013326251.1	<i>Rasamsonia emersonii CBS 393.64</i>	Forkhead transcription factor Fkh1/2
RKF64128.1	<i>Oidium neolycopersici</i>	Fork-head transcriptional regulator 2
RKF77835.1	<i>Golovinomyces cichoracearum</i>	Fork head protein-like protein 1
PHH52226.1	<i>Ceratocystis fimbriata CBS 114723</i>	Fork-head transcriptional regulator 2
OAA65932.1	<i>Sporothrix insectorum RCEF 264</i>	forkhead transcription factor fkh1
EPE10174.1	<i>Ophiostoma piceae UAMH 11346</i>	forkhead transcription factor fkh1
XP_003002403.1	<i>Verticillium alfalfae VaMs.102</i>	FKH1
ELA27359.1	<i>Colletotrichum fructicola Nara gc5</i>	forkhead transcription factor fkh1
KLP06325.1	<i>Fusarium fujikuroi</i>	forkhead protein
KOS19255.1	<i>Escovopsis weberi</i>	Fork-head transcriptional regulator 2
OEJ88296.1	<i>Hanseniaspora osmophila</i>	Fork-head transcriptional regulator 2
BAP73260.1	<i>Kluyveromyces marxianus</i>	fork head protein homolog 2
NP_012135.1	<i>Saccharomyces cerevisiae S288C</i>	forkhead family transcription factor FKH1
NP_014331.3	<i>Saccharomyces cerevisiae S288C</i>	forkhead family transcription factor FKH2
KTB19864.1	<i>Candida glabrata</i>	Fork head protein -like protein 2
ONH77267.1	<i>Pichia kudriavzevii</i>	Fork-head transcriptional regulator 2
CEP20962.1	<i>Cyberlindnera jadinii</i>	Fork head protein homolog 2
ONH68535.1	<i>Cyberlindnera fabianii</i>	Fork-head transcriptional regulator 2
XP_011275688.1	<i>Wickerhamomyces ciferrii</i>	Fork head protein
XP_003869703.1	<i>Candida orthopsilosis Co 90-125</i>	Fkh2 forkhead transcription factor
XP_026597550.1	<i>Candida viswanathii</i>	Fork-head transcriptional regulator 2

Appendix B Table 11 Protein BLAST Query Selected Plm2 Homologs

Accession #	Organism	Product
OEJ91465.1	<i>Hanseniaspora opuntiae</i>	Protein TOS4
OEJ92832.1	<i>Hanseniaspora uvarum</i>	Protein TOS4
OEJ86255.1	<i>Hanseniaspora osmophila</i>	Protein PLM2
NP_010789.1	<i>Saccharomyces cerevisiae S288C</i>	Plm2p
NP_013284.1	<i>Saccharomyces cerevisiae S288C</i>	Tos4p
BAP69274.1	<i>Kluyveromyces marxianus</i>	protein PLM2
AAO25598.1	<i>Nakaseomyces delphensis</i>	TOS4
KTB16471.1	<i>Candida glabrata</i>	Protein TOS4
XP_026594000.1	<i>Candida viswanathii</i>	Protein PLM2
XP_713801.2	<i>Candida albicans SC5314</i>	Tos4p
KJX99201.1	<i>Zymoseptoria brevis</i>	transcription factor tos4 like protein
XP_023457797.1	<i>Cercospora beticola</i>	Protein PLM2
XP_018003444.1	<i>Phialophora attae</i>	Protein PLM2
OAL72471.1	<i>Trichophyton violaceum</i>	transcription factor Tos4
OAT09918.1	<i>Blastomyces gilchristii SLH14081</i>	transcription factor Tos4
CRG89064.1	<i>Talaromyces islandicus</i>	Protein TOS4
GAE00042.1	<i>Byssochlamys spectabilis</i> No. 5	transcription factor Tos4
OOQ83841.1	<i>Penicillium brasilianum</i>	transcription factor Tos4
GAQ39696.1	<i>Aspergillus niger</i>	transcription factor Tos4
GCB17339.1	<i>Aspergillus awamori</i>	protein PLM2
RJE17536.1	<i>Aspergillus sclerotialis</i>	transcription factor Tos4
RAQ49397.1	<i>Aspergillus flavus</i>	transcription factor Tos4
KUI69091.1	<i>Valsa mali</i>	Protein PLM2
POS77472.1	<i>Diaporthe helianthi</i>	transcription factor Tos4
CZR66749.1	<i>Phialocephala subalpina</i>	related to transcription factor PLM2
XP_001728193.2	<i>Neurospora crassa OR74A</i>	transcription factor Tos4
KXX79720.1	<i>Madurella mycetomatis</i>	Protein PLM2
KOS20468.1	<i>Escovopsis weberi</i>	Protein PLM2
XP_016640323.1	<i>Scedosporium apiospermum</i>	Transcription factor tos4
RZR65456.1	<i>Pochonia chlamydosporia 123</i>	transcription factor Tos4
TDZ46160.1	<i>Colletotrichum trifolii</i>	Protein PLM2
TID06293.1	<i>Colletotrichum higginsianum</i>	Protein PLM2
TKW55910.1	<i>Colletotrichum tanacetii</i>	Protein PLM2

