Quantitative proteomic analysis of chromatin-bound proteins in the fission yeast *Schizosaccharomyces pombe*

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**QUANTITATIVE PROTEOMIC
ANALYSIS OF CHROMATIN-BOUND
PROTEINS IN THE FISSION YEAST
*SCHIZOSACCHAROMYCES POMBE***

WANG HAN

School of Biological Sciences

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
TABLE OF CONTENTS	ii
LIST OF FIGURES	vi
LIST OF TABLES	viii
ABBREVIATIONS	ix
SUMMARY	1
CHAPTER 1. INTRODUCTION.....	4
1.1. Overview	4
1.2. Chromatin background	5
1.2.1. Chromatin architecture.....	5
1.2.2. Histone chaperones	7
1.2.3. DNA-binding proteins	9
1.2.4. Significance and disease relevance of chromatin-associated proteins.....	18
1.2.5. Fission yeast as an ideal model for studying chromatin-associated proteins.....	19
1.3. Strategies to identify chromatin-associated proteins.....	21
1.3.1. Single-protein strategies.....	22
1.3.2. Proteomic approaches	24
1.4. Research objectives	34
1.4.1. Analysis of non-histone chromatin-associated proteins using SILAC approach	34
1.4.2. Discovery of novel histone H4-interacting proteins	37
CHAPTER 2. MATERIALS AND METHODS	38
2.1. Strains	38
2.1.1. Bacterial strains.....	38
2.1.2. Fission yeast strains	38
2.2. Culture media	40
2.3. Plasmids.....	41
2.4. Antibodies	44
2.5. General protein methods	45
2.5.1. Preparation of cell lysate.....	45

2.5.2. Protein quantification	45
2.5.3. SDS-PAGE	46
2.5.4. Western blotting.....	46
2.5.5. Coomassie blue staining	46
2.5.6. Preparation of tryptic peptides	47
2.5.7. NanoLC-MS/MS.....	47
2.6. Chromatin fractionation assay	49
2.7. SILAC method	50
2.7.1. Testing of SILAC incorporation	50
2.7.2. Validation of SILAC incorporation	51
2.7.3. Preparation of SILAC mixture.....	51
2.7.4. Data analysis	52
2.7.5. Statistical analysis	54
2.8. Growth assays.....	55
2.8.1. Mini-culture growth curve assay	56
2.8.2. Plating assay.....	57
2.9. Microscopy analysis	58
2.9.1. Indirect immunofluorescence microscopy	58
2.9.2. Nuclear and cell wall staining.....	59
2.10. Time course study	59
2.11. Fluorescence-activated cell sorting (FACS).....	60
2.12. Homology searches and alignments	61
2.13. Cloning, expression and purification of Histone H4	61
2.13.1. Cloning of carboxy-terminal TAP tagging vector pJK210-CTAP- <i>hhf1</i>	61
2.13.2. Transformation of pJK210-CTAP- <i>hhf1</i> into fission yeast.....	62
2.13.3. Protein expression of pJK210-CTAP- <i>hhf1</i>	62
2.13.4. Protein preparation by dual-step affinity purification.....	63
2.13.5. Silver staining	64
2.13.6. Sample preparation and LC-MS/MS analysis.....	65
2.13.7. Statistical analysis	65
CHAPTER 3. IDENTIFICATION OF NON-HISTONE CHROMATIN- ASSOCIATED PROTEINS USING SILAC-MEDIATED PROTEOMIC ANALYSIS	66

3.1.	Introduction	66
3.2.	Results	70
3.2.1.	Cells are fully incorporated with the heavy lysine after nine generations	70
3.2.2.	The observed and expected premix ratio of light versus heavy peptides/proteins are highly correlated in SILAC pre-mixed samples.....	74
3.2.3.	Chromatin fractionation assay is a valid method to identify chromatin-associated proteins.....	75
3.2.4.	SILAC analysis of proteins from chromatin preparation.....	78
3.2.5.	Phenotypic analysis of SILAC-enriched proteins responsible for maintaining genomic stability.....	87
3.3.	Discussion	97
CHAPTER 4.PHENOTYPIC ANALYSIS OF HIRA PROTEIN HIP4 ..		107
4.1.	Introduction	107
4.2.	Results	110
4.2.1.	<i>hip4</i> ⁺ is required for DNA damage resistance	110
4.2.2.	Disruption of <i>hip4</i> ⁺ results in growth defects and sensitivity to spindle damage.....	113
4.2.3.	Hip4 is localized to the nucleus and associated with chromatin..	116
4.2.4.	Hip4 is not responsible for G2/M DNA damage checkpoint.....	117
4.3.	Discussion	124
CHAPTER 5.PHENOTYPIC ANALYSIS OF ZINC FINGER PROTEIN		
BC530.05		129
5.1.	Introduction	129
5.2.	Results	133
5.2.1.	<i>BC530.05</i> ⁺ encodes a zinc finger fungal specific DNA binding protein	133
5.2.2.	<i>BC530.05</i> ⁺ is required for normal resistance to microtubule inhibitors	136
5.2.3.	<i>BC530.05</i> Δ displays normal cell division and septa formation at restrictive temperature	139
5.2.4.	<i>BC530.05</i> ⁺ is required for normal tolerance to caffeine	140
5.2.5.	Deletion of <i>BC530.05</i> ⁺ results in hypersensitivity to CPT	147

