STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF
THE YEAST DUN1 FHA DOMAIN

DISSERTATION

Presented in Partial fulfillment of the requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Hyun Lee, M. S.

The Ohio State University
2006

Dissertation Committee: Approved by
Prof. Ming-Daw Tsai, Advisor
Prof. Thomas Clanton
Prof. Pravin Kaumaya
Prof. Mark Foster

Graduate program in Biophysics
ABSTRACT

The Dunl protein of *Saccharomyces cerevisiae* plays a crucial role within cell signaling pathways responsible for coping with DNA damage. Dunl is a signal transducer kinase similar to *S. cerevisiae* Rad53 and human Chk2. DNA damage activates Mec1 kinase which, in association with Rad9 kinase, phosphorylates Rad53. Activated Rad53 then activates Dunl by phosphorylation. This Mec1/Rad53/Dunl pathway regulates ribonucleotide reductase (RNR) activity by modulating the concentration of the RNR inhibitor Sml1. When activated, Mec1/Rad53/Dunl decreases the intracellular concentration of Sml1 by inducing its degradation via phosphorylation. In addition, RNR gene transcription is regulated by Mec1/Rad53/Dunl kinases through inhibition of the Crt transcriptional repressor. Both mechanisms affect the intracellular concentration of dNTPs, thereby regulating DNA synthesis upon DNA damage.

The fork-head associated (FHA) domain of Dunl has an essential role in regulating direct kinase-kinase interaction between Rad53 and Dunl through the first SQ/TQ-rich cluster domain (SCD) of Rad53. However, exactly how Dunl FHA functions in this mechanism is unknown: neither the structure of Dunl FHA nor its phosphoprotein recognition specificity have been determined. Once activated, Rad53 amplifies DNA damage signaling by oligomerization-promoted autophosphorylation. In the case of two other transducer proteins, human Chk2 and *S. Pombe* Cds1, oligomerization prior to
autophosphorylation is regulated by interaction between the FHA domain and SCD. Rad53 has two FHAs and two SCDs. FHA1 and SCD1 appear to be primarily responsible for its oligomerization: the FHA1 domain of one Rad53 molecule binds SCD1 of another Rad53, and vice versa, resulting in both proteins being hyperphosphorylated. Importantly, the same SCD of Rad53 that regulates its oligomerization via its FHA domain prior to autophosphorylation is also responsible for interaction with Dun1 FHA.

The work described here was undertaken to determine exactly how SCD1 of Rad53 interacts with both Rad53 FHA1 and Dun1 FHA. Our working hypothesis was as follows: 1) Rad53 FHA1 preferentially binds singly phosphorylated SCD1, thereby promoting oligomerization and autophosphorylation, 2) Rad53 FHA1 subsequently releases the multiply phosphorylated SCD1, 3) Dun1 FHA specifically recognizes the multiply phosphorylated SCD1. Consistent with this hypothesis, the data presented here shows that FHA1 (14-164) has higher affinity for SCD1 than does Dun1 FHA when there is a single phosphorylation of any of the four SCD1 threonines. In contrast, Dun1 FHA has higher affinity for multiply phosphorylated SCD1 than does FHA1. We have also investigated, in detail, the number of SCD1 phosphorylations that are required for recognition by Dun1 FHA. According to nuclear magnetic resonance (NMR) titration and surface plasmon resonance (SPR) studies, double phosphorylations of SCD1 are sufficient for recognition by Dun1FHA. Additionally, we solved the solution structure of free Dun1 FHA by NMR. Structures of Dun1 FHA complexed with a doubly
phosphorylated SCD1 peptide and a singly phosphorylated SCD1 peptide are in progress. These two complex structures represent the first FHA structures with an SCD target.

Autophosphorylation of Dun1 in vitro was found to be concentration-dependent, and seven autophosphorylation sites were identified by mass spectrometry. Two of them (Ser4 and Ser10) could be key residues that initiate dimerization prior to autophosphorylation. In addition, new binding partners of Dun1 FHA were screened by pull down assays and confirmed by co-immunoprecipitation.
Dedicated to my parents
ACKNOWLEDGMENTS

I would like to thank the Lord for being my motivation, a driving force, and wisdom everyday of my life. He was the essence of my strength and hope under the difficult situations.

I am so grateful to my advisor Dr. Ming-Daw Tsai for his generosity, patience, wonderful guidance, and his support both in science and in person. He gave me chances to learn many different areas during my graduate studies. I have been privileged to have him as my advisor.

I wish to thank Dr. Heierhorst for his advice, discussions, and invaluable suggestions whenever I need help in doing yeast works. He also sent me yeast plasmids for my studies, and I truly appreciate it.

I also would like to thank Dr. Chunhua Yuan for solving free Dun1 FHA structure in collaboration with me. He taught me basic NMR studies and gave me good suggestions and comments on my writing. I am grateful to Dr. Chi-Fon Chang and Dr. Mei-I Su in Academia Sinica in Taiwan for helping me doing HSQC titrations and other NMR studies. I wish to thank Dr. Chung Liao and Eric Chen for collaborating with me in protein identification and phosphorylation site identification.
I can not express how thankful I am to my parents and my sister Baesuk. I especially wish to thank my father for praying for me everyday. He even woke up 2:00 am in the morning to pray for me while I was taking candidacy exam and final oral defenses due to the time differences between Unites States and Korea. My sister Baesuk came to take care of me when I got sick in the middle of my graduate studies, which is deeply appreciated from the bottom of my heart. I also would like to thank my special mentor Karen Oliver for constantly encouraging me and praying for me. She gave me so many wonderful advices when I was discouraged by troubles.

I would like to thank Dr.Brandon Lamarch, Kathy Wang, Shenjiang Tu, and other my lab members for sharing valuable information, open discussion, and wonderful friendships, which helped me so much. I also wish to thank Dr.Dongyan Qin for kindly teaching me many different techniques at the beginning of my study. I will remember him for a long time.

Finally, I wish to deeply thank Dr.K. Hun Mok not only for giving me invaluable guidance and advices, but also for understanding and encouragement.
VITA

1973............................... Born in Seoul, Korea

1992 - 1995..................... B.S., Agricultural Biology, Chonnam National University, Korea

1996 - 1997..................... M.S., Chemistry, Chonnam National University, Korea

1998 - 1999..................... Staff Scientist, Korea Research Institute of Bioscience and Biotechnology (KRIBB), DaeJeon, Korea

1999 - 2000..................... Science Teacher in private Academia for high school students

2001 - present................. Graduate Teaching and Research Associate,

Department of Chemistry, The Ohio State University

PUBLICATIONS

Research Publications


**FIELDS OF STUDY**

Major Field: Biophysics
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>Dedication</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>Vita</td>
<td>viii</td>
</tr>
<tr>
<td></td>
<td>List of tables</td>
<td>xiv</td>
</tr>
<tr>
<td></td>
<td>List of figures</td>
<td>xv</td>
</tr>
<tr>
<td></td>
<td>List of abbreviations</td>
<td>xviii</td>
</tr>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1 <em>Saccharomyces cerevisiae</em>  Cell cycle and checkpoints</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.2 Small modular domains</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.2.1 Src-homology-2 (SH2) domain</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.2.2 14-3-3 domain</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.2.3 Phosphotyrosine binding (PTB) domain</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.2.4 WW domain</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.3 FHA (fork head-associated) domains</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1.3.1 Identification and general information</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1.3.2 Human Chk2 FHA</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1.3.3 Human Ki67 FHA</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1.3.4 Yeast Rad53 FHA1 and FHA2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>1.3.5 <em>Caenorhabditis elegans</em>-chk2 FHA</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>1.4 SCDs (SQ/TQ-rich cluster domains)</td>
<td>23</td>
</tr>
</tbody>
</table>
1.5 Yeast Dun1 protein ................................................................. 26
  1.5.1 Background ................................................................. 26
  1.5.2 Interaction between Dun1 FHA domain and Rad53 SCD1 ..... 29
1.6 The goal of this study ............................................................. 30
  1.6.1 Characterization of interaction between Dun1 FHA and SCD1 .. 30
  1.6.2 Investigation of Dun1 autophosphorylation and identification of phosphorylation sites ............................................. 31
  1.6.3 Binding analysis of Dun1 FHA based on structure ............... 32
  1.6.4 Screening for new additional biological targets of Dun1 FHA .. 33

2. MATERIALS AND METHODS ....................................................... 37
  2.1 Materials ................................................................. 37
  2.2 Gene cloning, expression and purification .............................. 37
    2.2.1 Expression and purification of Dun1 FHA domain .......... 37
    2.2.2 Expression and purification of Rad53 FHA1 and FHA2 domains ................................................................. 40
    2.2.3 Gene cloning and site-directed mutagenesis of Dun1 and Rad53 ................................................................. 42
    2.2.4 Protein expression and purification of full length Rad53, Dun1 and their mutants by Immunoprecipitation .. 44
  2.3 Dissociation equilibrium constant (K_d) determination by SPR.. 47
  2.4 HSQC titration experiments by NMR ........................................ 48
  2.5 Kinase assays .................................................................. 50
  2.6 Western blotting ............................................................ 50
  2.7 Pull down assays ............................................................ 51
  2.8 Protein identification by mass spectrometry ............................ 52
  2.9 Coimmunoprecipitation .................................................... 52
3. DUN1 FHA PREFERENCES THE MULTIPLY PHOSPHORYLATED SCD1 OF RAD53, WHILE RAD53 FHA1 PREFERENCES SINGLY PHOSPHORYLATED SCD1. ................................................................................................................. 54

3.1 Objective and experimental plans ........................................................................... 54

3.2 Ligand specificity of Rad53 FHA1 and Dun1 FHA ............................................ 55

3.3 Dun1 FHA does not have recognition specificity at the +3 position of pThr. .......... 59

3.4 Dun1 FHA requires only a short SCD1 instead of a long extended and structured binding surface .................................................................................................................. 68

3.5 Binding affinity studies and dissociation constant (K_d) determination by SPR ................................................................................................................................. 74

3.6 Dun1 FHA requires at least two phosphorylations for its recognition on targets. .................................................................................................................................. 87

3.7 Summary and Discussion ....................................................................................... 94

4. DUN1 AUTOPHOSPHORYLATION IS CONCENTRATION DEPENDENT AND REQUIRES ITS FHA DOMAIN ................................................................. 96

4.1 Objective and experimental plans ........................................................................... 96

4.2 Autophosphorylation of S. cerevisiae Rad53, Human Chk2 and S. pombe Cds1 ........................................................ .......................................................... 97

4.3 Autophosphorylation of Dun1 is concentration-dependent ................................ 98

4.4 Dun1 FHA plays a crucial role in Dun1 autophosphorylation in vitro ............... 101

4.5 Identification of autophosphorylation sites in full length Dun1. ....................... 105

4.6 Summary and Discussion ....................................................................................... 113
5. BINDING ANALYSIS OF DUN1 FHA BASED ON STRUCTURE .......... 114
   5.1 Objective and experimental plans .................................................. 114
   5.2 Structure determination of free Dun1 FHA (19-159) and its description...... 115
   5.3 Structure comparison of three FHA domains (Dun1 FHA, FHA1, Ki67 FHA) ...... 118
   5.4 Binding surface mapping of Dun1 FHA based on HSQC spectra with 4P4 peptide ................................................................. 122
   5.5 Differences in Dun1 FHA binding pattern between singly and multiply phosphorylated SCD1 peptides by HSQC titrations ......................... 124
   5.6 Summary and Discussion ................................................................ 132

6. SCREENING FOR ADDITIONAL BIOLOGICAL TARGETS OF DUN1 FHA ................................................................................. 133
   6.1 Objective and experimental plans .................................................. 133
   6.2 Identification of Dun1 FHA interacting proteins under MMS condition by pull down assay and mass spectrometry ........................................ 134
   6.3 In vivo confirmation of interaction between Dun1FHA and Cdc28 by co-immunoprecipitation .............................................................. 138
   6.4 Co-immunoprecipitation with wild type and mutant Dun1 FHA and Cdc28 ....................................................................................... 142
   6.5 Summary and Discussion ................................................................ 148

Bibliography ................................................................................................................. 149

Appendix A HSQC titration spectra of Dun1 FHA with multiply phosphorylated peptides ........................................................................................................ 158

Appendix B Dissociation equilibrium constant determination by SPR ................. 163

Appendix C Peptide sequencing shown in MS spectra ............................................. 178
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Protein sequences of three FHA domains from yeast Dun1 and Rad53</td>
<td>41</td>
</tr>
<tr>
<td>2.2</td>
<td>Sequences of primers used for cloning and site-directed mutagenesis of full length Dun1 and Rad53 and their mutants</td>
<td>43</td>
</tr>
<tr>
<td>2.3</td>
<td>Purified wild type and mutant Dun1 and Rad53 proteins by immunoprecipitation</td>
<td>46</td>
</tr>
<tr>
<td>2.4</td>
<td>The phosphorylated peptide sequences used in this study</td>
<td>49</td>
</tr>
<tr>
<td>3.1</td>
<td>Sequences of tested pThr peptides for HSQC titrations</td>
<td>62</td>
</tr>
<tr>
<td>3.2</td>
<td>Sequences of peptide and proteins for HSQC titrations</td>
<td>70</td>
</tr>
<tr>
<td>3.3</td>
<td>The list of pT peptides tested with Dun1 FHA, FHA1, and FHA2 by SPR</td>
<td>77</td>
</tr>
<tr>
<td>3.4</td>
<td>Tested peptides and their dissociation constants from SPR</td>
<td>78</td>
</tr>
<tr>
<td>4.1</td>
<td>List of sequenced peptide fragments of Dun1</td>
<td>110</td>
</tr>
<tr>
<td>4.2</td>
<td>List of phospho-peptide fragments and identified autophosphorylation sites of Dun1</td>
<td>111</td>
</tr>
<tr>
<td>6.1</td>
<td>Dun1 FHA associated proteins identified by pull down assay followed by mass spectrometry</td>
<td>137</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>12</td>
</tr>
<tr>
<td>1.4</td>
<td>13</td>
</tr>
<tr>
<td>1.5</td>
<td>16</td>
</tr>
<tr>
<td>1.6</td>
<td>18</td>
</tr>
<tr>
<td>1.7</td>
<td>19</td>
</tr>
<tr>
<td>1.8</td>
<td>21</td>
</tr>
<tr>
<td>1.9</td>
<td>22</td>
</tr>
<tr>
<td>1.10</td>
<td>25</td>
</tr>
<tr>
<td>1.11</td>
<td>26</td>
</tr>
<tr>
<td>1.12</td>
<td>28</td>
</tr>
<tr>
<td>1.13</td>
<td>34</td>
</tr>
<tr>
<td>3.1</td>
<td>58</td>
</tr>
<tr>
<td>3.2</td>
<td>61</td>
</tr>
<tr>
<td>3.3</td>
<td>63</td>
</tr>
<tr>
<td>3.4</td>
<td>71</td>
</tr>
<tr>
<td>3.5</td>
<td>79</td>
</tr>
<tr>
<td>3.6</td>
<td>84</td>
</tr>
<tr>
<td>3.7</td>
<td>84</td>
</tr>
</tbody>
</table>
with four peptides........................................................................................................... 89
4.1 Concentration dependency of autophosphorylation of Dun1 .............................. 100
4.2 Autophosphorylation of wild type and mutants of full length Dun1 .................. 103
4.3 Autophosphorylation of wild type and mutants of full length Rad53................. 104
4.4 Identified phosphorylation sites in full length Dun1 by autophosphorylation
    in vitro...................................................................................................................... 107
4.5 Matched peptides in protein sequence of Yeast Dun1 and error range......... 108
4.6 Peptide sequencing shown in MS spectra.............................................................. 109
5.1 Ribbon diagram of Dun1 FHA................................................................. 116
5.2 Ribbon diagrams of representative Dun1 FHA, FHA1, and Ki67FHA
    structures solved by NMR.................................................................................... 120
5.3 Sequence alignments of Rad53 FHA1, FHA2 and Dun1FHA............................. 121
5.4 Binding surface mapping by HSQC spectra......................................................... 123
5.5 $^{15}$N-HSQC spectra of free Dun1-FHA (19-159) and complexed with pT
    peptides.................................................................................................................... 126
5.6 Chemical shift changes of the backbone amide groups of residues that are
    involved in Dun1 FHA interaction upon the binding ............................................ 130
6.1 Pull down assay results......................................................................................... 136
6.2 Co-immunoprecipitations of Dun1 FHA and Cdc28........................................ 140
6.3 Co-immunoprecipitation results of Dun1 FHA and RNR2................................. 141
6.4 Co-immunoprecipitation results for wild type and mutant Dun1 FHA
    and Cdc28.............................................................................................................. 144
6.5 Summary of all Co-IP results ............................................................................. 147
A.1 $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 3P1 peptide......................... 159
A.2 $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 3P4 peptide ........................... 160
A.3 $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 2P4 peptide........................... 161
A.4 $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 2P5 peptide........................... 162
B.1 Binding affinity determination of Dun1 FHA and 1P1 peptide......................... 164
B.2 Binding affinity determination of FHA1 and 1P1 peptide.............................. 165
B.3 Binding affinity determination of Dun1 FHA and 1P2 peptide......................... 166
B.4 Binding affinity determination of FHA1 and 1P2 peptide.............................. 167
B.5 Binding affinity determination of Dun1 FHA and 1P3 peptide......................... 168
B.6 Binding affinity determination of FHA1 and 1P3 peptide.............................. 169
B.7 Binding affinity determination of Dun1 FHA and 1P4 peptide......................... 170
B.8 Binding affinity determination of FHA1 and 1P4 peptide.............................. 171
B.9 Binding affinity determination of Dun1 FHA and 2P1 peptide......................... 172
B.10 Binding affinity determination of FHA1 and 2P1 peptide............................. 173
B.11 Binding affinity determination of Dun1 FHA and 2P6 peptide....................... 174
B.12 Binding affinity determination of FHA1 and 2P6 peptide............................. 175
B.13 Binding affinity determination of Dun1 FHA and 4P4 peptide....................... 176
B.14 Binding affinity determination of Dun1 FHA and 4P4 peptide....................... 177
C1 Peptide sequencing of YASSSSSTDIENDDEK shown in mass spectra............. 179
C2 Peptide sequencing of SYKNDDEVFK shown in mass spectra......................... 180
C3 Peptide sequencing of KPQISATSSQNATTSAAIR shown in mass spectra............ 181
C4 Peptide sequencing of TRPVSFFDK shown in mass spectra......................... 182
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>a</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetracetic acid</td>
</tr>
<tr>
<td>FHA</td>
<td>Forkhead associated</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>HBS-EP</td>
<td>Hepese buffer saline with EDTA and P20</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum correlation</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>m</td>
<td>mili</td>
</tr>
<tr>
<td>MMS</td>
<td>Methylmethane sulfonate</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>mol</td>
<td>mole(s)</td>
</tr>
</tbody>
</table>

xviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>X</td>
<td>19 amino acids including norleucine, except cysteine and methionine</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 *Saccharomyces cerevisiae* cell cycle and checkpoints

*S. cerevisiae* has two different forms, haploid and diploid. The haploid form, which is simpler, grows by mitosis and dies when it encounters a highly stressful environment. The diploid cell is more complicated than haploid. It also grows by a simple lifecycle similar to haploid cells under the normal conditions, but it sporulates and generates haploid spores under stressful conditions.

The budding yeast cell cycle is composed of four phases called G1 (Gap 1), S (Synthesis), G2 (Gap 2), and M (Mitosis) phase. G1 phase and G2 phase are gaps between M phase and S phase, respectively. S phase is a period of DNA synthesis prior to cell division, and nuclear division occurs in M phase followed by cytokinesis to produce two daughter cells (Figure 1.1). There are several checkpoints (such as the G1 checkpoint, the G2 checkpoint, and the Metaphase checkpoint) in the cell cycle. The cell cycle can be arrested when the cell encounters problems such as DNA damage by replication errors, alkylation, oxidation, ionization radiation, and UV irradiation. The yeast cell cycle is
mainly controlled in G1, which is the phase where cells decide whether to mate, divide or go into stationary status. Progression through, or blockage at, cell cycle checkpoints is dependent on the activation of proteins called cyclin-dependent kinases (Cdk). When phosphorylating other proteins, the specificity of a given Cdk is determined by which cyclin it is bound to. S. cerevisiae has only one Cdk named Cdc28, but it has nine cyclins named Cln1, Cln2, Cln3, and six B type cyclins (Clb1-Clb6). When Cdc28 forms a dimer with either Cln1 or Cln2, it regulates budding. Cln3, also known as the G1-cyclin, functions in the G1 checkpoint. Two B type cyclins, Clb1 and Clb2, are crucial for mitosis. Clb5 and Clb6 are involved in DNA synthesis in S phase.