Appendix B Table 12 Protein BLAST Query Selected Pml1 Homologs

Accession #	Organism	Product
GAM39712.1	<i>Talaromyces cellulolyticus</i>	FHA domain protein
GAQ04319.1	<i>Aspergillus lentulus</i>	smad nuclear-interacting protein 1
XP_025513955.1	<i>Aspergillus piperis</i> CBS 112811	SMAD/FHA domain-containing protein
PYI03093.1	<i>Aspergillus sclerotii</i> carbonarius CBS 121057	SMAD/FHA domain-containing protein
XP_959230.3	<i>Neurospora crassa</i> OR74A	FHA domain-containing protein SNIP1
KFH48208.1	<i>Acremonium chrysogenum</i> ATCC 11550	Smad nuclear-interacting protein-like protein
OTA05455.1	<i>Trichoderma parareesei</i>	FHA domain protein SNIP1
TDZ24932.1	<i>Colletotrichum orbiculare</i> MAFF 240422	FHA domain-containing protein DDL
XP_023428855.1	<i>Fusarium fujikuroi</i> IMI 58289	related to PML1 Subunit of the RES complex, which is required for nuclear retention of unspliced pre-mRNAs
XP_028470552.1	<i>Sodiomyces alkalinus</i> F11	SMAD/FHA domain-containing protein
CZT42512.1	<i>Rhynchosporium secalis</i>	related to PML1 Subunit of the RES complex, which is required for nuclear retention of unspliced pre-mRNAs
CZT01677.1	<i>Rhynchosporium commune</i>	related to PML1 Subunit of the RES complex, which is required for nuclear retention of unspliced pre-mRNAs
XP_024744438.1	<i>Meliniomyces bicolor</i> E	Meliniomyces bicolor E
CZR64395.1	<i>Phialocephala subalpina</i>	related to PML1 Subunit of the RES complex, which is required for nuclear retention of unspliced pre-mRNAs
TVY62780.1	<i>Lachnellula suecica</i>	Smad nuclear interacting protein
TVY91508.1	<i>Lachnellula willkommii</i>	Smad nuclear interacting protein
SHO80143.1	<i>Malassezia sympodialis</i> ATCC 42132	Similar to S.cerevisiae protein PML1 (Subunit of the RES complex)
PIA15820.1	<i>Coemansia reversa</i> NRRL 1564	SMAD/FHA domain-containing protein
KXS14160.1	<i>Gonapodya prolifera</i> JEL478	SMAD/FHA domain-containing protein
XP_016274201.1	<i>Rhodotorula toruloides</i> NP11	smad nuclear-interacting protein 1
XP_023467858.1	<i>Rhizopus microsporus</i> ATCC 52813	SMAD/FHA domain-containing protein
RXK40511.1	<i>Tremella mesenterica</i>	smad nuclear-interacting protein 1
KZT73758.1	<i>Daedalea quercina</i> L-15889	SMAD/FHA domain-containing protein
TEB39717.1	<i>Coprinellus micaceus</i>	SMAD/FHA domain-containing protein
KIY72508.1	<i>Cylindrobasidium torrendii</i> FP15055 ss-10	SMAD/FHA domain-containing protein
SJL07461.1	<i>Armillaria ostoyae</i>	related to PML1 Subunit of the RES complex, which is required for nuclear retention of unspliced pre-mRNAs
NP_013116.1	<i>Saccharomyces cerevisiae</i> S288C	Pml1p
XP_022675008.1	<i>Kluyveromyces marxianus</i> DMKU3-1042	pre-mRNA leakage protein 1
SMN18619.1	<i>Kazachstania saulgeensis</i>	similar to Saccharomyces cerevisiae YLR016C PML1 Subunit of the RES complex, which is required for nuclear retention of unspliced pre-mRNAs
AQZ12033.1	<i>Zygosaccharomyces parabailii</i>	PML1 (YLR016C)

Appendix B Table 13 Protein BLAST Query Selected Far10 Homologs

Accession #	Organism	Product
XP_002847852.1	<i>Microsporum canis</i> CBS 113480	vacuolar protein sorting-associated protein 64
XP_003176435.1	<i>Nannizzia gypsea</i> CBS 118893	vacuolar protein sorting-associated protein 64
EGE01826.1	<i>Trichophyton equinum</i> CBS 127.97	cytoplasm to vacuole targeting Vps64
KMU79125.1	<i>Coccidioides immitis</i> RMSCC 3703	factor arrest protein 10
XP_002629183.1	<i>Blastomyces gilchristii</i> SLH14081	cytoplasm to vacuole targeting Vps64
XP_028489842.1	<i>Byssoschlamys spectabilis</i>	cytoplasm to vacuole targeting Vps64
XP_013330151.1	<i>Rasamsonia emersonii</i> CBS 393.64	Cytoplasm to vacuole targeting Vps64
OOQ83074.1	<i>Penicillium brasilianum</i>	cytoplasm to vacuole targeting Vps64
XP_025510790.1	<i>Aspergillus piperis</i> CBS 112811	cytoplasm to vacuole targeting Vps64
GAQ39236.1	<i>Aspergillus niger</i>	cytoplasm to vacuole targeting Vps64
XP_018068267.1	<i>Phialocephala scopiformis</i>	cytoplasm to vacuole targeting protein-like protein Vps64
KXX76658.1	<i>Madurella mycetomatis</i>	Vacuolar protein sorting-associated protein 64
KYK58998.1	<i>Drechmeria coniospora</i>	cytoplasm to vacuole targeting Vps64
ELA23567.1	<i>Colletotrichum fruticola</i> Nara gc5	cytoplasm to vacuole targeting vps64
OAA52614.1	<i>Beauveria brongniartii</i> RCEF 3172	cytoplasm to vacuole targeting Vps64
XP_006670455.1	<i>Cordyceps militaris</i> CM01	cytoplasm to vacuole targeting Vps64
RGP74911.1	<i>Fusarium sporotrichioides</i>	vps64 vacuolar sorting
CVK93831.1	<i>Fusarium mangiferae</i>	related to VPS64 Vacuolar protein sorting
KLO85988.1	<i>Fusarium fujikuroi</i>	VPS64 Vacuolar protein sorting
OAQ67472.1	<i>Purpureocillium lilacinum</i>	cytoplasm to vacuole targeting Vps64
KHO01290.1	<i>Metarhizium album</i> ARSEF 1941	cytoplasm to vacuole targeting Vps64
XP_018137867.1	<i>Pochonia chlamydosporia</i> 170	cytoplasm to vacuole targeting Vps64
KID66005.1	<i>Metarhizium anisopliae</i> ARSEF 549	cytoplasm to vacuole targeting Vps64
KTA96261.1	<i>Candida glabrata</i>	Factor arrest protein 10
NP_013339.1	<i>Saccharomyces cerevisiae</i> S288C	Far10p
BAP72685.1	<i>Kluyveromyces marxianus</i>	factor arrest protein 10
NP_010486.3	<i>Saccharomyces cerevisiae</i> S288C	Vps64p
KTB00597.1	<i>Candida glabrata</i>	Vacuolar protein sorting-associated protein 64