5.2.6. <i>BC530.05</i> ⁺ is required for normal resistance to toxic cations.....	149
5.2.7. <i>Bc530.05</i> is not involved in oxidative stress pathways	153
5.2.8. <i>Bc530.05</i> might be involved in calcineurin-mediated signalling pathway	153
5.3. Discussion	154
CHAPTER 6. IDENTIFICATION OF HISTONE H4 INTERACTING PARTNERS USING TANDEM AFFINITY PURIFICATION.....	160
6.1. Introduction	160
6.2. Results	164
6.3. Discussion	175
CHAPTER 7. GENERAL CONCLUSION.....	180
7.1. Conclusion of Chapter 3.....	180
7.2. Conclusion of Chapter 4.....	182
7.3. Conclusion of Chapter 5.....	183
7.4. Conclusion of Chapter 6.....	184
7.5. Future perspectives.....	185
APPENDIX A. SUPPLEMENTAL MATERIALS AND METHODS.....	187
APPENDIX B. SUPPLEMENTAL DATA	200
APPENDIX C. CONTENTS OF THE DVD-R	214
PUBLICATION RELATED TO THIS STUDY	216
REFERENCES.....	217

LIST OF FIGURES

Figure 1.1. A schematic representation of nucleosome core.	6
Figure 1.2. Histone assembly factors CAF-1 and HIRA complex.	8
Figure 1.3. The DNA damage checkpoint response.	14
Figure 1.4. Pathway for DNA damage and replication checkpoints in fission yeast.	15
Figure 1.5. Cellular architecture and morphology of fission yeast.	20
Figure 1.6. Two phases of SILAC experiment.	27
Figure 2.1. The schematic view of SILAC labelling and further procedures for analysis of chromatin extract.	52
Figure 2.2. A schematic overview of the Trans-Proteomic Pipeline (TPP) workflow for filtering potential chromatin-associated proteins (CAPs).	54
Figure 2.3. Map of the cloning vector for tandem affinity purification (TAP) tagging in <i>S. pombe</i>	62
Figure 3.1. Analysis of heavy lysine incorporation.	73
Figure 3.2. Observed ratio of the light and heavy peptide levels is correlated with the expected ratio in the pre-mixed samples using SILAC.	75
Figure 3.3. The procedure and validation of chromatin fractionation assay.	77
Figure 3.4. The ribosomal proteins are depleted in the top 10% SILAC-ranked proteins.	80
Figure 3.5. The nucleus-localized proteins are enriched in the top 10% SILAC-ranked proteins.	82
Figure 3.6. The chromatin association of the SILAC-enriched and SILAC-depleted nucleus-localized proteins.	84
Figure 3.7. The chromatin association of the SILAC-enriched dual-localized proteins.	85
Figure 3.8. SILAC-enriched nucleus-localized proteins exhibit the high likelihood of requirement for growth fitness in MMS.	87
Figure 3.9. Phenotypic assessment of the deletion mutants lacking one of the SILAC-enriched proteins ($\text{Log}_2^{\text{SILAC ratio}} \geq 1.0$).	88
Figure 3.10. Venn diagram showing the occurrence of HU and MMS sensitivity in the SILAC analysis and in the genome-wide study.	91
Figure 3.11. The frequency of obtaining candidates related to DNA damage and repair from the SILAC-enriched proteins is much higher than from the two genome-wide studies.	92
Figure 3.12. Pie chart of function categories of the SILAC-enriched proteins whose deletions lead to sensitivity to HU, or MMS, or both.	97
Figure 4.1. <i>hip4</i> Δ cells are sensitive to DNA damaging agents.	112
Figure 4.2. <i>hip4</i> Δ cells display growth defects.	114
Figure 4.3. <i>hip4</i> Δ cells display elongated cell morphology.	115
Figure 4.4. <i>hip4</i> Δ cells are hypersensitive to spindle damaging agents.	115
Figure 4.5. Hip4 is localized to the nucleus and associated with chromatin. ..	117
Figure 4.6. Comparison of the phosphorylated Cdc2-Tyr15 protein level between wild-type and <i>hip4</i> Δ cells.	119
Figure 4.7. FACS analysis of S-phase DNA damage checkpoint in <i>hip4</i> Δ	120
Figure 4.8. Phenotypic analysis of double deletion mutants in response to DNA damaging agent MMS.	121