Checkpoint signaling pathways are very important because the cell maintains the integrity of the genome by regulating the cell cycle in response to DNA damage. Protein-interactions are a critical feature of these signaling processes. The interaction between two proteins is mediated by small modular domains that can be folded autonomously and recognize partner proteins. Several types of modular domains that have been identified in signaling proteins from yeast to human (Pawson and Scott 1997). These include SH2 (Src homology 2 domain) (Waksman, Kominos et al. 1992; Songyang, Shoelson et al. 1993; Marengere, Songyang et al. 1994; Cohen, Ren et al. 1995), SH3 (Src homology 3 domain) (Saxton, Cheng et al. 2001), PTB(Phosphotyrosine binding domain) (Cohen, Ren et al. 1995; Kavanaugh, Turck et al. 1995), and 14-3-3 domains (Muslin, Tanner et al. 1996; Yaffe, Rittinger et al. 1997).
Figure 1.1: Schematic representation of the *S. cerevisiae* cell cycle and its checkpoints. This figure was generated from the web site (http://mpf.biol.vt.edu/research/budding_yeast_model/pp/intro.php)
1.2 Small modular domains

1.2.1 The Src-homology-2 (SH2) domain

First identified in two oncoproteins named Src and Fps, the SH2 domain is now known to interact with phospho-Tyr containing target proteins (Sadowski, Stone et al. 1986). The known functions of SH2 domains are regulating tyrosine kinase directly or indirectly by prompting interaction between other proteins and tyrosine kinases. As shown in Figure 1.2, the SH2 domain contains about 100 amino acid residues that are predominantly folded into β-strands. The motif recognized by SH2 is primarily phospho-Tyr, followed by hydrophobic residues (Cohen, Ren et al. 1995).

On the basis of structural studies, currently known SH2 domains can be classified into three categories: Src-like SH2, phospholipase C ?1 (PLC- ?1)-like SH2, and growth-factor-receptor-bound protein 2 (Grb2)-like SH2 (Pawson, Raina et al. 2002). The ones that interact with pYXXH (pYEEI) belong to the Src-like SH2 group, and the second of group SH2 domains recognize multiple hydrophobic residues such as pYHHHHH (pYIIPLP). The SH2 domains that belong to the third group bind preferentially to pYXNX that has Asn at the +2 position of pY (Kuriyan and Cowburn 1997; Yaffe 2002). Note that in these recognition motif notations, “H” represents hydrophobic residues, and “X” stands for any amino acid. SH2 domains do not bind pSer or pThr containing target proteins, and most interact with their targets in a phosphorylation-dependent manner. Two exceptions are growth-factor-receptor-bound protein 10 (Grb10) and SH2-domain protein 1A (SAP) – is which display weak interaction with unphosphorylated targets (Sayos, Wu et al. 1998; Morrione, Plant et al. 1999).
1.2.2 The 14-3-3 domain

First identified in human brain cells, the 14-3-3 domain is a ubiquitous pSer/pThr-binding protein. The size of the 14-3-3 domain is approximately 30 kDa, and it usually forms either homo- or hetero-dimers in eukaryotic cells. As shown in Figure 1.2B, the structure of the 14-3-3 domain consists of nine a-helices in each monomer. There are three known binding motifs of this domain, which are RSX(pS)XP, RXSX(pS)XP and RXF/Y(pS)XP (Muslin, Tanner et al. 1996; Yaffe, Rittinger et al. 1997; Yaffe and Elia 2001).

The 14-3-3 domains interact with pSer/pThr-containing proteins and regulate protein-protein interactions as an allosteric cofactor or steric regulator (Fu, Subramaniam et al. 2000). One function of the 14-3-3 domain is to regulate Cdc25, which has important roles in cell signaling pathways. When DNA damage occurs, Chk1 phosphorylates Cdc25 - leading to interaction with 14-3-3 domain. This Cdc25-14-3-3 complex stays in the cytoplasm and prevents the downstream activation of Cdc2-cyclin B, which ultimately hinders mitosis. The 14-3-3 domain also has crucial roles in apoptosis, cell cycle control and gene transcription (Yaffe and Elia 2001).

1.2.3 Phosphotyrosine binding (PTB) domain

Along with SH2 domain, the phosphotyrosine binding (PTB) domain is another pTyr-binding modular protein. It is involved in regulating amplification of signals in cell signaling pathways, and was identified as an SH2 collagen homology (SHC) adaptor protein (Kuriyan and Cowburn 1997). The PTB domain is classified into two groups: insulin receptor substrate 1 (IRS1)-like PTB and SHC-like PTB domain (Kuriyan and
Cowburn 1997; Yaffe 2002). PTB domains share low sequence homology, while SH2 domains have high sequence homology.

Even though both SH2 and PTB domains can recognize pTyr containing target proteins, their recognition specificities are different. One of the biggest differences between these two interaction mechanisms is the position of pTyr. The surface to which the SH2 domain binds to the pTyr residue is deep inside the protein, and the interaction is supported by residues in the C-terminal region. In contrast, the PTB domain interacts superficially with the pTyr and is supported by interaction with N-terminal residues. The general recognition motif of PTB is NPX(pY) (containing asparagine and proline at the -3 and -2 positions of pY, respectively), where the main interaction force is hydrophobic (Trub, Choi et al. 1995). The structure of PTB shown in Figure 1.2C has a β-sandwich with seven β-strands and α-helices in the C-terminal region (Yaffe 2002).

1.2.4 WW domain

The name of this domain came from its two conserved tryptophan residues that are usually separated by 20 to 22 amino acid residues. The WW domain, which can recognize both phosphoserine and phosphothreonine containing targets, is involved in cell signaling pathways as an adaptor protein (Ilsley, Sudol et al. 2002). It has also been reported that the WW domain interacts with some signal transduction proteins. There are currently four known binding motifs for recognition by the WW domain: the PPXY, (pS)p/(pT)P, PPR, and PPLP motifs. In those binding motifs, the WW domain interacts with its target in either a phosphorylation-dependent or a phosphorylation-independent
manner. One of the tryptophans located at the end of a β-strand and a tyrosine are key residues involved in ligand binding by this domain (Verdecia, Bowman et al. 2000).
Figure 1.2: Structures of small modular domains shown in ribbon representations. A, the Src-homology-2 (SH2) domain from PDB access number 1P13; B, the 14-3-3 domain obtained from PDB access number 1A37; C, the phosphotyrosine binding (PTB) domain obtained from PDB access number 2NMB; D, the WW domain with its target peptide KVSVVR (pT)PPKSPS shown in ball-and-stick configuration; it was obtained from PDB access number 1I8H. All structures were generated using WebLab ViewerPro.
1.3 FHA (The fork head-associated) domains

1.3.1 Identification and general information

The FHA domain was first discovered by database screening. A region of high sequence homology was found in a subset of the family of fork head-type transcription factors (Shiloh 1997) such as Fhl1(yeast), Fkh1(yeast), Fkh2 (yeast), and FoxK1 (mammalian) (Hofmann and Bucher 1995). FHA domains have since been found in more than 400 unrelated proteins (see http://smart.embl-heidelberg.de, Schultz et al., 2000). The name "forkhead" derives from the two distinct spike head-like shapes in the structure of these domains (Weigel, Jurgens et al. 1989).

The function of the FHA domain varies in many different areas such as signal transduction and transcription, protein transport, DNA replication, DNA damage response and repair, and cell cycle regulation in normal and damaged cells from prokaryotes to mammals (Hofmann and Bucher 1995; Li, Lee et al. 2000; Durocher and Jackson 2002). It has been found in various proteins such as proteases, kinases (Chk2, Rad9, Rad53, Dun1, Cds1, and NBS1), phosphatases, and ring-finger proteins (Chfr).

On the basis of extensive experimental evidence, the FHA domain was suggested to be a new phosphoprotein-binding domain (Hofmann and Bucher 1995). It has been shown in vitro that FHA domains can bind to phosphothreonine-containing (pThr) peptides (Durocher, Henckel et al. 1999) with some exceptions. One of the exceptions is Rad53 FHA2, which interacted with phosphotyrosine-containing peptide (Liao, Byeon et al. 1999).
The typical FHA domain consists of approximately 55-75 amino acids as a core region and contains three highly conserved blocks separated by more divergent spacer regions shown in Figure 1.3. The FHA domain requires protective flanking regions on both sides of the domain in order for it to exist as a stable functional or structural unit (Sun, Hsiao et al. 1998; Li, Smith et al. 1999). Initially, the recognition sites of FHA were known to be mainly pTXX(D/I/L) according to combinatorial library screening followed by mutagenesis and structural studies of free FHA and complexes with short phosphopeptides (Liao, Byeon et al. 1999; Durocher, Taylor et al. 2000; Liao, Yuan et al. 2000; Wang, Byeon et al. 2000; Byeon, Yongkiettrakul et al. 2001; Yuan, Yongkiettrakul et al. 2001; Li, Williams et al. 2002; Stavridi, Huyen et al. 2002). The amino acid residue at the +3 position was considered as critical in recognizing target proteins for Rad53 FHA1, FHA2, and human Chk2. In the case of human Chk2, pTXXI was selected from the library screening, and its crystal structure was solved with a peptide containing the pTXXI motif. The Ile at the +3 position of pT interacted with four Chk2 residues (Thr138, Asn166, Ser192, and Leu193). While Rad53 FHA1 showed preference for pTXXD, FHA2 showed preference for pTXXI.

Out of the 400 FHA containing proteins have been identified, only seven structures have been solved by both NMR and X-ray crystallography. Those proteins are human Chk2 FHA, human Chfr FHA, human Ki67 FHA, budding yeast Rad53 FHA1 and FHA2, yeast Dun1 FHA, and Arabidopsis FHA (Liao, Byeon et al. 1999; Durocher, Taylor et al. 2000; Liao, Yuan et al. 2000; Li, Williams et al. 2002; Stavridi, Huyen et al. 2002; Lee, Ding et al. 2003; Li, Byeon et al. 2004). Rad53 FHAs were the first FHA structures determined by NMR (Liao, Byeon et al. 1999). Most FHA complex structures
were solved with short synthetic phosphopeptides. All currently known FHAs share high structural similarity even though they have low sequence homology and their three conserved regions are separated from each other as shown in Figure 1.3. The FHA domains have structures consisting of 10 to 11 β-strands that are folded into a β-sandwich, and some of them contain short α-helices between the β-strands. The crystal structures of human Chk2 FHA and Chfr FHA were solved as a tetramer and a dimer, respectively (Li, Williams et al. 2002; Stavridi, Huyen et al. 2002).
Figure 1.3: Schematic representations of various FHA-containing proteins. Some symbols adopted from the web site.
Figure 1.4: Sequence alignments of FHA-containing proteins. The green arrows on the top show three conserved regions among FHA domains. Only the core regions of the FHA domain are aligned, and residue numbers are shown on the right side of each protein name. Dun1, FHA1, and FHA2 are from *S. cerevisiae*, and Cds1 is from *S. pombe*. Chk2 and Ki67 are human proteins.
1.3.2 Human Chk2 FHA

The Chk2 protein is one of the checkpoint kinases that responds to DNA damage in the cell cycle. Chk2 is the human counterpart of *S. cerevisiae* Rad53 (Cds1 in *S. pombe*), with 26% sequence homology (Matsuoka, Huang et al. 1998; Blasina, de Weyer et al. 1999; Brown, Lee et al. 1999; Chaturvedi, Eng et al. 1999). It has an SCD (SQ/TQ-rich cluster domain) at the N-terminus, an FHA in the middle, and a kinase domain in the C-terminal region. The human Chk2 is classified as a serine/threonine kinase that has important roles in cell signaling pathways, which are mainly regulated by phosphorylation.

Figure 1.5 shows a summary of related proteins in the pathways based on current knowledge of the biological roles of *S. cerevisiae* Rad53 and human Chk2. The function of human Chk2 is linked to cancer and is ataxia telangiectasia mutated (ATM) dependent. It was noted as an important cell signal transduction protein after discovering two mutants, Ile157T and Arg145W, in patients with Li-Fraumeni syndrome and colon cancer, which suggested that Chk2 functions as a tumor suppressor (Bell, Varley et al. 1999; Wu, Webster et al. 2001). Unlike Rad53, human Chk2 has only one FHA domain, which corresponds to the FHA1 of Rad53. When DNA damage occurs, ATM kinase phosphorylates human Chk2 at Thr68 in SCD, and Chk2 itself can autophosphorylate in order to amplify signal through dimerization (Lee and Chung 2001). Chk2 then phosphorylates Cdc25C (Abraham 2001), Cdc25A (Bashkirov, Bashkirova et al. 2003), p53 (Chehab, Malikzay et al. 2000; Hirao, Kong et al. 2000; Shieh, Ahn et al. 2000), and BRCA1 (Bashkirov, Bashkirova et al. 2003) in DNA double strand breaks, leading to cell cycle arrest in the G1, G2/M and S phases. Unphosphorylated Cdc25C can activate the
mitotic kinase Cdc2. However, phosphorylated Cdc25C can not activate Cdc2. So, if the human Chk2 phosphorylates Cdc25C, the cell cycle can not exit mitosis and is arrested. Unstable tumor suppressor p53 can be stabilized by phosphorylation of human Chk2. Stable p53 can activate p21 and Bax. BRCA1 is a putative tumor suppressor linked to breast cancer (Bashkirov, Bashkirova et al. 2003).

The complex Chk2 FHA (64-219) structure with a short synthetic pThr peptide (RHFE (pT) YLIRR) was solved by X-ray crystallography (Figure 1.6A). The structure of human Chk2 is similar to that of other known FHAs, but Chk2 FHA selectively binds to pTXXI-containing target peptides by hydrophobic interactions.
The yeast model was generated mainly based on a paper (Hammer et al., 2002).

Figure 1.5: Models of cell signaling pathways in budding yeast and human cells. ATM, ATR, and Mec1 are considered as sensors.
1.3.3 Human Ki67 FHA

Human Ki67 was first identified as an antigen of the Ki67 antibody from proliferating cells. It regulates the maintenance of cell proliferation and the organization of chromosomes (Gerdes, Schwab et al. 1983; Schluter, Duchrow et al. 1993; Endl and Gerdes 2000; Scholzen, Endl et al. 2002). Two possible Ki67 target proteins were identified later on, human kinesin-like protein 2 (hKLP2) and human NIFK1. Ki67 is a huge protein (~320 kDa) that contains a FHA domain at the end of the N-terminal region from residues 27 to 76. The interactions between Ki67 FHA and its two targets, hKLP2 and hNIFK1, are phosphorylation-dependent, and interaction sites were reported at 1017-1237 and 226-269, respectively (Sueishi, Takagi et al. 2000; Takagi, Sueishi et al. 2001).

Combinatorial peptide library screening to determine the recognition specificity of Ki67 did not show any preference for the +3 position of pThr, unlike that of other well-studied FHAs (Rad53 FHA1, FHA2, human Chk2). The FHA domain of human Ki67 showed completely different recognition specificity. It bound tightly to a 44 amino acid long peptide from the biological target hNIFK1, which clearly showed that Ki67 FHA recognition involves both a pThr residue and an extended binding surface; this extended binding surface was shown to involve direct interaction between the ligand and one of the β-sheets of Ki67 FHA. In the absence of an extended binding surface, Ki67 FHA can not recognize its target properly even with a triply-phosphorylated short peptide (Li, Byeon et al. 2004; Byeon, Li et al. 2005). The NMR structures of both free and complexed Ki67 FHA are shown in Figures 1.6C and D. Key residues of Ki67 FHA are pThr234 and pThr238, both of which contain a proline at the +1 position, which is similar to the binding motif of another protein called Ki FHA.
Figure 1.6: FHA domain structures shown in ribbon representations. A, Human Chk2 FHA structure complexed with pThr peptide obtained from PDB access number 1GXC; B, Free Chfr FHA structure obtained from PDB access number 1LGP; C, Free human Ki67 FHA structure obtained from PDB access number IR21; D, Ki67 FHA complexed with a 45 amino-acid-long pThr peptide obtained from PDB access number 2AFF. All structures were generated with WebLab ViewerPro.
1.3.4 Yeast Rad53 FHA1 and FHA2

The *S. cerevisiae* kinase Rad53, which is an ortholog of human Chk2, was identified as a serine/threonine kinase. It functions in checkpoint pathways related to DNA replication and DNA damage response and repair (Allen, Zhou et al. 1994; Weinert, Kiser et al. 1994; Sun, Fay et al. 1996; Lopes, Cotta-Ramusino et al. 2001). Five different models have been proposed for the activation of Rad53 in both Mec1-dependent and Mec1-independent manners after a signal is generated by DNA damage (Sanchez, Desany et al. 1996; Sun, Fay et al. 1996; Longhese, Foiani et al. 1998; Kim, Jang et al. 2002; Zhao and Rothstein 2002; Ma, Lee et al. 2006). In four models out of five, another yeast protein Rad9 interacts with Rad53 and functions as a mediator, and Rad53 alone can also activate itself by autophosphorylation without both Mec1 and Rad9 in a concentration-dependent manner. All five cases appear to be possible *in vivo* responses to DNA damage (Emili 1998; Vialard, Gilbert et al. 1998; Ma, Lee et al. 2006).

![Gene diagram of budding yeast Rad53.](image)

**Figure 1.7: Gene diagram of budding yeast Rad53.** It contains two SCD, and two FHA domains along with a kinase in the middle.

As seen in the gene diagram of Rad53 (Figure 1.7), Rad53 uniquely has two FHA domains in both the N-terminal (residues from 66 to 116) and the C-terminal regions (residues from 601 to 661) along with two SCDs (residues from 1 to 20 and residues from
471 to 490) and a kinase (residues from 198 to 466). The protein sequence of Rad53 is shown in Table 1. FHA1 and FHA2 free structures are similar, but they have different recognition specificity. A phosphothreonine peptide from p53, APPLSQE (pT) FSDLWK can hinder interaction between FHA1 and phosphorylated Rad9, while it does not interfere with the binding of FHA2 to phosphorylated Rad9 (Durocher, Henckel et al. 1999). FHA1 apparently has a more crucial role than FHA2 in responding to inhibition of DNA replication (Pike, Yongkiettrakul et al. 2003; Schwartz, Lee et al. 2003). Free and complex structures of FHA1 and FHA2 with synthesized peptides are shown in Figure 1.9.
Figure 1.8: Protein sequence of yeast Rad53. The core residues of the FHA1 domain (66-116) are shown in light green, that of kinase domain (198-466) in blue, and that of FHA2 domain (601-664) in green. Also, there are two SCDs in Rad53, and SCD1 (1-20) is shown in red and SCD2 (471-490) is in orange. The theoretical pI of this protein is around 7.88, as calculated by the ProtParam Tool from ExPASy.
Figure 1.9: FHA domain structures of yeast Rad53 shown in ribbon representations. A, Free Rad53 FHA1 obtained from PDB access number 1J4Q; B, Rad53 FHA1 complexed with pThr peptide from Rad9 obtained from PDB access number 1J4P; C, Free Rad53 FHA2 obtained from PDB access number 1FHQ; D, Rad53 FHA2 complexed with pThr peptide from Rad9 obtained from PDB access number 1J4L. All structures were generated with WebLab ViewerPro.
1.3.5 *Caenorhabditis elegans*-Chk2 FHA

*Caenorhabditis elegans* is a useful model organism for studying the processes of cell development and differentiation, including meiosis. In the nematode *C. elegans*, whose genome has been completely sequenced, about 500 protein kinases among the 19000 predicted proteins have been identified (Chaturvedi, Eng et al. 1999). *C. elegans* Chk2 has one FHA domain and one kinase domain and was suggested to be a novel protein kinase, related to human Chk2 - which is required for either initiation of mitotic recombination or monitoring a specific subset of DNA damage lesions in multicellular organisms (Byeon, Li et al. 2005).

1.4 SCD (SQ/TQ-rich cluster domains)

SCDs were identified and named by Elledge and his co-workers in 1999 in the process of phosphorylation site identification in breast cancer-associated 1 (BRCA1) (Cortez, Wang et al. 1999). BRCA1 has 10 SQ/TQ motifs, and human Chk2 has 7 SQ/TQ motifs within a short range in both cases. A protein sequence is considered to be an SCD when there are at least three SQ/TQ motifs within 100 amino acid residues.