Appendix B Table 14 Protein BLAST Query Selected Tos4 Homologs

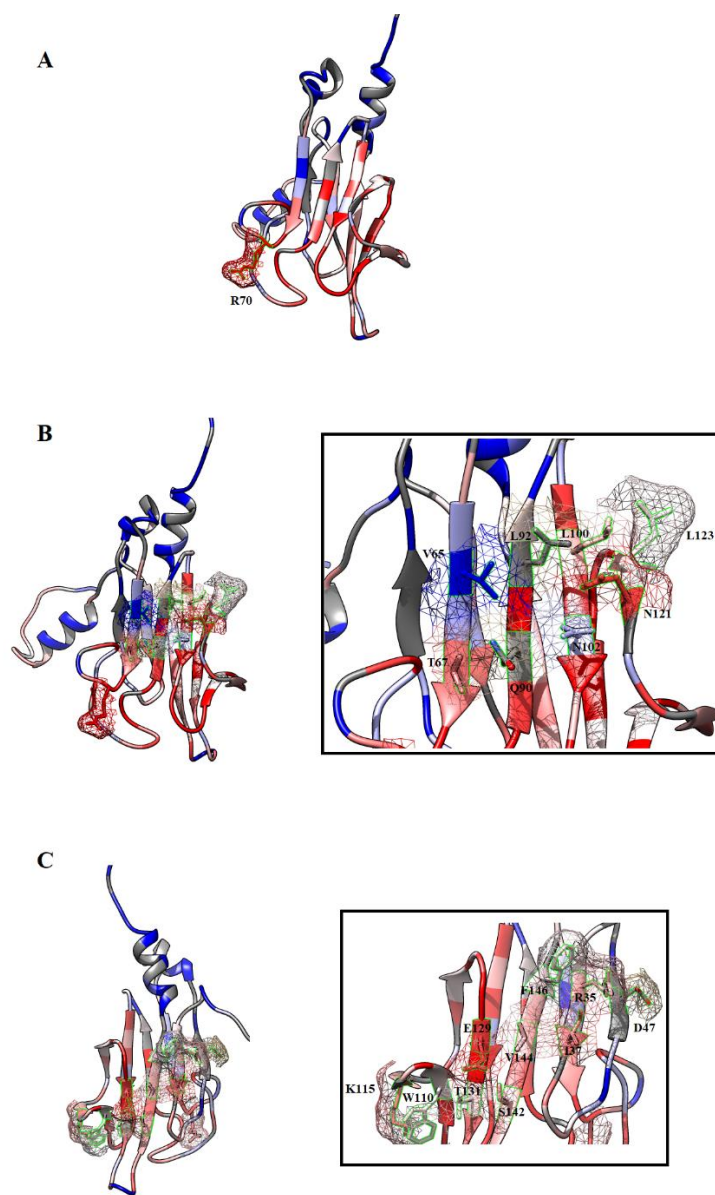
Accession #	Organism	Product
XP_018735662.1	<i>Sugiyamaella lignohabitans</i>	Tos4p
XP_020125578.1	<i>Diplodia corticola</i>	transcription factor tos4
OMP82222.1	<i>Diplodia seriata</i>	Protein PLM2
OAT09918.1	<i>Blastomyces gilchristii SLH14081</i>	transcription factor Tos4
EGC46565.1	<i>Histoplasma capsulatum H88</i>	transcription factor Tos4
OOQ83841.1	<i>Penicillium brasilianum</i>	transcription factor Tos4
GAE00042.1	<i>Byssosclamyces spectabilis</i> No. 5	transcription factor Tos4
XP_013329252.1	<i>Rasamsonia emersonii</i> CBS 393.64	Transcription factor Tos4
GAQ05806.1	<i>Aspergillus lentulus</i>	protein TOS4
RAQ49397.1	<i>Aspergillus flavus</i>	transcription factor Tos4
GAQ39696.1	<i>Aspergillus niger</i>	transcription factor Tos4
GCB17339.1	<i>Aspergillus awamori</i>	protein PLM2
POS77472.1	<i>Diaporthe helianthi</i>	transcription factor Tos4
KUI53891.1	<i>Valsa mali</i> var. <i>pyri</i>	Protein PLM2
EPE06528.1	<i>Ophiostoma piceae</i> UAMH 11346	transcription factor tos4
XP_016584722.1	<i>Sporothrix schenckii</i> 1099-18	transcription factor Tos4
RZR65456.1	<i>Pochonia chlamydosporia</i> 123	transcription factor Tos4
XP_007824368.2	<i>Metarhizium robertsii</i> ARSEF 23	transcription factor Tos4
KID95947.1	<i>Metarhizium majus</i> ARSEF 297	transcription factor Tos4
KID88956.1	<i>Metarhizium guizhouense</i> ARSEF 977	transcription factor Tos4
KXX79720.1	<i>Madurella mycetomatis</i>	Protein PLM2
XP_001728193.2	<i>Neurospora crassa</i> OR74A	transcription factor Tos4
NP_010789.1	<i>Saccharomyces cerevisiae</i> S288C	Plm2p
NP_013284.1	<i>Saccharomyces cerevisiae</i> S288C	Tos4p
KTb16471.1	<i>Candida glabrata</i>	Protein TOS4

Appendix B Table 15 Protein BLAST Query Selected Vps64 Homologs

Accession #	Organism	Product
XP_018068267.1	<i>Phialocephala scopiformis</i>	cytoplasm to vacuole targeting protein-like protein Vps64
CZT01908.1	<i>Rhynchosporium commune</i>	related to VPS64 Vacuolar protein sorting
PBP25970.1	<i>Diplocarpon rosae</i>	cytoplasm to vacuole targeting protein-like protein Vps64
OWO98954.1	<i>Marssonina coronariae</i>	cytoplasm to vacuole targeting protein Vps
KYK58998.1	<i>Drechmeria coniospora</i>	cytoplasm to vacuole targeting Vps64
ATY59613.1	<i>Cordyceps militaris</i>	cytoplasm to vacuole targeting Vps64
TQW08884.1	<i>Cordyceps javanica</i>	cytoplasm to vacuole targeting Vps64
OAA32964.1	<i>Moelleriella libera</i> RCEF 2490	cytoplasm to vacuole targeting Vps64
EQL01738.1	<i>Ophiocordyceps sinensis</i> CO18	cytoplasm to vacuole targeting Vps64
KPA44489.1	<i>Fusarium langsethiae</i>	vps64 vacuolar protein sorting
SCN88677.1	<i>Fusarium fujikuroi</i>	related to VPS64 Vacuolar protein sorting
XP_018178430.1	<i>Purpureocillium lilacinum</i>	cytoplasm to vacuole targeting Vps64
KDB14730.1	<i>Ustilaginoidea virens</i>	cytoplasm to vacuole targeting Vps64
KHO01290.1	<i>Metarhizium album</i> ARSEF 1941	cytoplasm to vacuole targeting Vps64
RZR69483.1	<i>Pochonia chlamydosporia</i> 123	cytoplasm to vacuole targeting Vps64
XP_014542950.1	<i>Metarhizium brunneum</i> ARSEF 3297	cytoplasm to vacuole targeting Vps64
TLD26015.1	<i>Venturia nashicola</i>	cytoplasm to vacuole targeting vps64
KMU79125.1	<i>Coccidioides immitis</i> RMSCC 3703	factor arrest protein 10
EGE84274.1	<i>Blastomyces dermatitidis</i> ATCC 18188	cytoplasm to vacuole targeting Vps64
GAQ39236.1	<i>Aspergillus niger</i>	cytoplasm to vacuole targeting Vps64
XP_013330151.1	<i>Rasamsonia emersonii</i> CBS 393.64	Cytoplasm to vacuole targeting Vps64
XP_028489842.1	<i>Byssoschlamys spectabilis</i>	cytoplasm to vacuole targeting Vps64
XP_020133666.1	<i>Diplodia corticola</i>	cytoplasm to vacuole targeting vps64
RMZ72247.1	<i>Pyrenophora seminiperda</i> CCB06	cytoplasm to vacuole targeting vps64
KNG44218.1	<i>Stemphylium lycopersici</i>	factor arrest protein 10
KTA96261.1	<i>Candida glabrata</i>	Factor arrest protein 10
NP_013339.1	<i>Saccharomyces cerevisiae</i> S288C	Far10p
NP_010486.3	<i>Saccharomyces cerevisiae</i> S288C	Vps64p
SJM83502.1	<i>Zygosaccharomyces bailii</i>	related to Vacuolar protein sorting-associated protein 64
BAP72685.1	<i>Kluyveromyces marxianus</i>	factor arrest protein 10