Figure 4.9. Validation of the expression vectors pREP1- <i>hip4</i> ⁺ and pREP41- <i>hip4</i> ⁺	122
Figure 4.10. Functional examination of the expression vectors pREP1- <i>hip4</i> ⁺ and pREP41- <i>hip4</i> ⁺	123
Figure 4.11. Genetic interactions between <i>hip4</i> ⁺ and DNA damage checkpoint kinases.	124
Figure 5.1. Cartoon representation of three major classes of eukaryotic zinc finger proteins.	130
Figure 5.2. Sequence alignment of Bc530.05 with zinc finger fungal specific proteins.	135
Figure 5.3. <i>BC530.05</i> ⁺ is required for normal tolerance to spindle damaging agent TBZ and MBC, and the protein is activated by TBZ.	138
Figure 5.4. Microscopic analysis of <i>BC530.05Δ</i> at restrictive temperature. ...	140
Figure 5.5. <i>BC530.05Δ</i> cells are sensitive to caffeine treatment.	142
Figure 5.6. Nuclear organization in <i>BC530.05Δ</i> upon caffeine treatment.	144
Figure 5.7. The protein level change of Bc530.05 following caffeine treatment.	145
Figure 5.8. The caffeine sensitivity of <i>BC530.05Δ</i> is not due to accumulation of cAMP.	146
Figure 5.9. The sensitivity of <i>BC530.05Δ</i> to DNA damaging agents.	148
Figure 5.10. <i>BC530.05</i> ⁺ is required for cellular resistance to the toxic cations.	151
Figure 5.11. Bc530.05 is not upregulated in response to toxic cation.	152
Figure 5.12. Bc530.05 is not involved in oxidative stress pathway.	153
Figure 5.13. Bc530.05 is responsible for regulating Ca ²⁺ homeostasis.	154
Figure 6.1. A schematic representation of tandem affinity purification.	164
Figure 6.2. C-terminal TAP tagging of Hhf1.	165
Figure 6.3. Protein expression and purification of TAP-tagged Hhf1.	167
Figure 6.4. Silver-stained SDS-PAGE gel of C-TAP-Hhf1 purification.	168
Figure 6.5. Proteins identified in the C-TAP-Hhf1.	169
 Figure A.1. A schematic overview of the IP2 workflow.	 191
Figure A.2. Plasmid maps of pREP1 and pREP41.	199
 Figure B.1. Analysis of chromatin extract via non-SILAC proteomic analysis.	 200
Figure B.2. Expression of HA-tagged fusion proteins used for chromatin fractionation assay.	201
Figure B.3. Mini-culture growth curve assays for sensitivity to 0.008% (v/v) MMS.	202
Figure B.4. A time course study to test conditions for spheroplasting.	204
Figure B.5. Analysis of ¹⁵ N labelling incorporation.	208
Figure B.6. The observed and expected ratio of the light and heavy peptide/protein levels in the pre-mixed samples are highly correlated in ¹⁵ N labelling.	209

LIST OF TABLES

Table 2.1. Fission yeast strains used in this study.	39
Table 2.2. Culture media used in this study.	42
Table 2.3. Plasmids used in this study.	43
Table 2.4. Antibodies used in this study.	44
Table 2.5. List of chemicals used in growth assays.	55
Table 3.1. Summary of growth phenotypes of the deletion mutants lacking one of the SILAC-enriched proteins.	89
Table 4.1. Comparison of the cell growth between wild-type and <i>hip4Δ</i> cells in liquid culture containing the indicated genotoxic agents.	112
Table 6.1. List of proteins identified by MS co-purifying with <i>S. pombe</i> C-TAP- Hhf1.	172
Table 6.2. Localizations and functions of the proteins identified by MS co- purifying with <i>S. pombe</i> C-TAP-Hhf1.	173
Table A.1. Bioneer deletion strains used in this study.	192
Table A.2. List of primers for construction and validation of HA-tagged strains and deletion mutant strains.	197
Table B.1. Quantification efficiencies obtained using Census within the IP2 for 7 ¹⁵ N pre-mixed samples analyzed using LTQ-Orbitrap.	209