Many SCD-containing proteins have been identified and most are involved in cell signaling pathways in response to DNA damage. It is for this reason that SCDs are now considered to be a third signature domain for identifying proteins that are responsible for DNA damage response and repair along with FHA and BRCT domains. These three signature domains play their roles through protein-protein interactions in response to DNA damage (Durocher and Jackson 2002; Hammet, Pike et al. 2003; Manke, Lowery et
al. 2003; Yu, Chini et al. 2003). Unlike some other modular domains, SCDs do not have high sequence similarity except that they contain many SQ/TQ motifs. In addition, there are no limitations in terms of their length or how many SQ/TQ motifs are present in SCDs, and SCDs can be located anywhere along the length of a protein.

Another unique characteristic of SCDs is that they do not have structural similarity to each other. Since SCDs have many SQ/TQ motifs within a short range, they often display highly disordered structures (Romero, Obradovic et al. 2001). A structure of an SCD complexed with an FHA domain is currently not available. However, one can predict how disordered SCDs are by using PONDR (Predictor of Natural Disordered Regions) program. According to PONDR, the entire SCD of human Chk2 is highly disordered. The structural disorder of SCDs is considered to be an important characteristic because other proteins can easily bind them without structural interference (Lakoucheva LM 2004).

SCDs have been found in many proteins that have diverse functions such as transducer kinases (human Chk1, Chk2 and DNA PK), mediators (human BRCA1, MDC1 and NBS1, Yeast Rad9 and Mrc1) and effectors in cell signaling pathways in response to DNA damage. Most human Chk2-like proteins contain at least one SCD. Chk2 has an SCD in its N-terminal region, S. cerevisiae Rad53 contains two SCDs: one in its N-terminus, and the other one between the kinase and FHA2 domains. An important known function of SCDs is the regulation of oligomerization through interaction with FHA domains on the same protein. SCDs have to be phosphorylated first by upstream kinases because interaction with FHA domains is phosphorylation-dependent.
Figure 1.10: Schematic representations of various SCD containing proteins. Most SCD containing proteins have one SCD except...
1.5 Yeast Dun1 protein

1.5.1 Background

The Dun1 protein of *S. cerevisiae* was first identified as a DNA damage response protein kinase in 1993 (Zhou and Elledge 1993). It contains both a kinase and a fork head-associated (FHA) domain, and its sequence is shown in Figure 1.10. Dun1 plays a crucial role in checkpoint signaling pathways in response to DNA damage. This checkpoint signaling pathways are highly conserved from mammals to yeasts and are involved in cell-cycle arrest or cell-cycle delay, which provide the opportunity to repair damaged DNA. A protein can participate in this process through direct activation of the DNA damage repair process, or through upstream activation of signal transducers, adaptors, or effectors in DNA repair pathways. The Dun1 protein is one of the transducers in *S. cerevisiae* and is a member of the human Chk2-like protein kinase family, which are important in Li-Fraumeni multicancer syndrome (Matsuoka, Huang et al. 1998; Bell, Varley et al. 1999; Brown, Lee et al. 1999).

![Figure 1.11: Gene diagram of yeast Dun1. It contains an FHA domain at the N-terminal region and a kinase domain.](image-url)
In the cell signaling pathway, a cascade of protein kinases is activated by phosphorylation when DNA damage occurs. The phosphoatidyl inositol kinase-like kinases (PIKK), Mec1/Tel1 in *S. cerevisiae* and ATM/ATR in mammals, are on top of this cascade acting as sensors of DNA damage. DNA damage activates Mec1 kinase, and Mec1 phosphorylates Rad53. The activated Rad53 is recognized by Dun1 FHA and subsequently activates Dun1 kinase by phosphorylation. Dun1 then phosphorylates a small ribonucleotide reductase (RNR) inhibitor protein, named sml1, causing its removal from the cell if DNA damage occurs during S phase (Zhao and Rothstein 2002). RNR is responsible for catalyzing the conversion of nucleoside diphosphates (NDP) to deoxynucleoside diphosphates (dNDP). Once the sml1 protein level is diminished, the amount of active RNR is increased, which maintains a high level of dNTP synthesis to improve DNA repair capacity (Zhao, Muller et al. 1998). Dun1 kinase phosphorylates sml1 at Ser56, Ser58, and Ser60 residues, which automatically leads to degradation of sml1. In addition, Dun1 is involved in gene transcriptional activation that is necessary for DNA synthesis as upstream protein of many pathways, and it also regulates G2/M phase arrest (Zhou and Elledge 1993; Pati, Keller et al. 1997; Gardner, Putnam et al. 1999).
Figure 1.12: Protein sequence of yeast Dun1. It contains an FHA domain from residues 56 to 112 shown in light green and a kinase domain from residues from 200 to 480 (shown in blue). Dun1 does not contain any SCDs.
1.5.2 Interaction between Dun1 FHA domain and Rad53 SCD1

Dun1 has an FHA domain and a kinase domain, and Rad53 has two FHA domains, two SQ/TQ-rich cluster domains (SCD), and a kinase domain. PIKKs usually phosphorylate Ser or Thr residues of the SCD in their substrates (Kim, Lim et al. 1999; O'Neill, Dwyer et al. 2000). SCDs have become known as one of the signature domains for proteins involved in DNA damage response along with FHA and BRCT domains (Durocher and Jackson 2002; Hammet, Pike et al. 2003; Manke, Lowery et al. 2003; Yu, Chini et al. 2003).

Interaction between FHA and SCD regulates autophosphorylation. After phosphorylation of the SCD of human Chk2 by ATM kinase at Thr68, its FHA domain recognizes pThr68 and forms a dimer. Oligomerization leads them to effect intermolecular autophosphorylation on other threonine or serine residues – which induces full Chk2 kinase activation. Additional trans-phosphorylation on Ser or Thr in the SCD also occurs in Chk2 oligomers (Ahn, Li et al. 2002; Xu, Tsvetkov et al. 2002). Rad53, Chk2-like kinase can also autophosphorylate to amplify the signal and its activity through FHAs and SCDs. Rad53 can even autophosphorylate without phosphorylation by Mec1 in a concentration-dependent manner in vitro (Gilbert, Green et al. 2001). SCD1 of Rad53 appears to play a more important role in activating Rad53 than does SCD2 even though both SCDs are phosphorylated by Mec1 (Lee, Schwartz et al. 2003). Similar to human Chk2 and S. cerevisiae Rad53, fission yeast Cds1 also contains SCD, FHA and kinase domains. Thr11 of Cds1 is phosphorylated by the upstream protein Rad3, an ataxia telangiectasia mutated and Rad3-related protein (ATR)-like kinase, and its FHA
domain can recognize pThr11 and dimerize followed by autophosphorylation to enhance the initial signal (Tanaka and Russell 2004).

Phosphorylated SCD1 can be recognized by Dun1 FHA and regulates kinase-kinase interaction between Rad53 and Dun1, which is an essential part of the kinase cascade in the Mec1/Rad53/Dun1 pathway. To date, human Chk2, the budding yeast Rad53, and fission yeast Cds1 have been studied intensively. However, little is known about Dun1 in terms of its recognition specificity, structural features, autophosphorylation, and many others.

1.6 The goals of this study

1.6.1 Characterization of interaction between Dun1 FHA and SCD1

The first goal was to determine how exactly Dun1 FHA plays a role in regulating the interaction between Rad53 and Dun1. So far, we have known only that it destroyed interaction between Dun1 and Rad53 when Dun1 FHA was mutated at Arg60 and Asn103. Ser74 and His77 were also reported as crucial residues for Dun1 FHA function (Bashkirov, Bashkirova et al. 2003). Our model for this mechanism is shown in Figure 1.13 in detail. After the signal is amplified in Rad53 by its autophosphorylation, Dun1 FHA recognizes multiply phosphorylated SCD1 of Rad53, which leads to the phosphorylation of Dun1 by Rad53 kinase. Once Dun1 is phosphorylated, it dimerizes through its FHA domain followed by autophosphorylation, which amplifies the signal. As shown in the model A (Figure 1.13), two proteins (FHA1 and Dun1 FHA) can recognize their targets properly because they have completely different ligand specificities. Our
hypothesis is that FHA1 recognizes a singly phosphorylated SCD1, whereas Dun1 FHA interacts with a multiply phosphorylated SCD1.

The second model (Figure 1.13B) shows two possible binding features of Dun1 FHA. Model 1 shows that Dun1 FHA binds to both SCD1 and FHA1 through a long extended binding surface. Model 2 shows Dun1 FHA binding to the short SCD1 only. Does Dun1 FHA require a long extended binding surface like Ki67 FHA? A short peptide from SCD1 and a long protein that contains both SCD1 and FHA1 were tested with Dun1 FHA in order to answer this question.

1.6.2 Investigation of Dun1 autophosphorylation and identification of phosphorylation sites

The second objective was to characterize the autophosphorylation of Dun1. Autophosphorylation amplifies checkpoint signals rapidly, and it has recently come to be understood as a general characteristic of many proteins that are involved in cell signaling pathways. Phosphorylation sites of Rad53 have been identified both under normal and MMS conditions. Its phosphorylation sites by in vitro autophosphorylation have also been identified. There were 17 phosphorylation sites in Rad53 treated with MMS, and more than thirty residues were phosphorylated in autophosphorylated Rad53 (Smolka, Albuquerque et al. 2005). Autophosphorylation was performed with Rad53 dephosphorylated by ?-phosphatase. Interestingly, 13 out of 17 phosphorylation sites identified in Rad53 under MMS treatment in vivo were also found in phosphorylation sites from in vitro autophosphorylation. This suggests that autophosphorylation of Rad53 in vitro can account for ~76% of the phosphorylation sites induced by DNA damage in
vivo and many more phosphorylations to enhance the cell signal. Therefore, identification of autophosphorylation sites in Dun1 is important to find key residues that have crucial roles on cell signaling and its autophosphorylation.

The autophosphorylation of human Chk2, budding yeast Rad53 and fission yeast Cds1 are well studied. However, autophosphorylation of budding yeast Dun1 has not been studied yet, and its mechanisms are unknown. The only information we know about Dun1 autophosphorylation is that it autophosphorylates and the presence of Rad53 might help to improve the level of Dun1 autophosphorylation (Bashkirov, Bashkirova et al. 2003). Therefore, we investigated Dun1 autophosphorylation in vitro followed by identification of autophosphorylation sites by mass spectrometry. In addition, the concentration dependency of autophosphorylation was tested, and mutagenesis of the Dun1 FHA domain was performed in order to elucidate its role in autophosphorylation.

1.6.3 Binding analysis of Dun1 FHA based on structure

Even though many FHA domains show high structural similarity, it was deemed important to solve the structure of Dun1 FHA because this domain appears to have completely different ligand specificity than other members of this domain family. Though the structure of free Dun1 FHA was previously determined by X-ray crystallography, it did not give a good diffraction pattern (Blanchard, Fontes et al. 2001). We therefore worked to solve the solution structure of free Dun1 FHA.
1.6.4 Screening for new additional biological targets of Dun1 FHA

The forth goal was to identify new additional biological targets for Dun1 FHA. It has been reported that Dun1 might be involved in other biological processes besides DNA damage response pathways (Ho, Gruhler et al. 2002). Ho et al. identified more than 20 proteins as Dun1 interacting proteins, and many of them are related to DNA damage repair and response. However, there are two proteins whose functions are unknown, which indicates that Dun1 might have functions in other areas other than DNA damage response. In addition, Dun1 and Mec1 participate in a Rad53-independent pathway that regulates gross chromosomal rearrangements (GRC) (Bashkirov, Bashkirova et al. 2003). This also suggests that Dun1 might have targets involved in other cell signaling pathways.
Figure 1.13: Models for Dun1 activation and Dun1 FHA interaction with SCD1 A. A model of Dun1 activation by Rad53 followed by its autophosphorylation; after activation of Rad53 through any of five possible models, signal is amplified by autophosphorylation, and then Rad53 kinase phosphorylates Dun1 on certain residues. Similarly, Dun1 also dimerizes and autophosphorylates. B, Two possible models for interaction between Dun1 FHA and Rad53. Our data support Model 2.
Figure 1.13

continued
Figure 1.13 continued

B

Model 1  
Model 2
2.1 Materials

Oligonucleotides for cloning were commercially synthesized by Integrated DNA Technologies. All enzymes were purchased from New England Biolabs, and antibodies were obtained from either Santa Cruz Biotechnology or Sigma-Aldrich. Phospho-peptides were either commercially synthesized by Genemed Synthesis or were synthesized by the Genomic Research Center of Academia Sinica in Taiwan. All other chemicals were purchased from Sigma-Aldrich.

2.2 Gene cloning, expression and purification

2.2.1 Expression and purification of Dun1 FHA domain

The pQE-60-Dun1-FHA expression vector, consisting of Dun1 residues 19-159 fused to a C-terminal His\textsubscript{6} tag, was constructed by Jorg Heierhorst (University of Melbourne, Australia). pQE-60-Dun1-FHA-containing BL21(DE3) E. coli cells were grown at 37 °C in an LB medium (5 g/L of yeast extract, 10 g/L Bacto-tryptone, and 10
g/L NaCl) overnight, and 1% of this seed culture was inoculated to fresh LB medium. At
OD$_{600}$ ~ 0.8, protein expression was induced by addition of IPTG to 0.5 mM. After an
additional 6 hours of incubation at 30 °C with shaking at 250 rpm, cells were pelleted by
centrifugation at 18,000 rpm, and stored at –20 °C.

For NMR studies, $^{15}$N-labeled and $^{15}$N, $^{13}$C-labeled and $^2$H, $^{15}$N, $^{13}$C-labeled Dun1 FHA (19-159) were expressed in M9 minimal media (12.8 g/L Na$_2$HPO$_4$•7H$_2$O, 3 g/L
KH$_2$PO$_4$, 0.5 g/L NaCl, 2 mM MgSO$_4$, 1 mM CaCl$_2$, and 100 mg/L ampicillin, pH 7.4).
The overnight seed culture was grown in LB medium and harvested at 5000 rpm in order
to remove all LB medium followed by resuspension with M9 medium. The sterilized 1
g/L $^{15}$NH$_4$Cl and 0.2% glucose were added into M9 for $^{15}$N-labeled Dun1 FHA (19-159).
For $^{15}$N, $^{13}$C-labeled Dun1 FHA (19-159), sterilized 1 g/L $^{15}$NH$_4$Cl and 2 g/L $^{13}$C-glucose
were used with M9. The series of seed cultures were grown with 20%, 50%, and 75%
D$_2$O for triply-labeled protein, and sterilized 1 g/L $^{15}$NH$_4$Cl and 2 g/L $^{13}$C-glucose were
added into M9 medium prepared in 95% D$_2$O. When OD$_{600}$ reached ~ 1.0, 0.5 mM IPTG
was added and incubated for 12 hours with shaking at 250 rpm for all three isotope-
labeled Dun1 FHA proteins.

Frozen cells were completely thawed and resuspended in a lysis buffer (5 mM
HEPES, pH 7.5, 500 mM NaCl, 10 mM β mercaptoethanol, 0.5% Triton X-100, 1 tablet
of protease inhibitor cocktail, and 1 mg/mL lysozyme from chicken egg white). The cell
suspension was incubated at 4 °C for 1 hour followed by four rounds of sonication with
five minutes cooling time in between. Cell debris was removed by centrifugation at
18,000 rpm for 1 hour, and soluble proteins were collected. The supernatant was applied
to Ni-NTA agarose (Qiagen) pre-equilibrated with five bed volumes of wash buffer (5 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM β mercaptoethanol). Then Ni-NTA agarose beads were thoroughly washed with at least 10 bed volumes of wash buffers containing 20 mM imidazole. Proteins were eluted stepwise using four elution buffers containing 100 mM, 200 mM, 300 mM, and 500 mM imidazole. Dun1 FHA proteins usually began to elute with 200 mM imidazole. Eluates were combined and diluted with 1 to 2 ratio of distilled water to reduce the ionic strength of the solution prior to ion-exchange chromatography. SP-sepharose beads were equilibrated with wash buffer 2 (20 mM imidazole, 100 mM NaCl and 10 mM β mercaptoethanol, pH 7.0) prior to loading the Ni-NTA eluate. After washing with 10 bed volumes of wash buffer 2, proteins were eluted with 300 mM, 500 mM and 1 M NaCl containing wash buffer 2. Proteins eluted mainly with 500 mM and 1 M NaCl. Eluates were combined and concentrated down to around 7 or 8 mL by Amicon Centriprep YM-10. The concentrated protein solution was further purified with S-100 gel filtration chromatography. S-100 gel (Pharmacia) was packed into a 500 mL column prior to its use and washed with final sample buffer (5 mM HEPES, 200 mM NaCl, 5 mM DTT, and 1 mM EDTA, pH 7.5). Later we changed the final buffer to phosphate buffer with lower amounts of DTT (10 mM Na₂HPO₄, 200 mM NaCl, 0.5 mM DTT, and 1 mM EDTA, pH 7.5). Dun1 FHA containing fractions were combined and concentrated up to 0.5 mM followed by lyophilization for long term storage at -80 °C.
2.1.2 Expression and purification of Rad53 FHA1 and FHA2 domains

Rad53 FHA1 and FHA2 were over-expressed as GST-fusion forms in the same manner as Dun1 FHA described above. Frozen cells were thawed and re-suspended with lysis buffer (50 mM Na$_2$HPO$_4$, pH 7.5, 500 mM NaCl, 10 mM ß mercaptoethanol, 1 mM EDTA, 0.5% Triton X-100, 1 tablet of protease inhibitor cocktail, and 1 mg/ml lysozyme from chicken egg white). Re-suspended cell solution was incubated at 4 °C for 1 hour followed by four rounds of sonication. Centrifugation at 18,000 rpm for 1 hour was used to spin down cell debris from soluble supernatant. The supernatant was applied to pre-equilibrated glutathione beads with wash buffer (50 mM Na$_2$HPO$_4$, pH 7.5, 500 mM NaCl, 10 mM ß mercaptoethanol, 1 mM EDTA), followed by gentle shaking on a rotator at 4 °C for 5 hours in order to promote efficient binding. Subsequently, washing was with at least 20 bed volumes of wash buffer. Thrombin (100 units /L) was added to the suspended beads along with two bed volumes of thrombin digestion buffer, and digestion was performed at room temperature for 4 hours and then at 4 °C overnight. Eluted proteins were concentrated down to 7 to 8 mL by Amicon Centriprep YM-10 and applied to S-100 gel filtration column as described above.
Table 2.1: Protein sequences of three FHA domains from yeast, Dnl1 and Rad53. The parentheses show residue numbers for each domain:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad53 FHA2</td>
<td>KFAYFIDPLNTLDEMYR NVRHSGTFLPFRGLTQEEHLPSEGL (793-730)</td>
</tr>
<tr>
<td>Rad53 FHA1</td>
<td>INNQRQGKTRGNIDPLNEGVTNGLNQFEEHLPSEGL (2175)</td>
</tr>
<tr>
<td>Dnl1 FHA</td>
<td>KGQSFSPSSSRDDINSPYTVLQGIDSLPPLFQEEHLPSEGL (19159)</td>
</tr>
</tbody>
</table>

**EXPA|**

Protein and these parameters (molecular weight, theoretical pI and extinction coefficient) were calculated by ProtParam tool from ExPaSy.
2.1.3 Gene cloning and site-directed mutagenesis of Dun1 and Rad53

Full length Dun1 was subcloned into a dual yeast expression vector named pESC-TRP that has two multiple cloning sites (MCS). The vector pESC-TRP has two different tags that are FLAG in MCS1 and myc in MCS2. Dun1 was cloned between EcoR1 and Not1 in MCS1 with FLAG tag. Full length Rad53 was subcloned into pYES2-CT yeast expression vector with V5 and His tags between Kpn1 and Xho1.

Two mutants of full length Dun1, R60AN103A-fDun1 and D328A-fDun1, were produced by site-directed mutagenesis with fdun1-FLAG in pESC-TRP vector as a template with mutagenic primers. Two Rad53 mutants, R70AN107A-fRad53 and 4AQs-fRad53, were also generated with fRad53-V5-His in pYES2-CT vector as a template. All primers are listed in Table 1. DNA sequences were confirmed by sequencing in Plant-Microbe Genomics Facility of The Ohio State University with a 3700 DNA Analyzer from Applied Biosystems Inc.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'-3' direction)</th>
</tr>
</thead>
</table>
| FMed23       | ATCTATTCCAAACCAAATACACCCTCCTCAAGATGCCACGAGCTCAGAGAGAGATATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATAAGAGAGAGATATA4
2.1.4 Protein expression and purification of full length Rad53, Dun1 and their mutants by Immunoprecipitation

Plasmids containing rad53, Dun1 and their mutants were transformed into yeast strain W303 and grown in synthetic deficient media plates. Rad53 and its mutants were selected by growth on uracil-deficient media plates (SD-U; 6.7 g/L yeast nitrogen base, 1.15 g/L -Uracil DO supplement, 2% glucose, and 2% bacto agar), and Dun1 and its mutants were grown on tryptophan-deficient media plates (SD-W; 6.7 g/L yeast nitrogen base, 1.15 g/L -Trp DO supplement, 2% glucose, and 2% bacto agar) at 30 °C for 4 days.