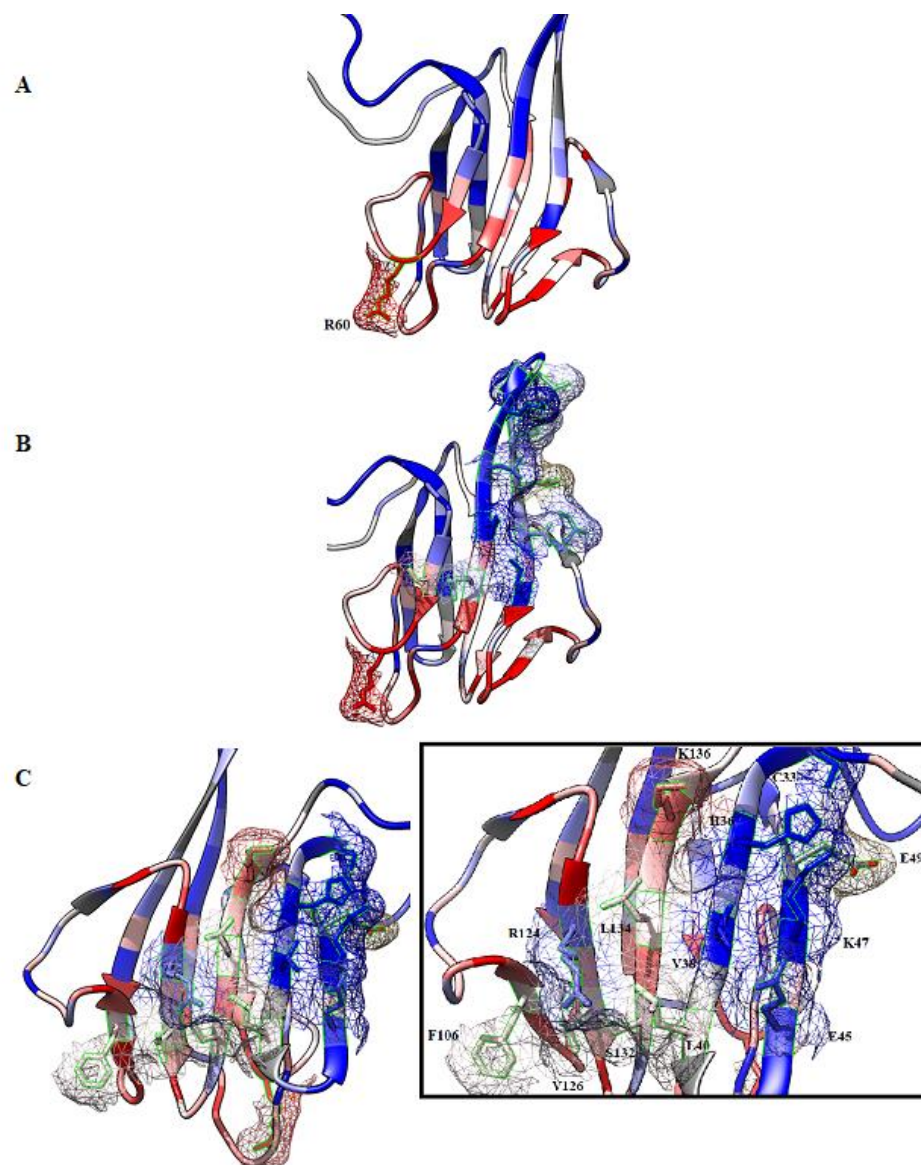
Appendix B Table 16 Protein BLAST Query Selected YGL081W Homologs

Accession #	Organism	Product
NP_011434.1	<i>Saccharomyces cerevisiae</i> S288C	uncharacterized protein YGL081W
EJT43122.1	<i>Saccharomyces kudriavzevii</i> IFO 1802	YGL081W-like protein
EJS43749.1	<i>Saccharomyces arboricola</i> H-6	YGL081W
XP_003685770.1	<i>Tetrapisispora phaffii</i> CBS 4417	hypothetical protein TPHA_0E02440
CDO94971.1	<i>Kluyveromyces dobzhanskii</i> CBS 2104	unnamed protein product
XP_454824.1	<i>Kluyveromyces lactis</i>	uncharacterized protein KLLA0_E19295g
XP_001645589.1	<i>Vanderwaltozyma polyspora</i> DSM 70294	hypothetical protein Kpol_1033p36
XP_003682728.1	<i>Torulaspora delbrueckii</i>	hypothetical protein TDEL_0G01500
SJM83710.1	<i>Zygosaccharomyces bailii</i>	uncharacterized protein ZBIST_1232
AQZ11229.1	<i>Zygosaccharomyces parabailii</i>	YGL081W
XP_002498361.1	<i>Zygosaccharomyces rouxii</i>	uncharacterized protein ZYRO0G08404g
GCE99785.1	<i>Zygosaccharomyces mellis</i>	hypothetical protein ZYGM_001210
SCW00478.1	<i>Lachancea fermentati</i>	LAFE_0C05072g1_1
CUS23205.1	<i>Lachancea quebecensis</i>	LAQU0S08e04434g1_1
XP_002556082.1	<i>Lachancea thermotolerans</i> CBS 6340	KLTH0H04620p
SCU93316.1	<i>Lachancea dasiensis</i> CBS 10888	LADA_0G02432g1_1
XP_022628659.1	<i>Lachancea lanzarotensis</i>	uncharacterized protein LALA0_S05e05556g
SCU80571.1	<i>Lachancea meyersii</i> CBS 8951	LAME_0B03774g1_1
SCU93697.1	<i>Lachancea nothofagi</i> CBS 11611	LANO_0E04566g1_1
XP_017988342.1	<i>Eremothecium sincaudum</i>	HER067Wp
NP_985476.1	<i>Eremothecium gossypii</i> ATCC 10895	AFL072Cp