ABBREVIATIONS

A-index	Relative Abundance Index
ATM	Ataxia Telangiectasia Mutated
ATR	ATM-Related
ASF1	anti-silencing factor 1
β-ME	β-mercaptoethanol
BRCA1	breast cancer gene 1
BSA	bovine serum albumin
bZIP	basic leucine zipper
CAF-1	Chromatin Assembly Factor-1
caffeine	3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione
cAMP	cyclic AMP
CAP	chromatin-associated proteins
CBP	calmodulin binding peptide
cdc	cell division cycle
CDK	cyclin-dependent protein kinase
<i>C. elegans</i>	<i>Caenorhabditis Elegans</i>
ChIP	Chromatin immunoprecipitation
Co-IP	Coimmunoprecipitation
CPT	Camptothecin
cs	cold-sensitive
CsA	cyclosporine A
CSC	checkpoint sliding clamp
C-TAP	carboxyl-terminal tandem affinity purification
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DSB	double strand break
DT	doubling time
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMM2	Edinburgh Minimal Medium
FACS	Fluorescence-activated cell sorting
FDR	false discovery rate
FK506	tacrolimus
4-NQO	4-Nitroquinoline-N-oxide
HA	haemagglutinin
HDAC	histone deacetylase
HIRA	histone regulatory complex A
HPLC	High-performance liquid chromatography
HP1	Heterochromatin protein 1
HRD	Hpc2-related domain
HRP	horseradish peroxidase
H ₂ O ₂	Hydrogen peroxide

HU	Hydroxyurea
ICAT	Isotope-coded affinity tag
IDV	Integrated Density Value
IgG	immunoglobulin G
IR	ionizing radiation
<i>kan</i> ⁺	kanamycin
LB	Luria Broth
LC	liquid chromatography
MAP kinase	Mitogen-activated protein kinase
MBC	Methyl 2-benzimidazolecarbamate
mChIP	modified ChIP
MDR	multi-drug resistance
MMS	Methyl methanesulfonate
MNase	micrococcal nuclease
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
m/z	Mass-to-charge ratio
9-1-1	Rad9-Rad1-Hus1
NP-40	Nonidet P-40
OD	optical density
1-DE	one-dimensional electrophoresis
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PI	propidium iodide
PiCh	proteomics of isolated chromatin segments
Pol II	RNA polymerase II
PTM	post-translational modification
PVDF	polyvinylidene fluoride
Rad	radiation
RFC	replication factor C
RNAi	RNA interference
RNR	ribonucleotide reductase
RT	room temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulfate
SIC	Single Ion Chromatogram
SILAC	Stable Isotope Labeling with Amino Acids in Cell Culture
SOC	Super Optimal broth with Catabolic repressor
SPB	spindle pole body
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SSB	single strand break
TAP	tandem affinity purification
TBZ	Thiabendazole
TCA	trichloroacetic acid
TEV	tobacco etch virus
Topo I	DNA Topoisomerase I
TPP	Trans-Proteomic Pipeline
TSA	trichostatin A

2-DE	two-dimensional electrophoresis
TX-100	Triton X-100
UPLC	Ultra performance liquid chromatography
<i>ura4⁺</i>	uracil
UV	Ultraviolet light
WCE	whole cell extract

SUMMARY

Chromatin-bound proteins, also known as chromatin-associated proteins (CAPs), play important roles in regulation of various biological processes. These include chromatin remodelling, replication, DNA damage and repair, and transcription during which physical interaction with the chromatin is required. Aberrations of these processes at the chromatin level are associated with many diseases, such as different types of cancers and neurodegenerative diseases. Proteomic analysis via Chromatin immunoprecipitation (ChIP) requires prior knowledge of the non-histone CAPs, and chromatin fractionation assay through the release of CAPs from the DNase I digested crude chromatin fails to effectively deplete the highly abundant ribosomal proteins.

The fission yeast *Schizosaccharomyces pombe* (*S. pombe*) is an excellent model to study CAPs. Through coupling chromatin fractionation assay with the Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)-based proteomics, we show that the relative abundance of most ribosomal proteins in chromatin fractionation assay is reduced when compared to the heavy lysine ($^{13}\text{C}_6$ -lysine)-labelled whole cell extract (WCE). We also find that the nucleus-localized proteins with chromatin-related functions are significantly overrepresented in the top 10% most enriched proteins. Chromatin fractionation assay further reveals that the SILAC-enriched but not SILAC-depleted nucleus-localized proteins are associated with the chromatin. Phenotypic assessment indicates that the SILAC-enriched nucleus-localized proteins are more likely to play a role in growth fitness under DNA damage stresses than the SILAC-depleted ones. Together, our results suggest that the SILAC-mediated proteomic approach is capable of determining non-histone

CAPs without prior knowledge. We propose this method is complementary to the proteomic analysis of protein complexes purified via ChIP with tandem affinity purification (TAP)-tagged CAPs for identification of CAP-interacting partners.