Single colonies from wild type and mutant Rad53 and Dun1 plate were suspended as small seed cultures in liquid SD-U (6.7 g/L yeast nitrogen base, 1.15 g/L -Uracil DO supplement, 2% sucrose) and SD-W (6.7 g/L yeast nitrogen base, 1.15 g/L -Trp DO supplement, 2% sucrose) media, respectively, at 30 °C with shaking at 200 rpm for 17 hours. 2% of overnight culture was inoculated to fresh medium and induced by 4% galactose for 12 hours at 30 °C with shaking at 200 rpm when \( A_{600} \) reached approximately 0.8. Cells were harvested by centrifugation at 5,000 rpm, washed with ice cold PBS buffer (10 mM \( \text{Na}_2\text{HPO}_4 \), 1.8 mM \( \text{KH}_2\text{PO}_4 \), 140 mM \( \text{NaCl} \), 2.7 mM \( \text{KCl} \)), and then flash frozen for storage.

Cells were thawed and suspended in four volumes of lysis buffer (10 mM \( \text{Na}_2\text{HPO}_4 \), 1.8 mM \( \text{KH}_2\text{PO}_4 \), 140 mM \( \text{NaCl} \), 2.7 mM \( \text{KCl} \), 10% glycerol, 0.5% Triton X-100, 0.5% NP-40, 5 mM EGTA, 5 mM EDTA, 50 mM sodium fluoride, 10 mM B-glycerophosphate, 5 mM sodium pyrophosphate, yeast protease inhibitor cocktail (Sigma), 2 mM PMSF). The cell suspension was transferred to several 2 mL tubes.
containing 1 mL of glass bead and vortexed at maximum speed four times for one minute each, with 5 minutes cooling on ice in between. Cell debris was removed by centrifugation at 14,000 rpm for 15 minutes at 4 °C. Pre-washed anti-FLAG agarose and anti-V5 agarose were added into supernatant of Dun1 and Rad53 respectively and incubated with gentle shaking at 4 °C for 4 hours to overnight. Supernatants were removed after centrifugation at 5,000 rpm for 1 min at 4 °C, and the beads were washed once with lysis buffer, twice with TBST (20 mM Tris-HCl, 140 mM NaCl, and 0.05% Tween 20, pH 7.5) and once with kinase assay buffers. Dun1 kinase assay buffer contains 50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT (without ^32P ATP), and Rad53 kinase assay buffer contains 25 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT (without ^32P ATP).
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Description</th>
<th>Mutated residue numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length Dun1-FLAG</td>
<td>Wild type Dun1</td>
<td>-</td>
</tr>
<tr>
<td>Full length Dun1-His</td>
<td>Wild type Dun1</td>
<td>-</td>
</tr>
<tr>
<td>R60A-fDun1-FLAG</td>
<td>FHA mutant Dun1-1</td>
<td>60</td>
</tr>
<tr>
<td>N103A-fDun1-FLAG</td>
<td>FHA mutant Dun1-2</td>
<td>103</td>
</tr>
<tr>
<td>R60AN103A-fDun1-FLAG</td>
<td>FHA mutant Dun1-3</td>
<td>60, 103</td>
</tr>
<tr>
<td>R62A-fDun1-FLAG</td>
<td>FHA mutant Dun1-4</td>
<td>62</td>
</tr>
<tr>
<td>R60AR62A-fDun1-FLAG</td>
<td>FHA mutant Dun1-5</td>
<td>60, 62</td>
</tr>
<tr>
<td>D328A-fDun1-FLAG</td>
<td>Kinase-dead Dun1</td>
<td>328</td>
</tr>
<tr>
<td>Full length Rad53-V5-His</td>
<td>Wild type Rad53</td>
<td>-</td>
</tr>
<tr>
<td>R70AN107A-fRad53-V5-His</td>
<td>FHA1-dead Rad53</td>
<td>70, 107</td>
</tr>
<tr>
<td>R70AN107AR605A-fRad53-V5-His</td>
<td>FHA1&amp;FHA2-dead Rad53</td>
<td>70, 107, 605</td>
</tr>
<tr>
<td>4AQs-fRad53-V5-His</td>
<td>SCD1-dead Rad53</td>
<td>5, 8, 12, 15</td>
</tr>
<tr>
<td>4AQs-R70AN107A-fRad53-V5-His</td>
<td>FHA1 and SCD1-dead Rad53</td>
<td>5, 8, 12, 15, 70, 107</td>
</tr>
<tr>
<td>K227AD339A-fDun1-V5-His</td>
<td>Kinase-dead Rad53</td>
<td>227, 339</td>
</tr>
</tbody>
</table>

**Table 2.3: Wild type and mutant Dun1 and Rad53 proteins purified by immunoprecipitation.** Two wild type Dun1 (with His-tag and FLAG-tag) and six mutants were overexpressed in yeast and purified by immunoprecipitation (IP) against either anti-His or anti-FLAG. Wild type Rad53 and four mutants were also purified by IP against anti-V5. The residue numbers for the mutation sites are listed in the last column.
2.3 The dissociation equilibrium constant (K\textsubscript{d}) determination by SPR

The K\textsubscript{d} between peptides and three FHA proteins were determined by surface plasmon resonance (SPR). All peptides were biotinylated and immobilized on SA sensor chip that has streptavidin on the sensor surface, which can interact strongly with biotin. All binding assays were performed on a BIAcore 3000 (Karlsson and Stahlberg 1995).

All peptides were biotinylated by incubating with EZ-Link\textsuperscript{TM} Sulfo-NHS-Biotin (Pierce) in non-amine buffer (0.3 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 8.0) for 2 hours to overnight at 4 °C or 30 minutes at room temperature. Reactions were stopped by adding 0.1 M Glycine for 30 minutes at room temperature. All biotinylated peptide solution were filtered with a 0.22 um syringe filter and diluted to 4 µM with commercial HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005 % Surfactant P20, pH 7.4) from Biacore.

Biotinylated peptides were immobilized at each channel using a 20 µL/min flow rate for 2 min on sensorchip SA that was pretreated with regeneration buffer (50 mM NaOH + 1 M NaCl) three times. A blank channel without any immobilization was used as a control. The resonance unit (RU) differences after immobilization were 50 to 350 RU. In order to remove unbound peptides, the sensor surface was washed and equilibrated with HBS-EP buffer for 1 or 2 hours until the baseline difference became less than 2 RU. Protein solutions in a series of increasing concentration (0.01 µM to 200 µM) were applied to all four channels at a flow rate of 25 µL/min for 180 seconds of dissociation time for peptide and protein interaction. Regeneration was performed with 10 mM NaOH/200 mM NaCl solution after each set of experiments.

The simple theory behind SPR is that resonance unit change is proportional to mass on sensor surface. At least seven different concentrations of proteins were studied to
obtain the dissociation equilibrium constant $K_d$ for each protein-peptide binding interaction. In order to get maximal response, excess proteins were passed through each channel, and the response units of the blank channel were subtracted from the rest of the interaction channels. SigmaPlot software was used to fit the data to a single rectangular hyperbolic curve in order to calculate the $K_d$. The hyperbola function shown below was used in order to plot maximal responses and corresponding concentration.

$$RU = c \times RU_{\text{max}} / (K_d + c)$$

### 2.4 HSQC titration experiments by NMR

Heteronuclear single quantum correlation (HSQC) titration was used to study the interaction between phosphorylated peptides and FHA proteins. All titration experiments were performed at 20 °C on a Bruker DMX-600 or DRX-800 with 15N-labeled Dun1 FHA (19-159) in elution buffer containing 5 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM DTT, 1 mM EDTA. The protein concentration was 0.5 mM in 95% $H_2O/5% D_2O$ containing elution buffer. 2D $^1H$-$^{15}N$ HSQC spectra were recorded in a series of increasing phospho-peptide concentration (0.125, 0.25, 0.5, 1.0, and 2.0 mM). Xwinmr from Bruker was used to process NMR spectra, and Xwinplot editor was used to generate overlapped HSQC spectra.
<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence</th>
<th>Phosphorylation sites</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTXXI-1</td>
<td>RYLVQL (pT) NVINS</td>
<td>410</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXI-2</td>
<td>SKINC (pT) NLIKFR</td>
<td>352</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXD-1</td>
<td>KHSNDTTAT (pT) INDVPPPIDV</td>
<td>42</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXD-2</td>
<td>APPLSQE (pT) FSDLWKL</td>
<td>18</td>
<td>Human p53</td>
</tr>
<tr>
<td>pTXXS</td>
<td>KREHSGDV (pT) DSSFKRQQR</td>
<td>14</td>
<td>Yeast Dun1</td>
</tr>
<tr>
<td>pTXXL</td>
<td>KPIMDL (pT) HVLRCN</td>
<td>631</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXL</td>
<td>DYVHQVDSNGKPIMDL (pT) HVLRCNLKDAGIQEKL</td>
<td>631</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>1P1</td>
<td>RMENI (pT) QPTQQSTQR</td>
<td>5</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>1P2</td>
<td>RMENIQP (pT) QQSTQR</td>
<td>8</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>1P3</td>
<td>RQPTQSS (pT) QATQRFR</td>
<td>12</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>1P4</td>
<td>RQQSTQA (pT) QRFLIER</td>
<td>15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>2P1</td>
<td>RENI (pT) QP (pT) QQSTQATQRFLR</td>
<td>5, 8</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>2P2</td>
<td>RENI (pT) QPTQQSS (pT) QATQRFLR</td>
<td>5, 12</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>2P3</td>
<td>RENI (pT) QPTQQSTQA (pT) QRFLR</td>
<td>5, 15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>2P4</td>
<td>RENITQP (pT) QQSTQA (pT) QRFLR</td>
<td>8, 12</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>2P5</td>
<td>RENITQP (pT) QQSTQA (pT) QRFLR</td>
<td>8, 15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>2P6</td>
<td>RENITQPT QQ (pT) QA (pT) QRFLR</td>
<td>12, 15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>3P1</td>
<td>RENI (pT) QP (pT) QQSTQA (pT) QRFLR</td>
<td>5, 8, 12</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>3P2</td>
<td>RENI (pT) QP (pT) QQSTQA (pT) QRFLR</td>
<td>5, 8, 15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>3P3</td>
<td>RENI (pT) QPTQQS (pT) QA (pT) QRFLR</td>
<td>5, 12, 15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>3P4</td>
<td>RENITQP (pT) QQSTQA (pT) QRFLR</td>
<td>8, 12, 15</td>
<td>Yeast Rad53</td>
</tr>
<tr>
<td>4P4</td>
<td>RENI (pT) QP (pT) QQSTQA (pT) QRFLR</td>
<td>5, 8, 12, 15</td>
<td>Yeast Rad53</td>
</tr>
</tbody>
</table>

Table 2.4: The phosphorylated peptide sequences used in this study. All peptides were commercially synthesized by Genemed Synthesis, and the phosphorylated threonines are shown in parenthesis. The last column shows the original proteins from which the peptide sequences came. Yeast represents *S. cerevisiae*.  

49
2.5 Kinase assays

Two Purified wild type full length Dun1s (fDun1-FLAG and fDun1-His) and six mutants (R60A-fDun1-FLAG, N103A-fDun1-FLAG, R60AN103A-fDun1-FLAG, R62A-fDun1-FLAG, R60AR62A-fDun1-FLAG, and Dun1-D32A) were incubated in a 30 uL reaction buffer containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 15 µCi of ³²P ATP for 30 minutes at 30 ºC. Wild type Rad53 and five mutants (R70AN103A-fRad53-V5-His, 4AQs-fRad53-V5-His, R70AN103AR605A-fRad53-V5-His, 4AQs-R70AN103A-fRad53-V5-His, K227AD339A-fRad53-V5-His) were incubated in a different kinase assay buffer (25 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, and 15 uCi ³²P ATP) for 30 minutes at 30 ºC. Kinase reactions were stopped by adding SDS sample buffer and analyzed with either 10% or 4-20% gradient SDS-PAGE (Bio-Rad). After the gels were washed thoroughly after electrophoresis and dried, they were exposed for 12 hours. A PhosphorImager was used to visualize radiolabeled proteins, and data were analyzed by ImageQuant.

2.6 Western blotting

Mini protein gels (Bio-Rad) or proper percentage gels were used for protein separation, and proteins were transferred to nitrocellulose membrane (Bio-Rad) by electrophoresis either for 3 hours at 130 mV or overnight at 22 mV in transfer buffer (4.8 g/L Tris base, pH 8.3, 0.37 g/L SDS, 2.9 g/L glycine, 200 ml/L methanol). TBST milk (5% nonfat dried milk, 25 mM Tris HCl pH 7.4, 140 mM NaCl, 2.5 mM KCl, 0.05% Tween-20) was used to block the membrane for 1 hour at room temperature. The first
antibody reaction (Santacruz Biotech, concentration- 1 to 1000 dilution) was performed with gentle shaking either for 1 hour at room temperature or overnight at 4 °C, and the membrane was washed twice with TBS buffer (25 mM Tris HCl pH 7.4, 140 mM NaCl, and 2.5 mM KCl) for at least five minutes with shaking. The secondary antibody was diluted to a 1 to 2000 ratio with TBS and incubated for 1 hour at room temperature followed by thorough washing with TBS three times for 10 minutes each. Precipitation or chemiluminescence was used as the detection method. For precipitation, BCP/TCIP (Pierce) one step visualizing reagent for AP-conjugated and CN/DAB (Pierce) for HRP-conjugated were used depending on the secondary antibodies. ECL film (Pharmacia) was used for chemiluminescence.

2.7 Pull down assays

Purified Dun1-FHA (19-159) prepared as described above was used to charge mini Ni-NTA columns (~ 1 mL bed volume), followed by washing with buffer consisting of 5 mM HEPES, pH 7.5, 150 mM NaCl, and 10 mM ß-mercaptoethanol; for control experiments the Ni-NTA beads were pre-incubated with buffer only. 10 mL of cell-free lysate, either from normal or DNA damaged yeast cells (exposed to methyl methane, MMS), was applied to the Ni-NTA columns followed by thorough washing with 20 mM imidazole in the buffer described above. Bound proteins were eluted with 500 mM imidazole and separated by SDS-PAGE with 4-20% gradient Mini gels (Bio-Rad) and sent to mass spectrometry for identification.
2.8 Protein identification by Mass spectrometry

After separation by electrophoresis, gels were fixed by soaking in the fixing solution (50% ethanol and 10% acetic acid in water) overnight followed by washing twice with washing solution (50% methanol, 5% acetic acid) for 30 minutes each time. Then gels were stained with GelCode Blue (PIERCE) for 1 hour at room temperature. After destaining for 1 or 2 hours, each protein band was excised and sent to Taiwan in storage solution (5% acetic acid in water). Tryptic digestion and peptide identification were performed at the Genomic Research Center of Academia Sinica in Taiwan.

2.9 Coimmunoprecipitation

Yeast strain W303 was grown at 30 °C in tryptophan deficient synthetic complete media (SC-W) and induced by 2% galactose for three hours when $A_{600}$ reached approximately 0.5. The DNA damaging reagents 0.05% MMS and 50 mM hydroxyurea (HU) were applied for 3 hours. Cells were harvested and lysed by glass bead bashing four times one minute each in ice cold lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM MgCl$_2$, 50 mM sodium fluoride, 10 mM B-glycerophosphate, 5 mM sodium pyrophosphate, yeast protease inhibitor cocktail (Sigma), 0.5 mM DTT and 0.5 % Triton X-100 pH 7.5. Cell debris was removed by centrifugation, and supernatant was pre-cleaned with protein-G sepharose for 1 hour at 4 °C. Each supernatant was divided into two 2 mL eppendorf tubes and incubated with monoclonal anti-FLAG and anti-myc at 4 °C for 4 hours and protein-G sepharose was added into each tube and
incubated two more hours. The protein-G sepharose beads were washed once with lysis buffer and twice with wash buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 50 mM B-glycerophosphate, 5% glycerol, 0.1% Triton X-100, 0.5 mM DTT, pH 7.5.
CHAPTER 3

DUN1 FHA PREFERENCES MULTIPLY PHOSPHORYLATED SCD1 OF RAD53, WHILE RAD53 FHA1 RELATIVELY PREFERENCES SINGLY PHOSPHORYLATED SCD1

3.1 Objective and Experimental Plans

Dun1 FHA was previously shown to recognize phosphorylated SCD1 of Rad53 upon hyperphosphorylation of Rad53 by autophosphorylation. The subsequent phosphorylation of Dun1 by Rad53 kinase passes the signal on to downstream proteins of the cell signaling pathway in budding yeast. Little is known about the recognition specificity of Dun1 FHA and the mechanism behind the interaction between the SCD1 of Rad53 and Dun1 FHA. Specific residues on the SCD1 that bind to Dun1 FHA are not known, either. This chapter focuses on investigating these questions, using the hypothesized models presented in Figure 1.13A and B as a starting point.

We investigated the characteristics of Dun1FHA recognition toward its targets through the use of both synthetic peptides and overexpressed proteins by using nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR). In addition, we also synthesized four singly phosphorylated, six doubly phosphorylated, four triply...
phosphorylated, and one quadruply phosphorylated peptide from the SCD1 of Rad53. Using these phosphorylated peptides, we were able to determine the number of SDC threonine phosphorylations required for recognition by Dun1 FHA.

### 3.2 Ligand specificity of Rad53 FHA1 and Dun1 FHA.

The structure Rad53 FHA1 has been characterized extensively, using both NMR (Byeon, Yongkiettrakul et al. 2001; Yuan, Yongkiettrakul et al. 2001) and X-ray crystallography (Durocher, Taylor et al. 2000; Li, Williams et al. 2002). The biological function of Rad53 FHA1 has also been studied extensively both in the presence and in the absence of DNA damage. In contrast, little information is available regarding the recognition specificity and structure of Dun1 FHA.

The binding target of Rad53 FHA1 was first identified by peptide library screening with pT, pS and pY libraries. Rad53 FHA1 clearly showed tight interaction with pTXXD containing peptides, and Rad53 FHA2 showed preference for pTXXI/L containing peptides (Durocher, Taylor et al. 2000; Liao, Yuan et al. 2000; Byeon, Yongkiettrakul et al. 2001). These peptide library screenings, coupled with mutagenesis studies, led us to believe that the +3 position of pT plays an important role in recognition by FHA domains; this came to be known as the "pT+3 rule". After library screening, several pTXXD containing peptides from Rad9, which is known to interact with Rad53 FHA1, were tested and complex structures were solved by NMR. However, people started to report some results that contradicted this "pT+3 rule". First, a new target sequence of Rad53 FHA1, identified in the budding yeast protein Mdt1, was shown to be pTXXI (NDPD pT LEIYS) instead of pTXXD (Pike, Yongkiettrakul et al. 2004). This
same peptide surprisingly does not interact with Rad53 FHA2 that is supposed to recognize pTXXI motif as a target. Second, Rad53 FHA1 also showed interaction with a pT(390)XXV motif from Rad9 both in vivo and in vitro (Schwartz, Duong et al. 2002). Third, human Ki67 FHA did not show any specific preference at the +3 position of pT, and was shown to require a long extended binding surface (Li, Byeon et al. 2004).

The ligand specificity of Rad53 FHA1 is summarized in Figure 4.3 based on structural studies followed by mutagenesis. Three residues (Arg70, Ser85 and Asn86) of FHA1 are responsible for interacting with pT by salt bridges or hydrogen bonds. A strong charge-charge interaction between Arg83 of FHA1 and the +3Asp of pTXXD is evident in Figure 3.1A. As highlighted in Figure 3.1C, hydrophobic interactions were suggested to be the main interaction force between FHA1 and the pTXXI motif (Mahajan, Yuan et al. 2005). Gly135 and Val136, located in the loop region between the ß9 and ß10 strands, are involved in hydrophobic interaction with the +3Ile residue of the peptide. Interestingly a hydrophobic interaction was shown between the aromatic ring of the +4Tyr (pT peptide) and Val136. Arg83 interacts with +4Tyr aromatic ring as well. Figure 4.3B shows how mutagenesis affected the interaction between FHA1 and its target peptide. Gly135 and Gly133 were mutated to Asp and Ile respectively, and it changed the interaction forces from charge-charge interaction (between Arg83 and +3Asp) to hydrophobic interaction between Ile133 of FHA1 and the +3Leu of peptide.