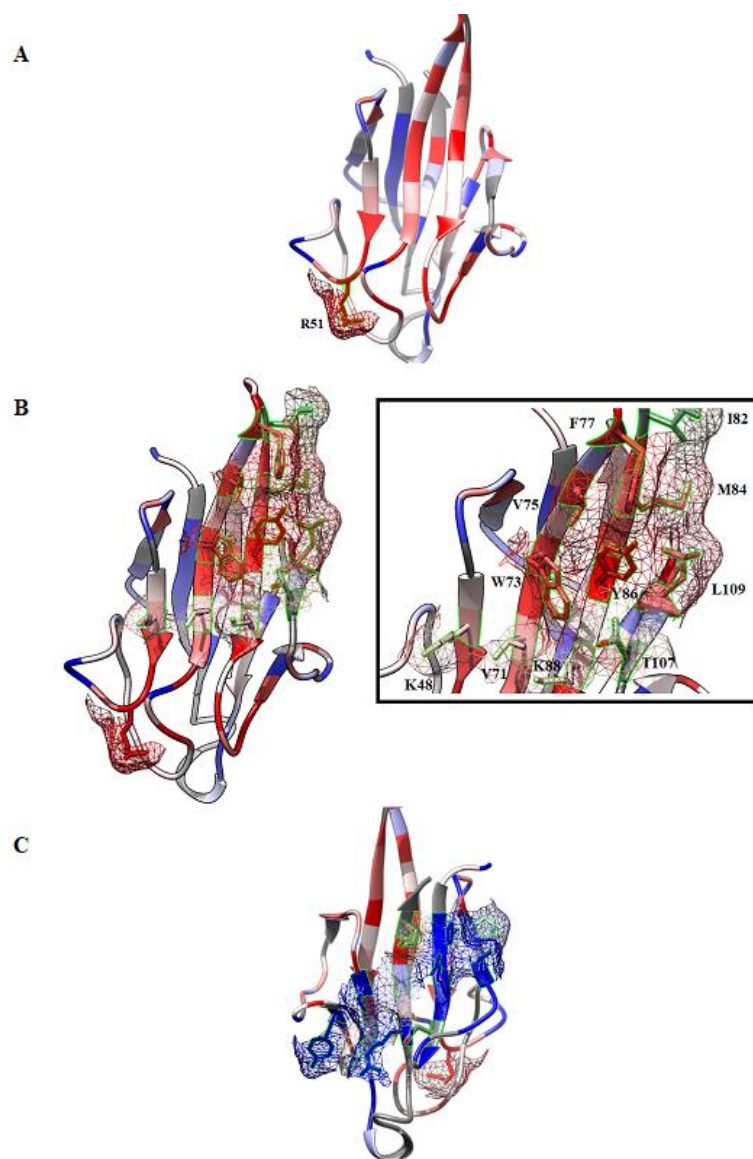
Appendix B Figure 1 Rad53 FHA1 Domain Conservation Mapping

3-D model of the Rad53 FHA1 domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Rad53 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-epitope binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



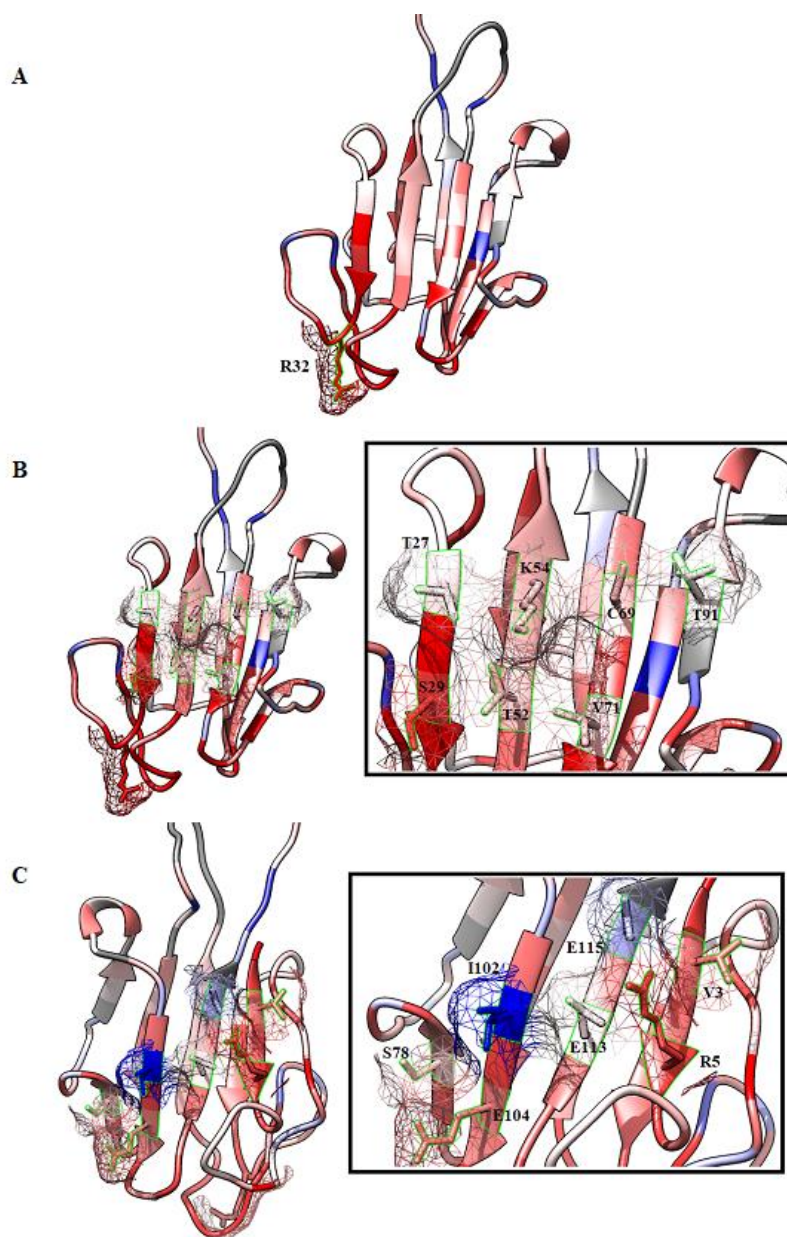
Appendix B Figure 2 Dun1 FHA Domain Conservation Mapping

3-D model of the Dun1 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Dun1 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



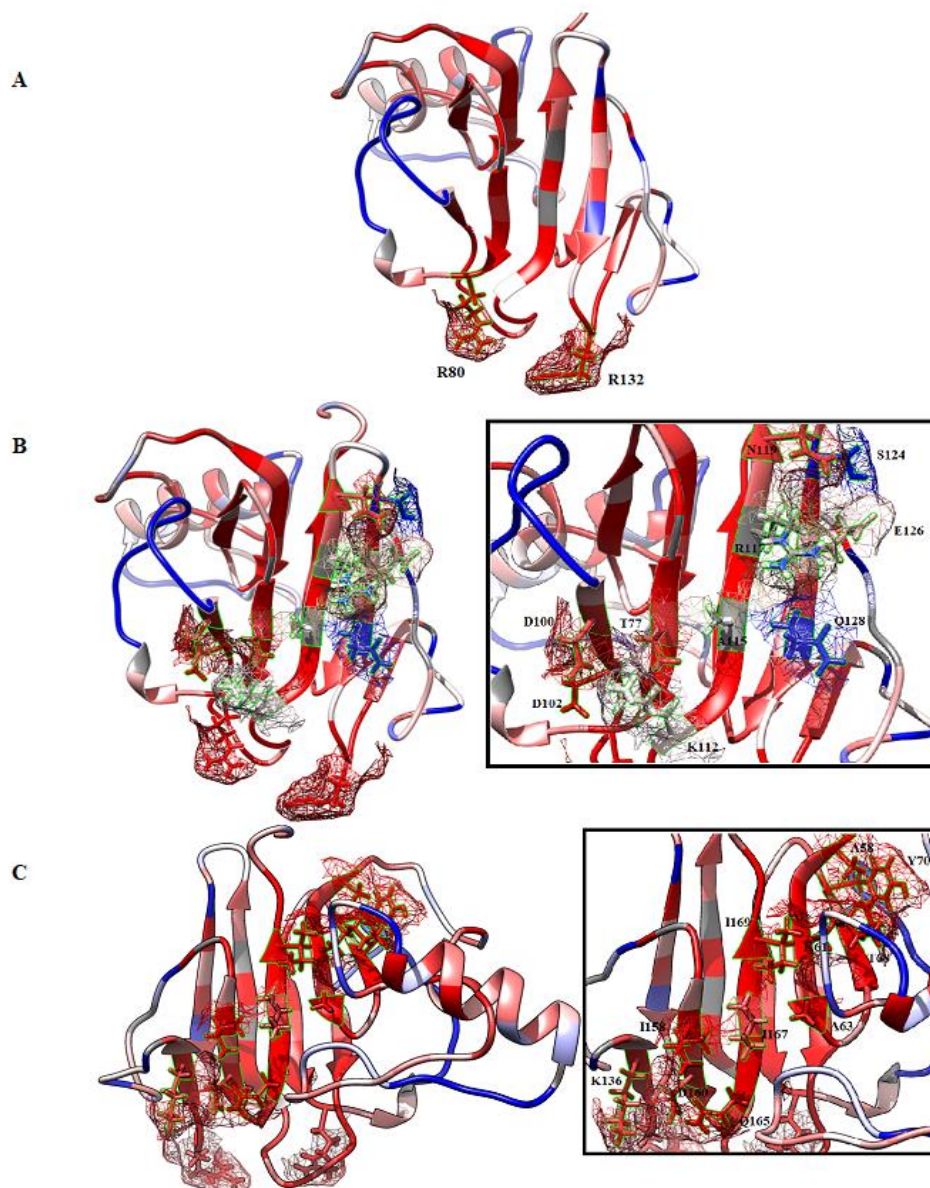
Appendix B Figure 3 Mek1 FHA Domain Conservation Mapping

3-D model of the Mek1 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Mek1 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



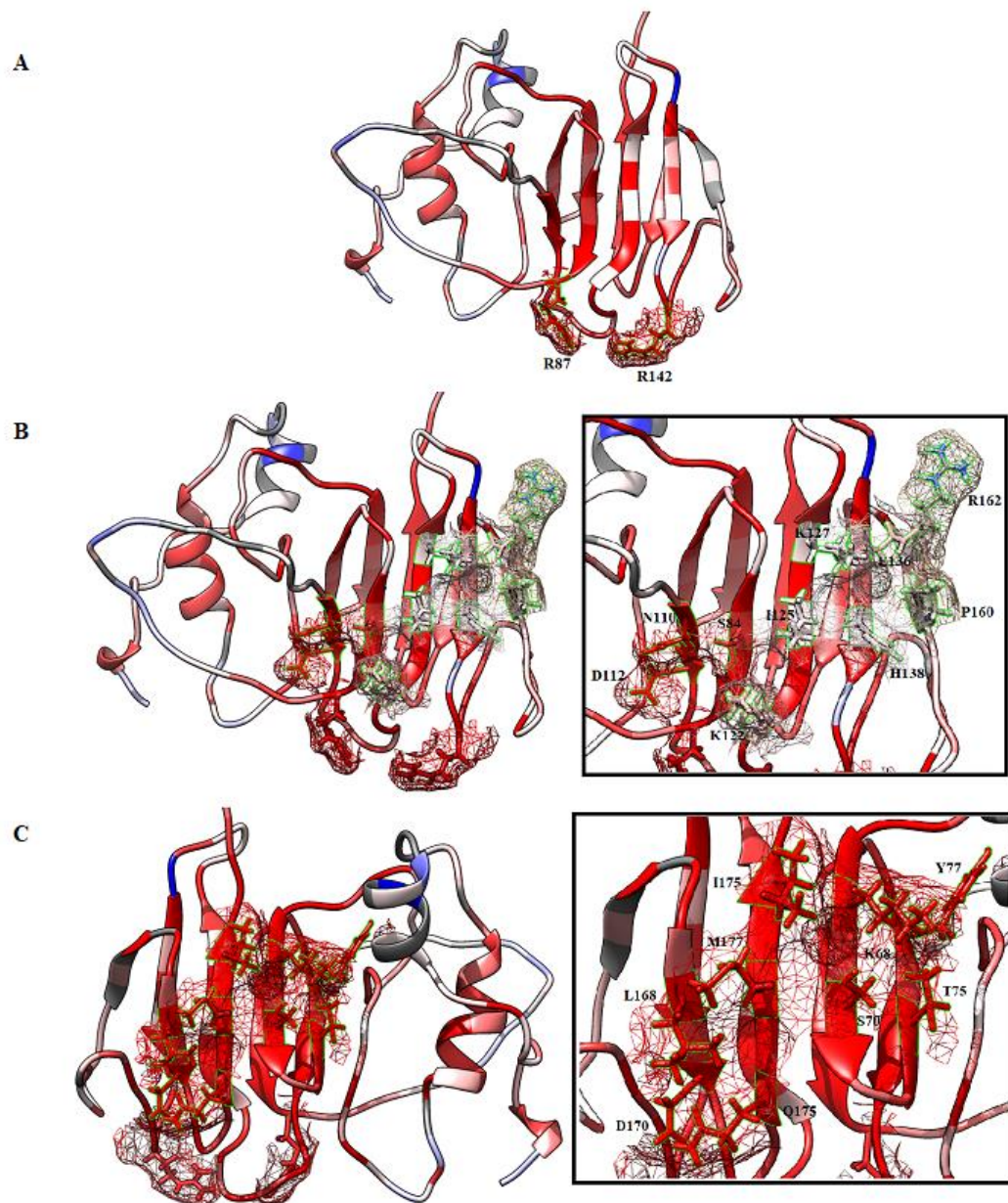
Appendix B Figure 4 Xrs2 FHA Domain Conservation Mapping

3-D model of the Xrs2 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Xrs2 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