Unlike the well studied FHA1, not much information is available for Dun1 FHA recognition specificity. Currently known biological targets of the Dun1 FHA domain are SCD1 of Rad53 and residues 2 to 237 of the yeast Pan3 subunit of the poly (A)-nuclease complex. The latter was identified by yeast-two hybrid screening (Hammet, Pike et al.
2002). Another case that was investigated with Dun1 FHA involved the biologically irrelevant pTXXD peptide from human p53, which appeared to have weak interaction (Ahn, Li et al. 2002).
Figure 3.1: Diagrams of interactions between Rad53 FHA1 and pT peptides. These diagrams compare the interactions of FHA1 with the +3 position in different peptides. A, Interaction with pTXXD from Rad9; B, Interaction with pTXXL when Gly135 and Gly133 were mutated to Asp135 and Ile133 respectively; C, Interaction with pTXXI from another biological target Mdt1. This figure was generated based on two papers (Yongkiettrakul, Byeon et al. 2004; Mahajan, Yuan et al. 2005)
3.3 Dun1 FHA does not have recognition specificity at the +3 position of pThr.

Three currently known factors that affect FHA recognition are pThr, pT+1 through pT+3, and long extended binding surfaces. Since we know little about the recognition specificity of Dun1 FHA, we examined these three known factors along with new factors. Dun1 FHA does require phosphothreonine as an essential factor for recognition similar to most FHA domains. In order to investigate the second known factor for Dun1 FHA recognition, peptide library screening and NMR HSQC titration were used. Peptide library screening against Dun1 FHA with pT library was performed as described (Liao, Byeon et al. 1999; Liao, Yuan et al. 2000). At the -2 position of pT, norisoleucine (X), a replacement for methionine was selected (Figure 3.2). Alanine and norisoleucine were equally selected for the -1 position of pT. At the +1 position, lysine, leucine and valine were almost equally selected, which is difficult to find general aspect from them because lysine is positively charged and the other two are hydrophobic residues. We also found arginine selected for the +2 and +3 positions of pT, but we know that these are not conclusive. In the case of human Chk2 FHA, arginines were selected at more than three positions near pT. However, it turned to be a nonspecific interaction (Qin, Lee et al. 2003).

In order to determine whether Dun1 FHA has recognition specificity on the pT+3 position, we tested interaction between Dun1 FHA and six singly phosphorylated peptides that contain Ile, Leu, Asp, and Ser at the +3 position by using HSQC titration experiments. These peptide sequences are listed in Table 3.1. Four of them came from yeast protein Pan3 known as a biological target of Dun1 FHA, and one peptide came from Dun1 itself. A pTXXD peptide (APPLSQE pT FSDLWKL) was from human p53 as
a control. Tested peptides were two pTXXI (RYLVQL pTNVINS and SKINC pT NLIKFR), two pTXXL (KPIIMDL pT HVLRC and DYVFHQVDSNGKPIIMDL pT HVRCLNKLDA GIQEK), two pTXXD (KHSN TTAT pT INDVPPPIDV and APPLSQE pT FSDLWK), and a pTXXS (KREHSGDV pT DSSFKRQR) containing peptides.

As shown in Figure 3.3, all HSQC titration spectra of Dun1 FHA with six phosphopeptides showed similar peak shift pattern even though they have different residues at the +3 position of pT, which indicates that pT+3 residue alone does not play a crucial role in selecting target of Dun1 FHA. Two pTXXI peptides showed slightly different peak shift pattern (Figure 3.3A and B). There are more shifted peaks involved in binding to pTXXI-1 than that of pTXXI-2, and chemical shift differences are also bigger in pTXXI-1. Two pTXXD containing peptides showed big differences in their binding patterns. A pTXXD-2 peptide from human p53 interacted with Dun1 FHA in a similar way to pTXXIs, but not many peaks were shifted upon binding to a pTXXD-1 from pan3. A pTXXL-1 binds to Dun1 FHA in a similar way to pTXXI-1 and pTXXD-2. One might expect to see similar peak shift pattern from pTXXI and pTXXL since both peptides have hydrophobic residues at the pT+3 position if it has the +3 position specificity. In case of Dun1 FHA, it showed a similar binding pattern with hydrophobic (Ile and Leu), negatively charged (Asp), and uncharged polar (Ser) residues at the pT+3 position.

Therefore, Dun1 FHA does not have preference at the +3 position rather it might be affected by overall sequence surrounding the pT residue.
Figure 3.2: Library screening results. This was done by Yong Ju. Y axis represents the number of occurrence at a certain position. X = norleucine (replacement for Met). A, B, C, D, E, and F show selected results for at the -2, -1, +1, +2, +3, and +4 position of pThr, respectively.
<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence</th>
<th>Phosphorylation sites</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTXXI-1</td>
<td>RYLVQL (pT) NVINS</td>
<td>410</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXI-2</td>
<td>SKINC (pT) NLIKFR</td>
<td>352</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXD-1</td>
<td>KHSDNTTAT (pT) INDVPPPIDV</td>
<td>42</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXD-2</td>
<td>APPLSQE (pT) FSDLWKL</td>
<td>18</td>
<td>Human p53</td>
</tr>
<tr>
<td>pTXXS</td>
<td>KREHSGDV (pT) DSSFKRQQR</td>
<td>14</td>
<td>Yeast Dun1</td>
</tr>
<tr>
<td>pTXXL-1</td>
<td>KPIMDL (pT) HVLRL</td>
<td>631</td>
<td>Yeast Pan3</td>
</tr>
<tr>
<td>pTXXL-2</td>
<td>DYVFHQVDNSNGKPIMDL (pT) HVLRLCN KLDAGIQEKL</td>
<td>631</td>
<td>Yeast Pan3</td>
</tr>
</tbody>
</table>

**Table 3.1: Sequences of tested pThr peptides for HSQC titrations.** All peptides have a single phosphorylation. The first and second columns show names of peptides and their sequences. The third column shows phosphorylation sites of each peptide, and the last column shows original proteins where these peptide sequences come from.
Figure 3.3: Overlaid $^1$H-$^{15}$N-HSQC spectra of Dun1-FHA (19-159) complexed with various peptides. In all HSQC spectra, free Dun1 FHA is shown in black, and 1 to 4 ratio of peptide complex is shown in pink. A, Dun1-FHA (19-159) complexed with pTXXI-1 peptide (RYLVQL pTNVINS) from Pan3; B, Dun1-FHA (19-159) complexed with pTXXI-2 (KPIIMDL pT HVLRCL) from Pan3; C, Dun1-FHA (19-159) complexed with pTXXD-1 (KHSDNTTAT pT INDVPPPIDV) from Pan3; D, Dun1-FHA (19-159) complexed with pTXXD (APPLSQE pT FSDLWKL) from Human p53; E, Dun1-FHA (19-159) complexed with pTXXL-1 (KPIIMDL pT HVLRCL) from Pan3; F, Dun1-FHA (19-159) complexed with pTXXL-2 (DYVFHQVDSNGKPIIMDL pT HVLRCLNKLDAGIQEKL) from Pan3; G, Dun1 FHA complexed with pTXXS (KREHSGDV pT DSSFKRQQR) from Dun1 itself.
A. Dun1 FHA (19-159) with pTXXI-1 from Pan3

B. Dun1 FHA (19-159) with pTXXI-2 from Pan3

Figure 3.3                 Continued
Figure 3.3 Continued

C. Dun1 FHA (19-159) with pTXXD-1 from Pan3

D. Dun1 FHA (19-159) with pTXXD-2 from Human p53
Figure 3.3 Continued

**F. Dun1 FHA (19-159) with pTXXL-1 from Pan3**

- Black: free dun1 (19-159)
- Pink: dun1 (19-159) with 1 to 4 pTXXL from Pan3

**F. Dun1 FHA (19-159) with pTXXL-2 from Pan3**

- Black: free dun1 FHA
- Pink: dun1 FHA with 1 to 4 pTXXL (31)
Figure 3.3 Continued

G. Dun1 FHA (19-159) with pTXXS from Dun1
3.4 Dun1 FHA requires only a short SCD1 instead of a long extended and structured binding surface

Dun1 FHA (19-159), FHA1 (2-175), and FHA2 (573-730) were overexpressed in *E. coli* and purified as described in methods. We determined that phosphothreonine is essential for the Dun1 FHA recognition, and it does not have specificity at the +3 position of pT. The remaining question now is whether Dun1 FHA requires a long extended binding surface in selecting its targets. In order to examine this, we have proposed two possible models for interaction between Dun1 FHA and SCD1-FHA1 shown in chapter 1 (Figure 1.13B). In Model 1, the interaction involves both the phosphorylated SCD1 and the FHA1 domain. In Model 2 it involves only the phosphorylated SCD1. The HSQC titration results between free Dun1 FHA (black) and complex with 4P4 (pink) are shown in Figure 3.4A, and 3.4B shows interaction with unphosphorylated SCD1-FHA1 (2-175) protein. Non-labeled SCD1-FHA1 (2-175) protein was expressed and purified and concentrated up to 5 mM and used for titration with $^{15}$N-labeled Dun1 FHA. There is no change between HSQC spectra of free Dun1 FHA and that of the complex (Figure 3.4B), which clearly indicates that Dun1 FHA does not interact with unphosphorylated SCD1-FHA1 (2-175). Two structurally and functionally important residues in Dun1 FHA, Ser74 and His77, were always shifted or disappeared upon binding to all peptides that have been tested, even with the peptides that have weak binding ($\sim$350 µM range of dissociation constant $K_d$). However, none of peaks was shifted upon interaction with unphosphorylated SCD1-FHA1. In the case of Ki67 FHA, it showed almost no interaction or a very weak interaction with short peptides. But, it showed a distinct interaction ($K_d = \sim$100 µM) with a 45 amino acid residue long
peptide from its biological target hNIFK even when it was not phosphorylated due to interaction between one of the β-sheets of Ki67 FHA. Even though threonines in SCD1 were not phosphorylated, it should show at least weak interaction if there is any interaction between Dun1 FHA and one of the β-sheets in FHA1. In addition, $^{15}$N-labeled SCD1-FHA1 protein was also titrated with non-labeled Dun1 FHA, which also showed no peak shift change just like $^{15}$N-labeled Dun1 FHA did not show any interaction with non-labeled SCD1-FHA1 protein (Figure 3.4D). According to NMR structures of FHA1 complexed with pT peptides from yeast protein Rad9 and Mdt1, there are five signature peaks that always were shifted upon binding, which are Ser85, Asn86, Arg70, Val134, Gly133, and Phe96. Asn107 was also involved in binding and also is known to be crucial for FHA1 functions. Gly133, Ser85, and Asn107 were marked in Figure 3.4D, which did not shift at all. Another unique feature of FHA1 is the appearance of a new peak for Asn86 upon binding with its target peptides. Residue Asn86 does not show up in free FHA1 HSQC spectra, but it always appeared upon binding in HSQC spectra of complexes. However, this new peak did not show up upon binding with Dun1 FHA, which could also be evidence that suggests there is no interaction between Dun1 FHA and unphosphorylated SCD1-FHA1 proteins. The blue rectangle in Figure 3.4D indicates where Asn86 peak is supposed to show up if they interact each other. FHA2 (573-730) protein was also tested with Dun1 FHA by titration as a negative control. As shown in Figure 3.4C, there is no peak shift in Dun1 FHA. Hence, we conclude that Dun1 FHA does not require a long extended binding surface for target to be recognized by it.
### Table 3.2: Sequences of peptide and proteins for HSQC titrations.

The first column (A, B, C, and D) correspond to HSQC titrations in Figure 4.4, and the second and third column show each pair of $^{15}$N-labeled protein and non-labeled peptide or protein sequences.

<table>
<thead>
<tr>
<th>$^{15}$N-labeled protein sequences</th>
<th>Non-labeled protein and peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A $^{15}$N-Dun1 FHA (19–159)</strong></td>
<td><strong>Rad53 FHA2 (573–730)</strong></td>
</tr>
<tr>
<td>KRQQRSNKPSSEYTCGLHVLNLPGEKQVE</td>
<td>GNGRFLTKLPLPSIIIQESLEIQQGVNPFFI</td>
</tr>
<tr>
<td>ITNNRNVTTIGRSGSCVILSEPDISTFHAEF</td>
<td>GRSEDNCNCKIEDNRSVLPHIFGKHKRAVGG</td>
</tr>
<tr>
<td>HLLQMDVDNFAQRLNVIDKSRNGTFINGNR</td>
<td>SMYESPAQGLDIDWYCHTVSNYVLNMMRI</td>
</tr>
<tr>
<td>LVKKDYILKNGDRIVFGKCSFLFKYASSSS</td>
<td>QTGKFLLQDGEIKI1WDKNKNFVIGKVEI</td>
</tr>
<tr>
<td>TDIENDDEKVSSESRSY</td>
<td>NDTNLGLFLNGLQEQRVVLQTAEEKDLV</td>
</tr>
<tr>
<td><strong>B $^{15}$N-Dun1 FHA (19–159)</strong></td>
<td><strong>Rad53 FHA1 (2–175)</strong></td>
</tr>
<tr>
<td>KRQQRSNKPSSEYTCGLHVLNLPGEKQVE</td>
<td>NITQPTQQSTQATQRFLIEKFSQEQIGENIV</td>
</tr>
<tr>
<td>ITNNRNVTTIGRSGSCVILSEPDISTFHAEF</td>
<td>CRVICTTGQIPRDLISADISQVLKEKRSIKK</td>
</tr>
<tr>
<td>HLLQMDVDNFAQRLNVIDKSRNGTFINGNR</td>
<td>VWTIFGRNPACDYHLGNISRLSNKHFQILLGE</td>
</tr>
<tr>
<td>LVKKDYILKNGDRIVFGKCSFLFKYASSSS</td>
<td>DGNLLDINDTNGTWLNGQKEVKNSNQLLSQ</td>
</tr>
<tr>
<td>TDIENDDEKVSSESRSY</td>
<td>GDEITGVGVEDILSLVLIFINDKFQCLEQ</td>
</tr>
<tr>
<td><strong>C $^{15}$N-Dun1 FHA (19–159)</strong></td>
<td><strong>Dun1 FHA (19–159)</strong></td>
</tr>
<tr>
<td>KRQQRSNKPSSEYTCGLHVLNLPGEKQVE</td>
<td>KRQQRSNKPSSEYTCGLHVLNLPGEKQVE</td>
</tr>
<tr>
<td>ITNNRNVTTIGRSGSCVILSEPDISTFHAEF</td>
<td>ITNNRNVTTIGRSGSCVILSEPDISTFHAEF</td>
</tr>
<tr>
<td>HLLQMDVDNFAQRLNVIDKSRNGTFINGNR</td>
<td>HLLQMDVDNFAQRLNVIDKSRNGTFINGNR</td>
</tr>
<tr>
<td>LVKKDYILKNGDRIVFGKCSFLFKYASSSS</td>
<td>LVKKDYILKNGDRIVFGKCSFLFKYASSSS</td>
</tr>
<tr>
<td>TDIENDDEKVSSESRSY</td>
<td>TDIENDDEKVSSESRSY</td>
</tr>
</tbody>
</table>

**4P4 Peptide**

RENIP (pT) QP (pT) QQS (pT) QA (pT) QRFL
Figure 3.4: Overlaid $^1$H-$^{15}$N-HSQC spectra of Dun1-FHA (19-159) complexed with 4P4 peptide and two proteins. A, $^1$H-$^{15}$N-HSQC spectra of yeast Dun1 FHA free (black) and in the presence of pT peptide 4P4 (RENI pT QP pT QQS pT QA pT QRFLR) from Rad53 (pink); B, $^1$H-$^{15}$N-HSQC spectrum of free yeast Dun1-FHA (black) and in the presence of non-labeled and unphosphorylated SCD1-FHA1 (2-175) protein (pink); C, $^1$H-$^{15}$N-HSQC spectrum of free yeast Dun1-FHA (black) and in the presence of non-labeled and unphosphorylated FHA2 (573-730) protein (pink); D, $^1$H-$^{15}$N-HSQC spectrum of free yeast SCD1-FHA1 (black) and in the presence of non-labeled and unphosphorylated Dun1 FHA (19-159) protein (pink).
Figure 3.4  
Continued
Figure 3.4 Continued

C. $^{15}$N-Dun1 FHA (19-159) with FHA2 (573-730) protein

D. $^{15}$N-SCD1-FHA1 (2-175) with Dun1FHA (19-159) protein
3.5 Binding affinity studies and dissociation equilibrium constant (Kd) determination by SPR

Seven phosphothreonine peptides from SCD1 were biotinylated before their use for SPR as described in Method. Three proteins (FHA1, FHA2, and Dun1 FHA) were investigated with seven peptides listed in Table 3.3. FHA2 protein was used as a negative control. The dissociation equilibrium constant ($K_d$) plots for individual Dun1 FHA and FHA1 binding to seven pT peptides are shown in Appendix B, and Figure 3.5 shows overlaid plots for each peptide with two proteins, Dun1 FHA and FHA1, in comparison to each other. All of them were fitted to a single rectangular hyperbola curve. FHA2 did not show any interaction with any of these peptides, which indicates that this interaction is specific between two proteins (Dun1 FHA and FHA1) and their target peptides. Peptide sequences and their phosphorylation sites of each peptide are listed in Table 3.3. 2P1 peptide has double phosphorylations on Thr5 and Thr8, and 2P6 is phosphorylated on Thr12 and Thr15. As shown in Figure 3.5, response unit changes (dRU) of Dun1 FHA with singly phosphorylated peptides (1P1, 1P2, 1P3, and 1P4) are much significant than that of FHA1 with the same peptides. Individual fitting curves of FHA1 and Dun1 FHA with those four peptides are shown in Appendix B. HSQC titration was performed to get the $K_d$ of FHA1 with 1P1 in order to confirm SPR results (data not shown), which was approximately 20 µM similar to the SPR result. The response unit changes of multiply phosphorylated peptides with both proteins are similar, which give nice fitting curves for all of them with small errors.

The obtained $K_d$ values are listed in Table 3.4. Dun1 FHA showed distinct differences in its affinity to pT peptides depending on the phosphorylation status, while
FHA1 showed similar binding affinity for all peptides with different phosphorylation status.

The $K_d$ of 1P1, 1P2, 1P3, and 1P4 with Dun1 FHA are 101.4 µM, 175.9 µM, 264.7 µM and 354.0 µM respectively. All of them showed very weak binding to Dun1 FHA. On the contrary, multiply phosphorylated peptides showed tight interaction with Dun1FHA by both HSQC and SPR. The $K_d$ of 2P1, 2P6, and 4P4 with Dun1 FHA are 0.96 µM, 4.77 µM, and 0.96 µM respectively.

For the singly phosphorylated peptides, FHA1 showed tighter binding than Dun1FHA. FHA1 binds to 1P1 more than 10 fold tighter than Dun1FHA. FHA1 binds to 1P2, 1P3, and 1P4 peptides 5.4 fold, 2.7 fold, and 5.6 fold respectively tighter than Dun1FHA. On the other hand, Dun1FHA binds to 2P1 peptide 24.3 fold tighter than FHA1, and 10 fold tighter to 2P6 than FHA1. As for 4P4, Dun1FHA has 30 fold tighter binding than FHA1. Therefore, these data suggest that FHA1 recognizes singly phosphorylated SCD1 relatively better than Dun1 FHA, whereas Dun1FHA binds to multiply phosphorylated SCD1.