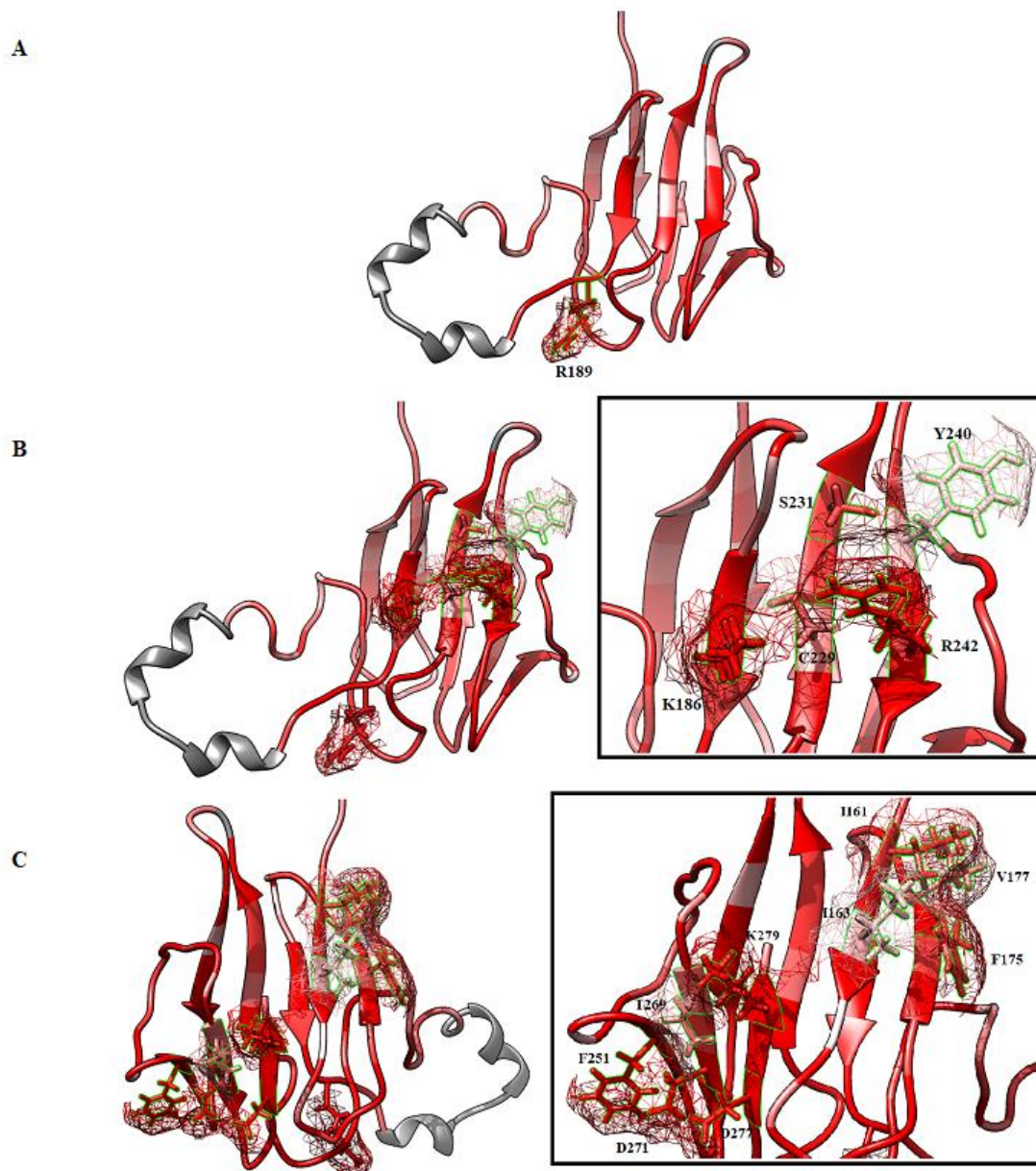
Appendix B Figure 5 Fkh1 FHA Domain Conservation Mapping

3-D model of the Fkh1 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Fkh1 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



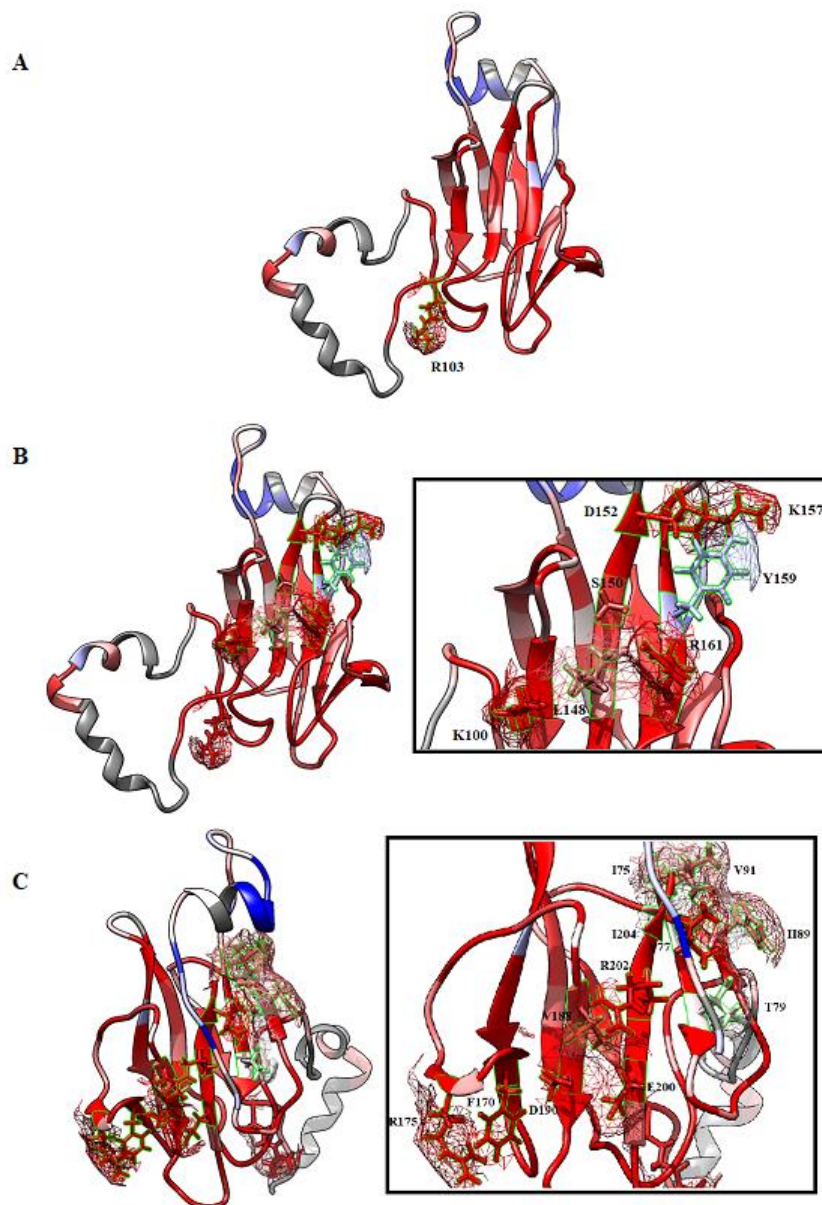
Appendix B Figure 6 Fkh2 FHA Domain Conservation Mapping

3-D model of the Fkh2 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Fkh2 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