Dun1 FHA showed tightest binding to 1P2 peptide among singly phosphorylated ones, and it also showed closer peak shift pattern in HSQC titration to multiply phosphorylated peptides than the rest of singly phosphorylated ones. An overlaid plot of Dun1 FHA with all singly phosphorylated peptides is shown in Figure 3.6A. 1P1 and 1P2 peptides showed tighter interaction than the rest, which might indicate that the first two threonines (Thr5 and Thr8) are more crucial than the other two (Thr12 and Thr15). 2P1 peptide has two phosphorylations on first two threonines, and it showed the same $K_d$ as that of a quadruply phosphorylated peptide 4P4. Figure 3.6B shows an overlaid plot of
Dun1 FHA with 2P1, 2P6, and 4P4 peptides. 2P1 and 4P4 peptides show similar fitting curves and Kds, which suggests us that Dun1 FHA recognizes only two phosphates even when there are four phosphorylations present. Figure 3.6C and D show interaction between FHA1 and all four singly phosphorylated peptides (Figure 3.6C) and multiply phosphorylated ones (Figure 3.6D). According to both NMR and SPR, FHA1 binds to all seven peptides with similar $K_d$ range, which is around 20~50 µM. This indicates that FHA1 recognizes only one phosphate no matter how many phosphorylations are present in the target sequences, while Dun1 FHA recognizes double phosphorylations.
<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence</th>
<th>Phosphorylation sites</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P1</td>
<td>RMENI pT QPTQQSTQATQR</td>
<td>5</td>
<td>1898</td>
</tr>
<tr>
<td>1P2</td>
<td>RMENITQP pT QQSTQATQR</td>
<td>8</td>
<td>1898</td>
</tr>
<tr>
<td>1P3</td>
<td>RITQPTQQS pT QATQRFLR</td>
<td>12</td>
<td>1913</td>
</tr>
<tr>
<td>1P4</td>
<td>RPTQQSTQA pT QRFLIER</td>
<td>15</td>
<td>1942</td>
</tr>
<tr>
<td>2P1</td>
<td>RENI pT QP pT QQSTQATQRFLR</td>
<td>5, 8</td>
<td>2563</td>
</tr>
<tr>
<td>2P6</td>
<td>RENITQPTQQS pT QA pT QRFLR</td>
<td>12, 15</td>
<td>2563</td>
</tr>
<tr>
<td>4P4</td>
<td>RENI pT QP pT QQS pT QA pT QRFLR</td>
<td>5, 8, 12, 15</td>
<td>2725</td>
</tr>
</tbody>
</table>

**Table 3.3: A list of pT peptides tested with Dun1 FHA, FHA1, and FHA2 by SPR.**

Four singly phosphorylated peptides, two doubly phosphorylated peptides, and a quadruply phosphorylated peptide were tested. The residue numbers of phosphorylation sites are shown in third column.
Table 3.4: Tested peptides and their dissociation constants $K_d$ from SPR. All peptides were biotinlyated by incubating with EZ-Link™ Sulfo-NHS-Biotin prior to their use. Dun1 FHA (19-159), FHA1 (14-164) and FHA2 (573-730) were overexpressed in *E. coli* and purified as described in methods. NI for FHA2 (573-730) stands for no interaction.
Figure 3.5: Dun1 FHA and Rad53 FHA1 Binding affinity determination with pT peptides by SPR. Four singly phosphorylated, two doubly phosphorylated and a quadruply phosphorylated peptides from SCD1 of Rad53 were biotinylated and immobilized on the SA sensor surface. All curves were fit to a single rectangular hyperbola ($dRU = c \times RU_{max}/(K_d + c)$). A, Interaction of 1P1 peptide (RMENI pT QPTQQSTQATQR) with Dun1 FHA (19-159) and FHA1 (14-164); B, Interaction of 1P2 peptide (RMENITQP pT QQSTQATQR) with Dun1 FHA (19-159) and FHA1 (14-164); C, Interaction of 1P3 peptide (RITQPTQQS pT QATQRFLR) with Dun1 FHA (19-159) and FHA1 (14-164); D, Interaction of 1P4 peptide (RPTQQSTQA pT QRFLIER) with Dun1 FHA (19-159) and FHA1 (14-164); E, Interaction of 2P1 peptide (RENI (pT)QP(pT )QQSTQATQRFLR) with Dun1 FHA (19-159) and FHA1 (14-164); F, Interaction of 2P6 peptide (RENITQPT QQSP(pT )QA( pT)QRFLR) with Dun1 FHA (19-159) and FHA1 (14-164); G, Interaction of 4P4 peptide (RENI(pT)QP(pT )QQS(pT )QA( pT) QRFLR) with Dun1 FHA (19-159) and FHA1 (14-164).
A. 1P1 with Dun1FHA and FHA1

B. 1P2 with Dun1FHA and FHA1

Figure 3.5  
Continued
C. 1P3 with Dun1FHA and FHA1

\[ K_d \text{ of Dun1FHA} = 264.76 \pm 4.41 \]
\[ K_d \text{ of FHA1} = 96.59 \pm 11.58 \]

D. 1P4 with Dun1FHA and FHA1

\[ K_d \text{ of Dun1FHA} = 354.05 \pm 32.18 \]
\[ K_d \text{ of FHA1} = 63.31 \pm 16.82 \]

Continued
Figure 3.5 Continued

E. 2P1 with Dun1FHA and FHA1

\[ K_d \text{ of Dun1FHA} = 0.97 \pm 0.03 \]
\[ K_d \text{ of FHA1} = 23.28 \pm 3.46 \]

F. 2P6 with Dun1FHA and FHA1

\[ K_d \text{ of Dun1FHA} = 4.77 \pm 0.53 \]
\[ K_d \text{ of FHA1} = 48.43 \pm 4.92 \]

Continued
Figure 3.5 Continued

G. 4P4 with Dun1FHA and FHA1

\[ K_d \text{ of Dun1FHA} = 0.96 \pm 0.08 \]
\[ K_d \text{ of FHA1} = 28.0759 \pm 3.04 \]
Figure 3.6: Overlaid Plots of Dun1 FHA and Rad53 FHA1 with singly and doubly phosphorylated peptides by SPR. Four singly phosphorylated, two doubly phosphorylated and a quadruply phosphorylated peptides from SCD1 of Rad53 were biotinylated and immobilized on the SA sensor surface. All curves were fit to a single rectangular hyperbola (dRU = c x RU_{max} / (K_{d} + c)). A, Interaction of Dun1 FHA (19-159) with four singly phosphorylated peptides (1P1 peptide: RMENI pT QPTQQSTQATQR, 1P2 peptide: RMENITQP pT QQSTQATQR), 1P3 peptide: RITQPTQQS pT QATQRFLR, 1P4 peptide: RPTQQSTQA pT QRFLIER); B Interaction of Dun1 FHA (19-159) with two doubly phosphorylated peptides and a quadruply phosphorylated peptide (2P1 peptide: RENI (pT) QP(pT )QQSTQATQRFLR, 2P6 peptide: RENITQPT QQ(pT )QA(pT )QRFLR, 4P4 peptide: RENI(pT)QP(pT )QQS(pT )QA(pT )QRFLR); C, Interaction of FHA1 (4-164) with four singly phosphorylated peptides (1P1, 1P2, 1P3, 1P4 peptides); D Interaction of FHA1 (4-164) with two doubly phosphorylated peptides and a quadruply phosphorylated peptide (2P1, 2P6, 4P4 peptides)
A. Dun1FHA with 1P1, 1P2, 1P3, and 1P4

B. Dun1FHA with 2P1, 2P6, and 4P4

Figure 3.6  Continued
Figure 3.6 Continued

C. FHA1 with 1P1, 1P2, 1P3, and 1P4

![Graph showing dRU vs Concentration for 1P1, 1P2, 1P3, and 1P4 with regression lines and Kd values.]

- Kd of 1P1 = 6.62 +/- 5.27
- Kd of 1P2 = 18.93 +/- 4.57
- Kd of 1P3 = 96.59 +/- 11.58
- Kd of 1P4 = 63.31 +/- 16.82

D. FHA1 with 2P1, 2P6, and 4P4

![Graph showing dRU vs Concentration for 2P1, 2P6, and 4P4 with regression lines and Kd values.]

- Kd of 2P1 = 23.28 +/- 3.46
- Kd of 2P6 = 48.43 +/- 4.92
- Kd of 4P4 = 28.07 +/- 3.04
3.6 Dun1 FHA requires at least two phosphorylations for its recognition of targets

As we have already discussed above with SPR results, HSQC titration also showed that multiple phosphorylation is necessary for Dun1 FHA recognition. If we compare Figure 3.7A and B, overall peak shift pattern looks similar, but there are more peaks involved in binding of Dun1 FHA complexed with 4P4 than singly phosphorylated peptides (1P1, 1P2, 1P3, and 1P4). In addition, there are two distinct new peaks that showed up only in the HSQC of the complex with 4P4 but not with any of the singly phosphorylated peptides. Those same new peaks started showing up with doubly phosphorylated peptide 2P1 along with arginine side chain peaks that we observed in 3P2 (RENI pT QP pT QQSTQA pT QRFLR) as we expected (Figure 3.7C and E). The first loop region sequence of Dun1 FHA is ^RSRSCD_65^, which is located between ß3 and ß4 strands of Dun1 FHA structure. The Arg60 is the residue highly conserved in all FHA domains and responsible for recognizing phosphate according to sequence homology and other FHA structure. Dun1 first loop has one more arginine at the 62 position, the peak for which we can see shift in multiply phosphorylated peptide complex, but no peak shift is observed in any of singly phosphorylated peptide complexes. By comparison of titrations, Arg62 and Lys100 might be involved in binding to the second phosphate of multiply phosphorylated peptides. It appears that the first two threonines (Thr5 and Thr8) of SCD1 are more crucial than the rest since 2P1 that has two phosphorylations at first two threonines interact with Dun1 FHA tighter than 2P6 that contains two phosphorylations on the last two threonines (Thr12 and Thr15). As shown in Figure 3.7, the general pattern of peak shifts of doubly phosphorylated peptide 2P1 is similar to its 3P1 and 4P4, which indicate that double phosphorylation on the first (Thr5) and second...
Thr8) threonines are sufficient for interaction between Dun1FHA and SCD1. HSQC spectra of 2P3 (Figure 3.7D) and 2P5 (Appendix A3) with Dun1 FHA confirmed this conclusion. Peptide 2P3 has double phosphorylations on the first TQ (Thr5) and the third TQ (Thr12), and its peak shift pattern is similar to that of a singly phosphorylated peptide. 2P5 phosphorylated on Thr8 and Thr15 also showed the same peak shift pattern as 2P3. This clearly indicates that Dun1 FHA requires both Thr5 and Thr8 to be phosphorylated in its recognition. Therefore, both SPR and NMR titration lead us to the same conclusion. Dun1FHA recognizes SCD1 when it is doubly phosphorylated. Dun1 FHA recognition sites on SCD1 were not known, and we now propose that it might be Thr5 and Thr8.
Figure 3.7: Overlaid $^1$H-$^{15}$N-HSQC spectra of Dun1-FHA (19-159) complexed with four peptides. A, $^1$H-$^{15}$N-HSQC spectra of yeast Dun1 FHA free (black) and in the presence of 1P1 peptide (RMENI pT QPTQQSTQATQR); B, $^1$H-$^{15}$N-HSQC spectra of yeast Dun1 FHA free (black) and in the presence of 4P4 (RENI pT QP pT QQ pT QA pT QRFLR) from Rad53 (pink); C, $^1$H-$^{15}$N-HSQC spectrum of free yeast Dun1-FHA (black) and in the presence of 2P1 peptide (RENI pT QP pT QQSTQATQRFLR); D, $^1$H-$^{15}$N-HSQC spectrum of free yeast Dun1-FHA (black) and in the presence of 2P3 peptide (RENI pT QP pT QQSTQATQRFLR); E, $^1$H-$^{15}$N-HSQC spectrum of free yeast Dun1-FHA (black) and in the presence of 3P2 (RENI pT QP pT QQSTQA pT QRFLR)
Figure 3.7 Continued
Figure 3.7 Continued

C. Dun1 FHA (19-159) with 2P1 peptide
Figure 3.7 Continued

D. Dun1 FHA (19-159) with 2P3 peptide
Figure 3.7 Continued

E. Dun1 FHA (19-159) with 3P2 peptide

Titration with 3P2
Black: Free dun1FHA(19–159), 0.4 mM
Pink: with 1 to 4
3.7 Summary and Discussion

Studies in this chapter show that Dun1 FHA prefers to bind multiply phosphorylated SCD1 after hyperphosphorylation of Rad53 by autophosphorylation, whereas FHA1 prefers to bind singly phosphorylated SCD1. In Figure 1.13, our model shows that one of the possible ways of Rad53 activation is by autophosphorylation after it was activated by the upstream protein Mec1 with help from adaptor protein Rad9. When SCD1 is phosphorylated on one of threonines among four threonines (Thr5, Thr8, Thr12, and Thr15), FHA1 can recognize it and those four threonines functions are redundant according to in vivo study (Lee, Schwartz et al. 2003). The important question we wanted to answer here was to figure out why only FHA1 recognizes phosphorylated SCD1 first when both FHA1 and Dun1 FHA can interact with phosphorylated SCD1. According to our studies on this chapter, Dun1 FHA has unique recognition specificity different from other FHA domains, which is to recognize two phosphates on its target proteins. Dun1 FHA showed weak interaction with singly phosphorylated SCD1 (K\textsubscript{d} range; 106 µM to 369 µM), while it showed tighter interaction with multiply phosphorylated SCD1 (K\textsubscript{d} range; 0.96 µM to 4.77 µM). As we already mentioned, FHA1 binds to 1P1, 1P2, 1P3, and 1P4 peptides 10 fold, 5.4 fold, 2.7 fold, and 5.6 fold respectively tighter than Dun1FHA. The data suggests that the third threonine (Thr12) has the weakest interaction with FHA1 among four singly phosphorylated SCD1s, which actually supports the result from Lee SJ et al. They tested their redundant function of 1-4 threonines in SCD1 of Rad53 by doing a sensitivity test with hydroxyurea (HU) that prevents DNA synthesis. In order to determine which threonine is more crucial for Rad53 function, they performed "add-back" alleles, which is to put threonines back one by one to their alanine mutants. What
they observed was that Rad53 function was fully recovered when they put threonines
back individually, but they saw a slight defect of its function when they put only third
threonine (Thr12) back to SCD1. Our affinity study by SPR showed that binding affinity
between FHA1 and 1P3 (phosphorylation on Thr12) was ~100 µM, which is the weakest
interaction among four singly phosphorylated peptides. It appears that three threonines
(Thr5, Thr8, and Thr15) except third threonine (Thr12) can be recognized by FHA1 tight
enough to make dimer prior to autophosphorylation. The third TQ might also be able to
make dimer with weak interaction with FHA1, but it seems like not that efficient.
Therefore, the conclusion of this chapter is that Dun1 FHA recognizes doubly
phosphorylated SCD1, whereas FHA1 interacts with singly phosphorylated SCD1
preferentially. The recognition sites of Dun1 FHA might be Thr5 and Thr8, and that of
FHA1 could be all four threonines.
CHAPTER 4

DUN1 AUTOPHOSPHORYLATION IS CONCENTRATION DEPENDENT AND REQUIRES ITS FHA DOMAIN

4.1 Objective and experimental plans

It has been known that the role of autophosphorylation is to amplify checkpoint signals rapidly and effectively. Autophosphorylation has become recognized as a general characteristic of many proteins that are involved in cell signaling pathways in response to DNA damage. Unlike some other FHA containing proteins such as human Chk2, budding yeast Rad53 and fission yeast Cds1, autophosphorylation of budding yeast Dun1 has not been studied yet, and its mechanisms are unknown. Therefore, this chapter focuses on characterizing autophosphorylation of Dun1, and studying the importance of the FHA domain in regulating autophosphorylation and its concentration-dependency.

Mutagenesis was performed in Dun1 FHA and kinase domains, followed by kinase assays with $\alpha^{-31}$P-ATP in order to see how they affect autophosphorylation of Dun1. Similar kinase assays were performed with wild type, FHA1 mutant, and SCD1 mutant of Rad53 to see how much these two domains affect autophosphorylation of
Rad53, so that they could be compared to Dun1. Large scale Dun1 autophosphorylation was also performed, so that mass spectrometry could be used to identify phosphorylation sites on full length Dun1.

4.2 Autophosphorylation of *S. cerevisiae* Rad53, Human Chk2 and *S. pombe* Cds1

Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and Rad3-related protein (ATR) are protein kinases, which play essential roles in DNA damage checkpoints (Zhou and Elledge 2000; Abraham 2001; Nyberg, Michelson et al. 2002; Shiloh 2003). The Mec1/Tel1 are counterparts of mammalian ATM/ATR, which are responsible for keeping genome integrity when there is DNA damage in *S. cerevisiae*. Many targets of these kinases contain SQ/TQ-rich cluster domains (SCDs), and serines and threonines in SCDs are phosphorylated by ATM/ATR-like protein kinases. Human Chk2, budding yeast Rad53 and fission yeast Cds1 can autophosphorylate in both *trans* and *cis*-phosphorylation, and autophosphorylation *in vitro* was concentration dependent. Autophosphorylation of Rad53 could occur even without phosphorylation by Mec1 when there is high concentration of Rad53 (Sun, Hsiao et al. 1998; Xu, Tsvetkov et al. 2002; Lee, Schwartz et al. 2003; Ma, Lee et al. 2006).

In the case of human Chk2, once Thr68 in its SCD is phosphorylated by ATM kinase, it can be recognized by the FHA domain of another inactive Chk2 and make an oligomer, which lead them to do trans-autophosphorylation on threonine residues. This autophosphorylation seems to fully activate Chk2 as a kinase (Ahn, Li et al. 2002; Xu, Tsvetkov et al. 2002). In addition, fission yeast Cds1 undergoes two step activations. Cds1 is first activated by its upstream kinase Rad3 with help from Mrcl, and then it
dimerizes through interaction between phospho-Thr11 in SCD and its FHA followed by autophosphorylation in the second step (Xu, Davenport et al. 2006). However, for Rad53 and Dun1, the threonines that are crucial for initiating dimerization and autophosphorylation are still unknown. Previous studies showed that Rad53 activation by Mec1 was not enough to fully activate Rad53 in vivo (de la Torre-Ruiz, Green et al. 1998; Emili 1998), and autophosphorylation of Rad53 itself contributed for Rad53 activation.

The dissociation mechanism of SCD and FHA domain after autophosphorylation is still not understood in detail. In the case of human Chk2, it seems like pThr68 in SCD dissociates from dimerization when FHA itself is phosphorylated by autophosphorylation (Ahn, Li et al. 2002). The autophosphorylation of Rad53 depends on dimerization through its FHAs and SCDs. Also, human Chk2 and fission yeast Cds1 showed similar characteristics regarding their autophosphorylations. All these three transducer proteins in cell signaling pathways contain at least one SCD, FHA and kinase domain. Dimerization prior to autophosphorylation appears to be crucial through interaction between phosphorylated SCD and FHA domain. However, Dun1 does not have any SCD, but it still clearly does autophosphorylate. Therefore, it is interesting to study oligomerization and autophosphorylation of Dun1.

4.3 Autophosphorylation of Dun1 is concentration-dependent

The human Chk2 and yeast Rad53 can autophosphorylate in both trans and cis-phosphorylation, and trans-phosphorylation is concentration dependent (Sun, Hsiao et al. 1998; Xu, Tsvetkov et al. 2002; Lee, Schwartz et al. 2003). The autophosphorylation of
these two proteins has been well studied, but that of Dun1 is still unknown and has not been studied yet.

In order to investigate autophosphorylation of Dun1, FLAG-tagged full length Dun1 (fDun1) was overexpressed in yeast and purified by immunoprecipitation against anti-FLAG antibody. Kinase assays were performed in six different Dun1 concentrations ranging from 0.05 nM to 2 nM. Immunoblot was also performed with exactly the same concentration as Dun1 used in the kinase assay in order to visualize its amount (Figure 4.1). Autophosphorylation was detected starting at 0.25 nM concentration, and its level was increased as the concentration was increased. Dun1 autophosphorylation occurs very effectively at high concentration starting 1 nM, and it shows an almost exponential curve when we plotted volume of ImageQuant (y axis) vs log Dun1 concentration (x axis) shown in Figure 4.1. The volume of ImageQuant represents the level of autophosphorylation. Dun1 was unable to autophosphorylate when its concentration was lower than 0.1 nM. The possible reason for this is that Dun1 might not be able to oligomerize at concentration lower than 0.1 nM. It might also indicate that Dun1 autophosphorylation is oligomerization-dependent, and it occurs through trans-phosphorylation rather than cis-phosphorylation. Therefore, Dun1 autophosphorylation is highly concentration dependent.
Figure 4.1: Concentration dependency of autophosphorylation of Dun1. Full length Dun1-FLAG was purified by immunoprecipitation. A series of 30 µL reactions were performed with different Dun1 concentrations from 0.05 nM to 2 nM in kinase reaction buffer containing 15 µCi of ?-32P ATP for 30 min at 30°C. (upper panel) Immunoblot was performed with the same concentrations as the kinase assay against anti-FLAG. (middle panel) Dun1 autophosphorylation was quantified using ImageQuant and plotted using SigmaPlot.
4.4 Dun1 FHA plays a crucial role in Dun1 autophosphorylation \textit{in vitro}

FLAG-tagged full length Dun1 (fDun1), kinase-dead Dun1 (D328A-fDun1) and FHA-dead Dun1 (R60N103A-Dun1) were overexpressed in yeast and purified by immunoprecipitation. In addition, V5-His-tagged full length Rad53, FHA1-dead Rad53 (R70N107A-Rad53), and SCD1-dead Rad53 (4AQs-Rad53) were also overexpressed in yeast and purified in order to be compared to Dun1. Dun1 autophosphorylation \textit{in vitro} was completely eliminated by mutating the FHA domain at Arg60 and Asn103. These two residues are located in the loop regions, which is structurally easy to access to its targets.

Upper panel of Figure 4.2 shows a gel picture of the kinase assay results with wild type and mutant Dun1s, and a graph on the bottom shows the quantification of kinase activity by ImageQuant. The first lane shows a negative control purified from yeast cell lysate without any protein expression against anti-FLAG. Wild type Dun1 can autophosphorylate, and the autophosphorylation level of Dun1 treated with 1% methylmethane sulfonate (MMS) was higher than the one purified from the normal cells (lane 2 and 3 in Figure 4.2). Under the normal environments, Dun1 seems to be able to autophosphorylate without any other proteins present, and it become more effective when DNA damage occurs by alkylating agent MMS. An interesting result here is that FHA-dead Dun1 (R60AN103A-fDun1) was unable to autophosphorylate similar to the kinase-dead Dun1 (D328A-fDun1) shown in lane 4 and 5 in Figure 4.2. This indicates that Dun1 FHA is crucial for its autophosphorylation. It is likely because Dun1 FHA is required for dimerization prior to autophosphorylation.
In the case of Rad53, FHA1-dead Rad53 (R70N107A-Rad53) showed autophosphorylation even though its level was quite low compared to SCD1-dead Rad53 (4AQs-Rad53) (Figure 4.3). The ability of autophosphorylation of the FHA1-dead Rad53 decreased down to approximately 19.3% of wild type Rad53, and that of SCD1-dead Rad53 decreased down to 37.2% of wild type. Therefore, both mutants showed less ability to autophosphorylate than wild type Rad53, but FHA1-dead mutant was affected much more than the SCD1-dead one. This suggests that FHA1 is more important than SCD1 in regulating its autophosphorylation. The autophosphorylation level of Rad53 was less affected by mutation of the FHA1 domain than that of Dun1 when Dun1 FHA was mutated. This happens probably because Rad53 has two FHA domains and two SCDs. FHA2 domain and SCD2 might be able to accomplish similar tasks when FHA1 and SCD1 are not functional. However, Dun1 has only one FHA domain and does not have any SCD. This simple fact probably makes the role of Dun1 FHA so important for the regulation of its autophosphorylation.
Figure 4.2: Autophosphorylation of wild type and mutants of full length Dun1. Gel picture of the kinase assay result of wild type and mutant Dun1s is shown in upper panel, and the graph on the bottom shows the quantification of kinase activity by ImageQuant. The x-axis numbers in graph on the bottom correspond to the lane numbers of the gel.
Figure 4.3: Autophosphorylation of wild type and mutants of full length Rad53. Gel picture of the kinase assay result of wild type and mutant Rad53 proteins is shown in upper panel, and the graph on the bottom shows the quantification of kinase activity by ImageQuant. The x-axis numbers in graph on the bottom correspond to the lane numbers of the gel.
4.5 Identification of autophosphorylation sites in full length Dun1

It has been reported that Rad53 has more than 30 phosphorylation sites by its autophosphorylation in vitro, but none of phosphorylation sites on Dun1 is known. Full length Dun1 with a FLAG tag was overexpressed in yeast and purified by immunoprecipitation. Autophosphorylation was performed as described in Method. Autophosphorylated Dun1 was sent to the mass facility at the Genomic Research Center in Taiwan for identification of phosphorylation sites. Sequenced tryptic peptides covered 43% of the whole Dun1 Sequence, and those peptide fragments are highlighted in red color in Figure 4.4A. Residue numbers and corresponding sequences are listed in Table 4.1. Mascot was used as a database searching tool. Eight peptides are identified as phosphopeptides, and they are $^2$SLSTKR$^7$, $^8$EH pS GDVTDSSF$^19$, $^{130}$SC pS FLFK$^{136}$, $^{137}$YA pS SSSTDIEFDEK$^{151}$, $^{137}$YASSS pS TDIENDE$^{151}$, $^{158}$pS YKNDDEVFK$^{167}$, $^{168}$KPQISATSSQNA T pT SAAIR$^{186}$, and $^{191}$TRPV pS FF DK$^{199}$. Eight newly identified phosphorylation sites are Ser4, Ser10, Ser132, Ser139, Ser142, Ser158, Thr181, and Ser195 (Listed in Table 4.2). Two representatives of MS spectra of peptide sequencing are shown in Figure 4.5, and the rest of MS spectra for five sequenced peptides are shown in the Appendix C.

As shown in Figure 4.6, the two identified phosphorylation sites sites (Ser4 and Ser10) are located at the end of N-terminal region of Dun1. Five phosphorylation sites were found in between FHA domain core region and its kinase domain. It has been known that FHA domain requires flanking region in both end of the core domain in order to be fully functional, and residues from 19 to 159 has been proposed to be functional Dun1 FHA
domain. Therefore, four phosphorylation sites (Ser132, Ser139, Ser142 and Ser158) are actually located at the end of C-terminus of Dun1 FHA domain (Figure 4.6).

Two identified phosphorylation sites on Ser4 and Ser10 might be responsible for its dimerization. As shown in Figure 4.6, human Chk2 makes a dimer via interaction between pThr68 and its FHA domain. Phosphorylation on Thr11 of S. pombe Cds1 is also crucial for dimerization. Interestingly, those two key residues (Thr11 of Cds1 and Thr68 of Chk2) are separated from their FHA core domains by ~ 50 amino acid residues. Out of seven phosphorylation sites, Ser4 and Ser10 residues are located ~ 50 residues apart from Dun1 FHA core domain. Therefore, we propose that these two residues might be key residues in dimerization of Dun1 FHA.
Figure 4.4: Matched peptides in protein sequence of Yeast Dun1. Peptide sequences matched in the MS studies are shown in red color.
Figure 4.5: Peptide sequencing shown in MS spectra. A, Sequenced peptide residue numbers are from 8 to 19, and the sequence is EHpSGDVTDSSFK. The serine at position 10 was phosphorylated; B, Sequenced peptide residue numbers are from 137 to 151, and the sequence is YA pS SSSTDIEINDDEK. The serine 139 was phosphorylated.
Figure 4.5
<table>
<thead>
<tr>
<th>Residue numbers</th>
<th>Sequenced tryptic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-7</td>
<td>SLSTKR</td>
</tr>
<tr>
<td>8 - 19</td>
<td>EHSGDVTDDSFK</td>
</tr>
<tr>
<td>45-53</td>
<td>EQKVEITNR</td>
</tr>
<tr>
<td>54-60</td>
<td>NVTTTIGR</td>
</tr>
<tr>
<td>130-136</td>
<td>SCSFLFK</td>
</tr>
<tr>
<td>137 - 151</td>
<td>YASSSSTDIENDDEK</td>
</tr>
<tr>
<td>152-157</td>
<td>VSSESR</td>
</tr>
<tr>
<td>158 – 167</td>
<td>SYKNDDEVFK</td>
</tr>
<tr>
<td>168-186</td>
<td>KPQISATSSQNATTSAAIR</td>
</tr>
<tr>
<td>191-199</td>
<td>TRPVSSFDFK</td>
</tr>
<tr>
<td>205-215</td>
<td>ELGAGH YALVK</td>
</tr>
<tr>
<td>221-229</td>
<td>KTGQQVAVK</td>
</tr>
<tr>
<td>230-240</td>
<td>IFHAQQNDDQK</td>
</tr>
<tr>
<td>247-254</td>
<td>EETNILMR</td>
</tr>
<tr>
<td>274-284</td>
<td>SQIQKYLVLEK</td>
</tr>
<tr>
<td>285-293</td>
<td>IDDGELFER</td>
</tr>
<tr>
<td>311-327</td>
<td>QLLTGLKYLH EQNIHR</td>
</tr>
<tr>
<td>396-401</td>
<td>EVLTKKGYTSK</td>
</tr>
<tr>
<td>432-438</td>
<td>EQILQAK</td>
</tr>
<tr>
<td>495-502</td>
<td>LELQRLQITDNK</td>
</tr>
</tbody>
</table>

**Table 4.1: List of sequenced peptide fragments of Dun1.** 43% of Dun1 sequence was covered. The residue numbers corresponds to the sequenced residues are listed on the left and the sequences in the right column.
<table>
<thead>
<tr>
<th>Residue numbers</th>
<th>Fragment sequences</th>
<th>Score</th>
<th>The phosphorylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-7</td>
<td>SL pS TKR</td>
<td>23</td>
<td>Ser4</td>
</tr>
<tr>
<td>8 - 19</td>
<td>EH pS GDVTSSFK</td>
<td>59</td>
<td>Ser10</td>
</tr>
<tr>
<td>130 - 136</td>
<td>SC pS FLFK</td>
<td>42</td>
<td>Ser132</td>
</tr>
<tr>
<td>137 - 151</td>
<td>YA pS SS pS TDIENDDEK</td>
<td>75</td>
<td>Ser139, Ser142</td>
</tr>
<tr>
<td>158 – 167</td>
<td>pS YKNDDEVFK</td>
<td>48</td>
<td>Ser158</td>
</tr>
<tr>
<td>168-186</td>
<td>KPQISATSSQNAT pT SAAIR</td>
<td>89</td>
<td>Thr181</td>
</tr>
<tr>
<td>191-199</td>
<td>TRPV pS FFDK</td>
<td>41</td>
<td>Ser195</td>
</tr>
</tbody>
</table>

**Table 4.2: List of phosho-peptide fragments and identified autophosphorylation sites of Dun1.** Residue numbers are listed along with the sequences, and third column shows scores from the searching tool Mascot for each phosphorylation site.
Figure 4.6: Identified phosphorylation sites on full length Dun1 by autophosphorylation *in vitro*. A, Gene diagram of human Chk2, T68 is the key residue responsible for oligomerization of Chk2; B, A, Gene diagram of *S. pombe* Cds1, T11 is the key residue responsible for oligomerization of Cds1; C, Newly identified seven phosphorylation sites in Dun1 are shown in Red, T5 and S10 might be residues essential for dimerization of Dun1.
4.6 Summary and Discussion

Results in this chapter have shown that the FHA domain is essential for Dun1 autophosphorylation *in vitro* because its autophosphorylation was completely eliminated in FHA-dead mutant (R60AN103A-fDun1). This result also supports our model (Figure 1.13A). In our model, Dun1 FHA recognizes another Dun1 to make a dimer followed by autophosphorylation. Dun1 FHA mutant could not dimerize and autophosphorlylate. This result clearly showed the significance of dimerization and the role of Dun1 FHA. Dun1 autophosphorylation occurs in concentration-dependent manner similar to Rad53.

Key phosphorylation sites of Dun1 required for its oligomerization prior to autophosphorylation has not yet been determined. Eight phosphorylation sites by autophosphorylation have been identified. Seven out of eight were on serine residues, and only one threonine residue (T181) was identified as phosphothreonine. We found two residues (Ser4 and Ser10) that could be responsible for that function. Phosphorylations on both Ser4 and Ser10 might be necessary since Dun1 FHA recognize double phosphorylations instead of single phosphorylation on its targets.
CHAPTER 5

BINDING ANALYSIS OF DUN1 FHA BASED ON STRUCTURE

5.1 Objective and experimental plans

Unlike other well studied FHA domains, structure of Dun1 FHA has not been solved yet due to some difficulties in expression and stability. X-ray crystallography was used in attempt of solving free Dun1 FHA structure before, but its diffraction was not good to produce 3D structure (Blanchard, Fontes et al. 2001). Our goal was to solve structure of Dun1 FHA by NMR.

Two constructs, Dun1 FHA (27-144) and Dun1 FHA (19-159), were cloned and expressed, and only Dun1 FHA residue from 19 to 159 was stable enough to pursue further study. It was stable for at least 10 days at room temperature in buffer containing 5 mM HEPES, pH 7.5, 200 mM NaCl, 5 mM DTT, and 1mM EDTA. NMR structure determination and all HSQC titration experiments were performed in this buffer condition. Dun1 FHA (19-159) construct was used for all studies in this chapter even though it contains a long random-coiled region on both N- and C-terminus. Purification of a smaller construct (residues from 27-144) was unsuccessful due to precipitation. Dun1
FHA showed precipitation easily when its concentration was increased over 0.5 mM. Hence, 0.4 ~ 0.5 mM concentration was used for all studies in this chapter. $^{15}$N-HSQC titrations were performed with many different synthesized peptides from biological targets in order to study binding site mapping.

5.2 Structure determination of free Dun1 FHA (19-159) and its description

Free Dun1 FHA structure has been solved by Dr. Chunhua Yuan. I overexpressed and purified non-labeled, $^{15}$N-labeled, $^{15}$N, $^{13}$C-labeled, and $^{15}$N, $^{13}$C, $^2$H-labeled Dun1 FHA proteins, and involved in early stage of solving structure by NMR, which was to read chemical shifts of $H_n$, $H_N$, $C_a$, and $C_\beta$ from $^{15}$N, $^1$H-HSQC and triple resonance NMR experiments (HNCA, HN(CO)CA, CBCANH, and CBCA(CO)NH). It was handed over to Dr. Yuan and he solved free Dun1 FHA structure.

Overall structure of free Dun1 FHA (19-159) is similar to other known FHA domain structures. It consists of two large twisted anti-parallel $\beta$-sheets with 10 $\beta$-strands and no $\alpha$-helices shown in Figure 5.1. It has two long random coil regions on both ends from residue 19 to 29 and from 142 to 159, which was excluded from the structure. Those 10 $\beta$-strands are numbered from $\beta_1$ to $\beta_9$, and $\beta_3'$ represents a short one that exists right after $\beta_3$ strand. The overall Dun1 FHA structure is similar to human Ki67 FHA structure (Figure 5.2A and C). Both FHA domains do not have any $\alpha$-helices unlike FHA1 and FHA2. There are five highly conserved residues in FHA domains, which are Gly59, Arg60, Ser74, His77 and Asn103 in Dun1 FHA. All of them are located in the loop regions. Gly59 and Arg60 are in the first loop between $\beta_3$ and $\beta_3'$, and Ser74 and His77
are located in the loop between β3' and β4. The last conserved residue Asn103 is in the loop between β5 and β6, and it is known to be crucial for Dun1 FHA function.
**Figure 5.1: Ribbon diagram of Dun1 FHA.** A. Stereoview showing the backbone atoms of 20 Dun1 FHA structures (19-159). The backbone atoms of residues 19-159 were used for superposition; B, Stereoview of the ribbon diagram of a representative Dun1 FHA structure.
5.3 Structure comparison of three FHA domains (Dun1 FHA, Rad53 FHA1, and Ki67 FHA)

Free FHA1 (14-164) structure and two FHA1 complexed with pT peptides from another yeast protein Rad9 were determined by Dr. Chunhua Yuan (Liao, Yuan et al. 2000; Yuan, Yongkiettrakul et al. 2001). Another FHA1 complex structure was also solved by Anjali Mahajan with different pT peptide from Mdt1 identified as biological target protein of Rad53 later on. Both free and complex of human Ki67 FHA structure were solved by Dr. Byeon In-Ja (Li, Byeon et al. 2004; Byeon, Li et al. 2005).

FHA1 interacts with phosphothreonine containing target peptides, but it does not bind to phosphotyrosine or phosphoserine containing ones. The first two target peptides were selected based on peptide library screening. According to library screening, FHA1 showed high selectivity for pTXXD that has aspartic acid at the +3 position of phosphothreonine. Therefore, two pTXXD peptides were selected from Rad9 known to have interaction with Rad53. Those two peptides were $^{188}$SLEV(pT)EADATFVQ$^{200}$ and $^{148}$KKMTFQ(pT)PTDPLE$^{160}$. Five amino acid residues from FHA1 (Ser82, Arg83, Ser85, Thr106, and Asn107) showed intermolecular NOEs with three amino acid residues in peptides (pT, pT+1, and pT+2) in both complexes. In these two structures, interaction between phosphothreonine and side-chains of Asn86 and Arg70 contributed mainly for the binding selectivity along with interaction between side-chain of Arg83 and Asp at the +3 position of peptide.

Ki67 FHA had different ligand specificity, which is to require a long extended binding surface. After testing many short peptides, a long peptide (44 amino acid residues from hNIFK) was selected as a tight interacting target. The peptide was originally
unstructured and random-coiled, but it has become well-structured upon binding to Ki67 FHA. Interestingly, a β-strand was adopted from hNIFK peptide when it interacts with β4-strand of Ki67 FHA, which is why Ki67 FHA required a long extended binding surface to recognize its target. This is a unique characteristic of Ki67 FHA, which did not apply to Dun1 FHA according to our studies in chapter 3.

The Ki67 FHA structure is the most similar one to Dun1 FHA structure among all known FHA domain structures. However, their recognition specificities are completely different. The most important factor for the recognition of Ki67 FHA appears to be a long extended binding surface, whereas that of Dun1 FHA is double phosphorylations. In case of Dun1 FHA, the length of target does not affect in recognition, but it requires multiple phosphorylations to be bound. Dun1 FHA binds to a doubly phosphorylated 20 amino acid long peptide 2P1 (RENI pTQP pT QQSTQATQRFLR) from SCD1 of Rad53. The dissociation equilibrium constant $K_d$ was 0.96 µM. As shown in 2P1 sequence above, it is composed of many uncharged polar residues. Hence, interaction forces between Dun1 FHA and 2P1 peptide might be mainly hydrogen bonding.
Figure 5.2: Ribbon diagrams of representative FHA (a), FHA1 (b), and K97 FHA structures (c) solved by NMR. The letter N represents N-termini and C for C-termini region. All structures were generated by using WebLab ViewerPro program.
Figure 5: Sequence alignments of Rad53 FHA1, FHA2 and Dun1 FHA domains. A-helices are shown in red and secondary structural elements are shown in orange (a-helices) and green (b-strands). Dun1 FHA does not have any a-helices.
5.4 Binding surface mapping of Dun1 FHA based on HSQC spectra with 4P4 peptide

In order to map out binding surfaces of Dun1 FHA, free Dun1 FHA structure and a known binding target (4P4) were used. $^{15}$N-HSQC spectra of free Dun1 (Black) and complexed with a quadruply phosphorylated peptide 4P4 (Pink) are shown in Figure 5.4A. There are about 30 peaks shifted upon binding, and two new peaks showed up only in $^{15}$N-HSQC of Dun1 FHA complexed with 4P4 peptide. All 30 shifted peaks are assigned and marked their names right next to peaks shown in red color in structure (Figure 5.4B). As shown in ribbon diagram of structure, all 30 shifted peaks are located in four loop regions also shown in red. All four loop regions appear to be involved in the binding directly or indirectly. Those loop sequences are RSRSCD (located between β3 and β3’), SEPDISTFH (between β3’ and β4), KSRNG (between β5 and β6), and LKNGD (between β8 and β9). Dun1 FHA contains many arginines and lysines in those loop regions except the second loop when we compare these loop sequences to that of Rad53 FHA1 and FHA2 (See Figure 5.3). These additional arginines (Arg62 and Arg102) and lysines (Lys100 and Lys120) in the binding surface could give a unique ligand specificity of Dun1 FHA, which is to recognize doubly phosphorylated targets. As for two new peaks showed up upon the binding to 4P4 peptide, one of them could be Asn103 since it did not show in $^{15}$N-HSQC of free Dun1 FHA similar to Asn87 of FHA1, which also did not show in its free HSQC and showed up in complex HSQC upon binding. Four functionally important residues are Arg60, Ser74, His77, and Asn103 shown in ball and stick in green color. These four residues are highly conserved in all FHA domains (Figure 1.4 and 5.4).
and stick (green). Structure was generated by WebLab ViewerPro program.