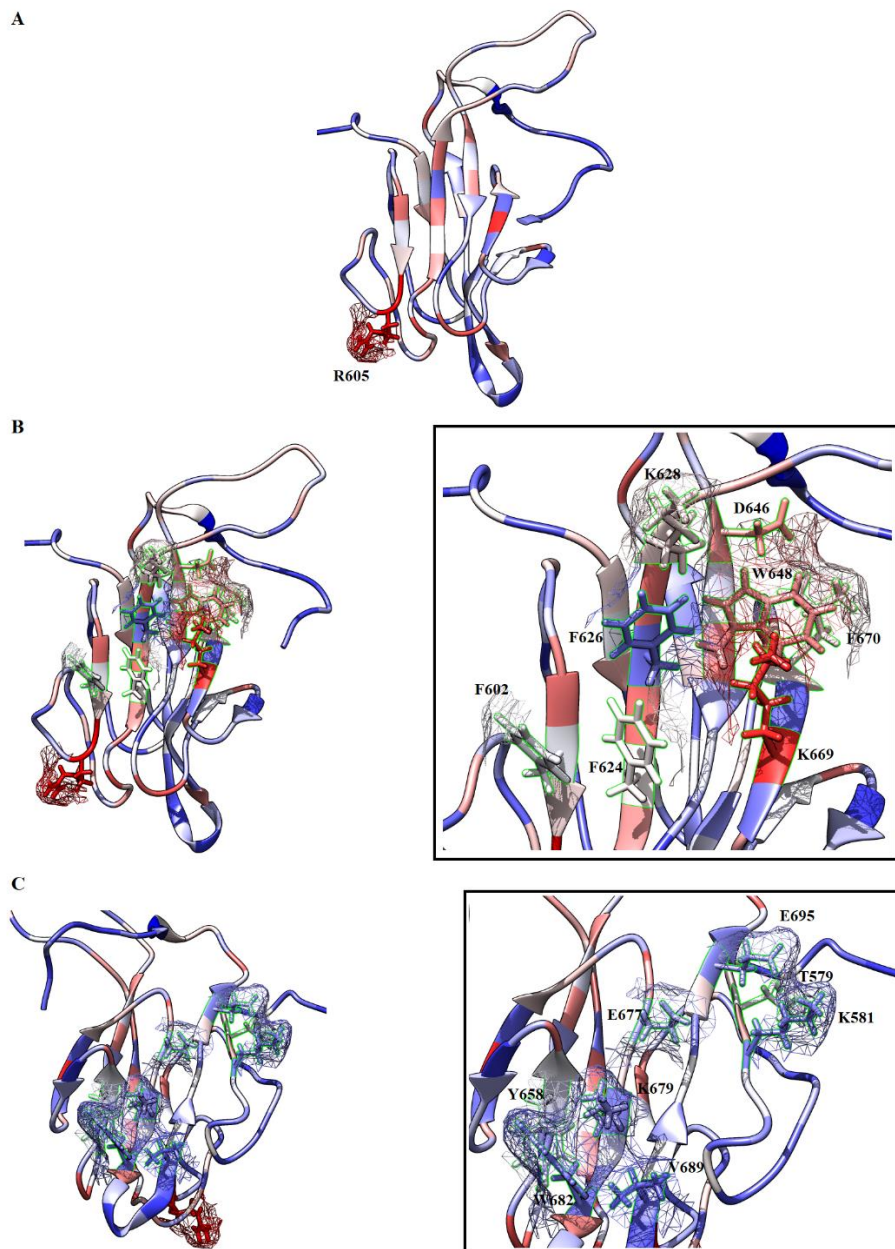
Appendix B Figure 7 Vps64 FHA Domain Conservation Mapping

3-D model of the Vps64 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Vps64 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



Appendix B Figure 8 Far10 FHA Domain Conservation Mapping

3-D model of the Far10 FHA domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Far10 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.



Appendix B Figure 9 Rad53 FHA2 Domain Conservation Mapping

3-D model of the Rad53 FHA2 domain with conservation mapping of conserved residues according to MUSCLE-generated MSAs for Rad53 homologs. Mesh outline indicates lateral surface space of externally facing amino acid side chains. Conservation depiction ranges from blue (no conservation) to red (high conservation). Residues or regions of interest were depicted for the pThr-binding site (A), the proximal lateral surface (B), and the distal lateral surface (C) to illustrate residues identified as possible contributors to protein-protein interactions relative to the pThr-binding site on the apical surface.

Appendix C

Chapter 4 Supplementary Table

Appendix C Table 1 Protein BLAST Query Selected Mek1 Homologs

Accession #	Organism	Product
ONH69372.1	<i>Cyberlindnera fabianii</i>	Meiosis-specific serine/threonine-protein kinase MEK1
XP_026597502.1	<i>Candida viswanathii</i>	Meiosis-specific serine/threonine-protein kinase MEK1
RLV93349.1	<i>Spathaspora sp. JA1</i>	Meiosis-specific serine/threonine-protein kinase MEK1
OEJ92352.1	<i>Hanseniaspora osmophila</i>	Meiosis-specific serine/threonine-protein kinase MEK1
BAP72958.1	<i>Kluyveromyces marxianus</i>	meiosis-specific serine/threonine-protein kinase MEK1
SSD60567.1	<i>Saccharomycodes ludwigii</i>	related to Meiosis-specific serine/threonine-protein kinase MEK1
KTBO1294.1	<i>Candida glabrata</i>	Meiosis-specific serine/threonine-protein kinase MEK1
SMN20722.1	<i>Kazachstania saulgeensis</i>	similar to <i>Saccharomyces cerevisiae</i> YOR351C MEK1 Meiosis-specific serine/threonine protein kinase
EJT41423.1	<i>Saccharomyces kudriavzevii</i> IFO 1802	MEK1-like protein
EJS41534.1	<i>Saccharomyces arboricola</i> H-6	mek1p
NP_014996.3	<i>Saccharomyces cerevisiae</i> S288C	serine/threonine protein kinase MEK1
AQZ09139.1	<i>Zygosaccharomyces parabailii</i>	MEK1 (YOR351C)
GCE97546.1	<i>Zygosaccharomyces mellis</i>	meiosis-specific serine/threonine-protein kinase mek1
OLL26992.1	<i>Neolecta irregularis</i> DAH-3	Meiosis-specific serine/threonine-protein kinase mek1
XP_002174585.1	<i>Schizosaccharomyces japonicus</i> yFS275	CAMK/RAD53 protein kinase Mek1
XP_017996945.1	<i>Phialophora attae</i>	Meiosis-specific serine/threonine-protein kinase mek1
GBF67102.1	<i>Trichophyton mentagrophytes</i>	meiosis-specific serine/threonine-protein kinase
XP_002845045.1	<i>Microsporium canis</i> CBS 113480	mek1
PIG87779.1	<i>Aspergillus arachidicola</i>	serine/threonine protein kinase
GCB24254.1	<i>Aspergillus awamori</i>	meiosis-specific serine/threonine-protein kinase MEK1