All of them are located in loops. Four residues (K60, S74, H77, and N103) are critical for Dnu1-FA function and shown in ball and stick (pink). All residues shield upon binding are shown in red. Dnu1-FA structure. A1. Residues shielded upon binding are shown in red. Dnu1-FA (black) and complexed with 4Pb1-Phe (green).
5.5 Differences in Dun1 FHA binding pattern between singly and multiply phosphorylated SCD1 peptides by HSQC titrations

\(^{15}\)N-Dun1 FHA (19-159) was prepared as described in Materials and Methods, and all phosphopeptides were purchased from the company (Genemed Synthesis). Dun1 FHA was titrated with a series of peptide concentration from 0.25 mM to 2 mM. 20 mM peptide stocks for all peptides were used for HSQC titrations. As shown in the Figure 5.5, all four singly phosphorylated peptides show similar peak shift patterns, which indicate that they interact with Dun1 FHA in a similar manner. About 20 peaks were shifted or missing during the titrations. Also, most of peaks gradually shifted upon binding to phosphopeptides, and some of them showed gradual weakening of signal, which indicate intermediate exchanges. In HSQC spectra of 1P1, 1P3 and 1P4, we can see more gradual peak shifts than peak weakening or disappearing, while in that of 1P2, some of peaks weakened as pT peptide concentration were increased. Therefore, binding upon 1P2 peptide generates different exchange status of certain amino acid residues (such as Ser74 and His77) from the rest of singly phosphorylated peptides. When we look at HSQC of 4P4 with Dun1 FHA, almost all 30 peaks disappeared at first several titrations and reappeared as concentration was increased to 1 to 1 ratio of protein and peptide. This indicates that most of them showed intermediate exchange upon its binding. In FHA1 case, Asn86 does not show in its free HSQC spectrum, but it appeared as a new peak upon binding to its target peptides. There are five conserved residues among FHA domains, which are Gly59, Arg60, Ser74, His77, and Asn103 in Dun1 FHA. Among these residues, Dun1 FHA loses its function when it was mutated at Arg60 and Asn103 at the same time. In addition, it has been shown that double mutant at Ser74 and His77 also
could not perform its function properly (Bashkirov, Bashkirova et al. 2003). Both Ser74 and His77 peaks were either gradually shifted or weakened at different levels in HSQC spectra of Dun1 FHA complexed with singly phosphorylated peptides, and there is no peak showed up in all four peptides. On the other hand, Dun1 FHA complexed with quadruply phosphorylated peptide 4P4, Ser74 and His77 were disappeared first and reappeared when 1 to 1 ratio concentration of peptide was added.
Figure 5.5: $^{15}$N-HSQC spectra of free Dun1-FHA (19-159) and complexed with pT peptides. Free Dun1 FHA is shown in black, and Dun1 FHA complexed with 1 to $\frac{1}{2}$ ratio (green), 1 to 1 ratio (blue), 1 to 2 ratio (yellow) and 1 to 4 ratio (pink) ratio of peptides are shown in colors in parentheses. A, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with peptide 1P1 (RMENI pT QPTQQSTQATQR); B, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with peptide 1P2 (RMENITQP pT QQSTQATQR); C, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with 1P3 peptide (RITQPTQQS pT QATQRFLR); D, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with 1P4 peptide (RPTQQSTQA pT QRFLIER); E, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with peptide 4P4 (RENI pT QP pT QQS pT QA pT QRFLR).
A. Dun1 FHA (19-159) with 1P1

B. Dun1 FHA (19-159) with 1P2

Figure 5.5  Continued
Figure 5.5 Continued

C. Dun1 FHA (19-159) with 1P3

D. Dun1 FHA (19-159) with 1P4

Continued
Figure 5.5 Continued

E. Dun1 FHA (19-159) with 4P4
Figure 5.6: Chemical shift changes of the backbone amide groups of residues that are involved in Dun1 FHA interaction upon the binding. These are partial $^{15}$N-HSQC spectra from free Dun1-FHA (19-159) and complexed with five pT peptides. Free Dun1 FHA is shown in black, and Dun1 FHA complexed with 1 to $\frac{1}{2}$ ratio (green), 1 to 1 ratio (blue), 1 to 2 ratio (yellow) and 1 to 4 ratio (pink) ratio of peptides are shown in colors in parentheses. A, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with peptide 1P1 (RMENI pT QPTQQSTQATQR); B, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with peptide 1P2 (RMENITQP pT QQSTQATQR); C, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with 1P3 peptide (RITQPTQQS pT QATQRFLR); D, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with 1P4 peptide (RPTQQSTQA pT QRFLIER); E, Overlaid spectra $^{15}$N-HSQC of Dun1 FHA with peptide 4P4 (RENI pT HQ pT QQS pT QA pT QRFLER).
Figure 5.6
5.6 Summary and discussion

The overall structure of free Dun1 FHA is similar to other FHA domain structures. Four loop regions are involved in its binding to a quadruply phosphorylated peptide 4P4, and those loop sequences uniquely contain more arginines and lysines than Rad53 FHA1 or FHA2. This could be the reason why Dun1 FHA interacts with multiply phosphorylated targets since Arg60, a conserved residue in all FHA domains, is mainly responsible for interacting with phospho-Thr. Those extra arginines (Arg62 and Arg102) and lysines (Lys100 and Lys120) in binding surfaces of Dun1 FHA might interact with the second phospho-Thr of the 4P4 peptide. Despite structural similarity, ligand specificity of Dun1 FHA is completely different from other FHA domains. We are in the process of solving structures of the complexes of Dun1 FHA with two small peptides, NI (pT)QP (pT) QQST and SL (pS) TKREH (pS) GDVT. The first peptide is from SCD1 of Rad53, which will show the interaction between SCD1 of Rad53 and Dun1 FHA. The second peptide is from N-terminus of Dun1 itself, which will give some insight for Dun1 dimerization prior to autophosphorylation.
CHAPTER 6

SCREENING FOR ADDITIONAL BIOLOGICAL TARGETS OF DUN1 FHA

6.1 Objective and experimental plans

Dun1 has been known to be involved with many proteins that are related to not only DNA damage response, but also cell cycle regulation under normal conditions and in the times of cell stress. There is strong proof that Dun1 interacts with other yeast proteins, which might lead to a direction for alternative pathways of DNA repair in yeast. In addition, Dun1 also might have new functions in areas other than DNA damage response since the functions of some proteins screened as Dun1 interacting proteins are still unknown. Therefore, this chapter was focuses on finding binding partners of Dun1 FHA in the presence of DNA damage and under normal condition.

In order to study the last objective, pull down assays were performed with whole yeast cell lysates from both methylmethane sulfonate (MMS)-treated and normal cells. Mass spectrometry was used to identify Dun1 FHA interacting proteins. In order to
confirm this interaction by an in vivo method, co-immunoprecipitation was done after co-expression of both Dun1 FHA and possible target proteins.

6.2 Identification of Dun1-FHA-interacting proteins under MMS condition by pull down assay and mass spectrometry

More than 30 proteins were identified as targets of full length Dun1 (Ho, Gruhler et al. 2002). Here we report some proteins that interact with Dun1 FHA specifically. Roughly 20 different Dun1 FHA interacting proteins were identified by in vitro pull down assay followed by mass spectrometry. Identified proteins are listed in Table 6.1. Not all of them belong to the list identified for full length Dun1. Some proteins interact with Dun1 FHA constitutively, but some of them interact with Dun1 FHA only under MMS treatment. As shown in the Figure 6.1, we were able to see distinct cdc28 band around 35 kDa in both healthy and DNA damaged cells. This suggests that interaction between Dun1 FHA and Cdc28 is not limited to DNA damage response. It might be involved in the regular cell cycle regulation.

Cdc28 is not only required for regulating cell cycle progression, but also for a number of DNA damage responses. Swi6 is a good target for G1 cycle delay after DNA damage, although Dun1 has been known to play more important roles for G2/M checkpoint based on currently known data. Rad50 is important for all facets of genome stability such as checkpoint activation, initiation and execution of non-homologous end-joining and homologous recombination, DNA damage detection, and telomere maintenance. Rnr1 is a large chain of ribonucleoside reductase (RNR), and other RNR subunits are already known to interact with Dun1. Ssa1 and Ssa2 are heat shock protein,
and some other heat shock proteins have recently been shown to play a role in checkpoint regulation. eIF3a, EF1A, and Ils1 are translational proteins. Tbb could reflect a role in delaying spindle elongation by G2/M checkpoint, and this function is defective in Dun1 mutants. There are some metabolic enzymes identified, which could be linked to checkpoints.
**Figure 6.1: Pull down assay results.** Dun1 FHA (19-159) was immobilized and whole yeast lysate was passed through followed by thorough washing with 50 mM imidazole solution. Yeast cells applied to lane 1 and 2 were not treated with MMS, and the ones applied to lane 3 and 4 were treated with 1% MMS for an hour in order to induce DNA damage. Cdc28 (34 kDa) is shown in the darkest bands in both lane 1 (from healthy cells) and 3 (from DNA damaged cells) between 37 kDa and 25 kDa.
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC28 (CDC28, CDK1)</td>
<td>Cell division control protein</td>
</tr>
<tr>
<td>SWI6</td>
<td>Regulatory protein SWI6 (Cell-cycle box factor, chain SW)</td>
</tr>
<tr>
<td>RAD50</td>
<td>DNA repair protein</td>
</tr>
<tr>
<td>RNR1, RNR2</td>
<td>Ribonucleoside-diphosphate reductase large chain 1</td>
</tr>
<tr>
<td>EIF3A</td>
<td>Eukaryotic translation initiation factor 3 110 kDa suunit</td>
</tr>
<tr>
<td>EF1A</td>
<td>Elongation factor 1-alpha</td>
</tr>
<tr>
<td>VMA1</td>
<td>vacuolar ATPase V1 domain</td>
</tr>
<tr>
<td>YGL7</td>
<td>Hypothetical 30.8 kDa protein in ABC1-CDC20 intergenic region</td>
</tr>
<tr>
<td>NSR1 (P67)</td>
<td>Nuclear localization sequence binding protein</td>
</tr>
<tr>
<td>SNF4</td>
<td>Nuclear protein Snf4</td>
</tr>
<tr>
<td>ILS1</td>
<td>Isoleucyl-tRNA synthetase</td>
</tr>
<tr>
<td>HS71(SSA1), HS72(SSA2)</td>
<td>Heat shock protein SSA1 (Heat shock protein YG100)</td>
</tr>
<tr>
<td>ASN1</td>
<td>Asparagine synthetase</td>
</tr>
<tr>
<td>ADH1</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>DHA6 (ALD6)</td>
<td>Mg-activated aldehyde dehydrogenase</td>
</tr>
<tr>
<td>KPY1</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>TBB</td>
<td>Tubulin beta chain</td>
</tr>
</tbody>
</table>

**Table 6.1:** Dun1 FHA associated proteins identified by pull down assay followed by mass Spectrometry. Identified proteins are listed and full name of each protein is shown on the right side, which also summerizes their main functions.
6.3 *In vivo* confirmation of interaction between Dun1FHA and cdc28 by Co-immunoprecipitation

Three proteins, Swi6, Rnr2, and Cdc28, were chosen and subcloned into yeast expression vector pESC-TRP. The pESC-TRP has two multiple cloning sites (MCS), and we put Dun1 FHA in MCS1 and targets in MCS2. In order to verify the pull-down assay results, co-immunoprecipitation was performed. We were able to co-express Rnr2 and Cdc28 with Dun1 FHA. For some reason, co-expression of Swi6 with Dun1 FHA was unsuccessful.

Two chemicals, MMS and hydroxyurea (HU), were used to induce DNA damage directly or indirectly. MMS directly damages DNA by alkylation in bases, which leads single strand break. In contrast, HU indirectly affects DNA by inhibiting RNR. As I mentioned in chapter 1, RNR protein is an enzyme that is responsible for converting ribonucleotides to deoxyribonucleotides. By this mechanism hydroxyurea reduces the overall concentration of dNTPs in the cell, and therefore it ends up blocking or reducing the efficiency of DNA replication.

Expression of both Dun1 FHA and Cdc28 under MMS and basal conditions are visible in the cell lysate (Figure 6.2). Figure 6.2A shows interaction between Cdc28 and Dun1 FHA under normal and MMS conditions by Co-IP, and Figure 6.2B shows Co-IP result under MMS and HU conditions. Co-IP against myc-Cdc28 captures Dun1FHA-FLAG in both normal and MMS conditions, but not in HU condition. Dun1 FHA and Cdc28 interact with each other even without DNA damage, which is the same result as in the pull down assay. However, Dun1 FHA does not interact with Cdc28 under HU condition. In other words, HU abolishes the interaction between Cdc28 and Dun1 FHA.
One possible explanation could be because HU indirectly cause the dephosphorylation of Cdc28, which might hinder interaction with Dun1 FHA since interaction is phosphorylation-dependent.

Any kind of interaction between Dun1 FHA and Rnr2 could hardly been detected under normal, MMS, and HU conditions (Figure 6.3). Expression level of Rnr2 was not as high as that of Dun1 FHA. Co-IP against Dun1 FHA does not capture Rnr2 in both control and under damaged conditions (MMS and HU). Also, Co-IP against Rnr2 does not capture Dun1 FHA either, which can give us conclusion that Dun1 FHA and rnr2 does not interact at any conditions in vivo. According to Co-IP results, Dun1 FHA does interact with Cdc28, but it does not bind to Rnr2.
Figure 6.2: Co-immunoprecipitations of Dun1 FHA and Cdc28. A, Co-IP between wild type Dun1 FHA and wild type full length cdc28 under normal and MMS conditions; B, Co-IP between wild type Dun1 FHA and cdc28 under MMS and HU conditions. Co-IPs were performed against monoclonal anti-myc and anti-FLAG. The letter M and H represent MMS and HU, respectively.
Figure 6.3: Co-immunoprecipitations results of Dun1 FHA and Rnr2. Wild type Dun1 FHA-FLAG and Rnr2-myc were co-expressed in yeast under normal, MMS, HU conditions. Co-IPs were performed against monoclonal anti-myc and anti-FLAG. The letter C, M, and H represent control, MMS, and HU, respectively.
6.4 Co-immunoprecipitation with wild type and mutant Dun1 FHA and Cdc28

Three Dun1 FHA mutants (R60AN103A-Dun1 FHA, S74AH77A-Dun1 FHA, and R60AN103AS74AH77A-Dun1 FHA) and three Cdc28 mutants (T18AY19A-Cdc28, T169A-Cdc28, and T230A-Cdc28) were generated by mutagenesis. Arg60 and Asn103 are conserved residues in all FHA domains, which are known to be responsible for the interaction with phosphate on Thr of target proteins. In addition, S74 and H77 are also known be significant in Dun1 FHA functions. Using wild type Cdc28 and three different mutants of Dun1 FHA we see that the interaction between these proteins is completely abolished in all three Dun1 FHA mutants shown in Figure 6.4A. According to these Co-IP results, all four residues are involved in binding to cdc28, or those residues might distrubt Dun1 FHA structure, which hinders interaction between these two proteins.

Cdc28 is inactivated when either T18 or Y19 is phosphorylated, which is why mutagenesis on these residues were done in order to see if Dun1 FHA recognizes this region (Booher, Deshaies et al. 1993). The T18AY19A-Cdc28 double mutant still expressed well. Similar to wild type Cdc28, this double mutant is not captured at a detectable level during Co-IP against Dun1 FHA. Co-IP against T18AY19A-Cdc28 showed that a very small quantity of Dun1 FHA is captured under normal conditions, but not under MMS conditions (Figure 6.4B). The amount of Dun1 FHA captured by this mutant appears to be dramatically lower than that captured by wild type Cdc28. This suggests that the association of Dun1 FHA with Cdc28 is dependent, at least in part, on the phosphorylation of one or both residues Y18 and T19 of Cdc28 under normal conditions.
The second mutant of cdc28 is T169A because of the opposite reason from first mutant. The phosphorylation on T169 activates cdc28 function. The third mutant T230 of cdc28 has been chosen for mutation because we see interaction in healthy cells without DNA damage, and in many cases basal phosphorylation occurs in S/T followed by proline. T230 is the only (S/T)(P) in entire cdc28. Both T169A-Cdc28 and T230A-Cdc28 mutants expressed well similar to wild type Cdc28. Both mutants were not captured at a detectable level during Co-IP against Dun1 FHA. Co-IP against T169A-Cdc28 showed that Dun1 FHA was not captured at all under either normal or MMS conditions, but Co-IP against T230A-Cdc28 showed that a very small quantity of Dun1-FHA was captured under normal conditions. Summarized Co-IP results are illustrated in Figure 6.5. Under the normal condition, interaction between Dun1 FHA and Cdc28 depends on all four residues (R60, N103, S74, and H77) on Dun1 FHA and T169 on Cdc28. All four residues on Dun1 FHA and all three residues on Cdc28 are involved in interaction between two proteins under MMS condition.
Figure 6.4: Co-immunoprecipitation results with wild type and mutant Dun1 FHA and Cdc28. A, Co-IP of wild type Cdc28 and four Dun1 FHAs (Wily type, R60AN103A-Dun1 FHA, S74AH77A-Dun1 FHA, and R60AN103AS74AH77A-Dun1); B, Co-IP of wild type Dun1 FHA and four Cdc28s (T18AY19A-Cdc28, T169A-Cdc28, and T230A-Cdc28). Wild type and mutants of both Dun1 FHA and Cdc28 were co-expressed in yeast under normal and MMS conditions. All Dun1 FHAs has FLAG-tag, and Cdc28 has myc-tag. Co-IPs were performed against monoclonal anti-myc and anti-FLAG.
Figure 6.4

Continued
Figure 6.4 continued

B

<table>
<thead>
<tr>
<th>MMS</th>
<th>Lysate</th>
<th>Co-IP anti-FLAG</th>
<th>Co-IP anti-myc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- **T18Y19A** cdc28-myc
- **WT Dun1 FHA-FLAG**
- **T169A** cdc28-myc
- **WT Dun1 FHA-FLAG**
- **T230A** cdc28-myc
- **WT Dun1 FHA-FLAG**
Figure 6.5: Summary of all CO-IP results. Interactions between Duntl FHA and Cdc28 under normal conditions are shown in left panel, and right panel shows results under DNA damaged conditions. Bold arrows (→) and dotted arrows (→) represent strong and weak interactions, respectively.
6.5 Summary and Discussion

Eighteen proteins were identified as putative binding targets of the Dun1 FHA domain. Some of them (Cdc28, Swi6, Rnr1, Rnr2, and Rad50) are involved in cell cycle regulation, cell cycle checkpoint, and DNA damage detection. Three of them (eIF3a, EF1A, and Ils1) have functions in translation. Interaction between Dun1 FHA and Cdc28 was confirmed by an *in vivo* method. Not all of them may be real binding partners of Dun1 FHA, but it is still useful information to be aware of them since Dun1 FHA could directly or indirectly regulate biological function of these identified proteins. In addition, they might be working together as large protein complexes in some cases. Ultimately, identifying new targets could open a door for different directions that have not been discovered yet. In order to explore more about this, further studies are necessary.
BIBLIOGRAPHY


APPENDIX A

HSQC TITRATION SPECTRA OF DUN1 FHA WITH DOUBLY PHOSPHORYLATED PEPTIDES
Figure A.1: $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 3P1 peptide
Figure A.2: $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 3P4 peptide
Figure A.3: $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 2P4 peptide
Figure A.4: $^{15}$N-HSQC spectra of Dun1 FHA (19-159) with 2P5 peptide
APPENDIX B

DISSOCIATION EQUILIBRIUM CONSTANT DETERMINATION BY SPR
Figure B.1: Binding affinity determination of Dun1 FHA and 1P1 peptide
1P1 with FHA1 (14-164)

$K_d = 6.62 \pm 5.27$

$R = 0.9849$

**Figure B.2:** Binding affinity determination of FHA1 and 1P1 peptide
Figure B.3: Binding affinity determination of Dun1 FHA and 1P2 peptide
1P2 with FHA1 (14-164)

\[ K_d = 18.93 +/- 4.57 \]
\[ R = 0.9958 \]

**Figure B.4:** Binding affinity determination of FHA1 and 1P2 peptide
**Figure B.5:** Binding affinity determination of Dun1 FHA and 1P3 peptide
Figure B.6: Binding affinity determination of FHA1 and 1P3 peptide
Figure B.7: Binding affinity determination of Dun1 FHA and 1P4 peptide

1P4 with Dun1FHA (19-159)

Concentration (µM)

dRU

\[ K_d = 354.05 \pm 32.18 \]

\[ R = 0.9997 \]
Figure B.8: Binding affinity determination of FHA1 and 1P4 peptide
**Figure B.9:** Binding affinity determination of Dun1 FHA and 2P1 peptide
Figure B.10: Binding affinity determination of FHA1 and 2P1 peptide
Figure B.11: Binding affinity determination of Dun1 FHA and 2P6 peptide
Figure B.12: Binding affinity determination of FHA1 and 2P6 peptide
**Figure B.13:** Binding affinity determination of Dun1 FHA and 4P4 peptide
Figure B.14: Binding affinity determination of Dun1 FHA and 4P4 peptide
APPENDIX C

PEPTIDE SEQUENCING SHOWN IN MS SPECTRA
Figure C1: Peptide sequencing of YASSSSpSTIEHNDDEK shown in mass spectra.
Figure C2: Peptide sequencing of SYKNDDEVK shown in mass spectra
Figure C3: Peptide sequencing of KPOIISAITSQNSATSAIR shown in mass spectra.
Figure C4: Peptide sequencing of TRPVSFK shown in mass spectra.