Bc947.08c (or Hip4) and Bc530.05 are two novel non-histone CAPs that are significantly enriched in our SILAC analysis. To understand functional roles of these two proteins, we performed a series of phenotypic analysis in the fission yeast mutants deleted for *BC947.08c*⁺ (or *hip4*⁺) and *BC530.05*⁺. Our results conclude that *hip4*⁺ is responsible for maintaining genomic integrity. However, deletion of *hip4*⁺ does not lead to inactivation of G2/M DNA damage checkpoint. Genetic analysis shows that *hip4*⁺ is epistatic to the DNA damage checkpoint kinases, further suggesting this gene is not responsible for G2/M DNA damage checkpoint control. Bc530.05 is a putative fungal-specific zinc finger transcription factor. We show that *BC530.05*⁺ is responsible for normal tolerance of specific DNA damaging and spindle damaging agents. The activity of this potential non-histone CAP is also essential for cellular resistance to caffeine and cation toxicity. We propose Bc530.05 might be associated with the activity of calcineurin, which is important in regulating cell integrity. Given that both proteins are novel, deep analysis of these phenotypes and further characterization of their interactions with chromatin are essential to determine their precise functions in eukaryotic cells.

Histone-interacting proteins play critical roles in cell cycle regulation and chromatin structure maintenance, and there has been no systematic analysis using proteomic approach in fission yeast. To this end, we perform a pilot experiment by expressing histone H4.1 (i.e., Hhf1) in a TAP assay to identify

potential histone H4-interacting partners in fission yeast cells. All four core histones (H2A, H2B, H3 and H4) were pulled out of the cell lysates by Hhf1 and identified with high sequence coverage. We also find that Sap1 and Rim1 are potential histone-binding proteins. Besides Sap1 and Rim1, most of the targeted proteins possess chromatin-related functions, including chromosome assembly, DNA replication and repair, and cell cycle regulation. These miscellaneous functions might depict the synergy of these proteins to maintain an organized chromatin structure through interacting with histone proteins.

Overall, these efforts will help us to find new regulators of chromatin and thus provide mechanistic insights into the potential biomarkers for disease diagnosis and treatment.

CHAPTER 1.INTRODUCTION

1.1. Overview

The DNA content of a cell is packaged into proteinaceous structures called chromosomes. Chromatin, the basic structural matrix of chromosomes, affects many aspects of chromosome function. Chromatin is composed of DNA, histones and non-histone proteins. Many different types of proteins are known to be associated with chromatin, and the histones are the ones with most intimate contact with DNA. CAPs are involved in a variety of essential cellular events such as gene transcription or silencing, DNA replication and repair, during which physical interaction with the chromatin is essential. Although the highly abundant histones have been extensively studied for many decades, many other CAPs have just been reported or have not been discovered. Therefore, discovery of these proteins is able to make the whole picture of chromatin more complete. CAPs are extensively associated with specific human diseases, mainly cancer. Given that many CAPs are evolutionarily conserved from fission yeast to human, functional studies of these proteins in fission yeast may shed lights on human disease development.

The development of chromatin techniques and analysis has greatly accelerated the progress of functional discovery of important structural proteins and enzymes associated with chromatin. The extensive employment of proteomic strategies significantly broadens our knowledge about protein crosstalks and protein-DNA contacts in the chromatin. Development of ChIP assays means a milestone in chromatin biology. This method has been modified

and extended to a proteome-wide scale by coupling with affinity purification and mass spectrometry (MS) for identifying the interacting partners of known CAPs. However, this kind of analysis requires prior knowledge about chromatin, and does not apply to high-throughput screening. Chromatin fractionation assay has been a classical strategy to determine whether a particular protein is associated with chromatin. By coupling Chromatin fractionation assay with MS-based proteomic approach, multiple protein complexes without direct interactions will be isolated simultaneously. Theoretically, this strategy allows systematic analysis of proteins in the whole chromatin macrocomplex. Furthermore, this method does not rely on the availability of antibodies, and circumvents the problems that epitope tagging affects the physical or chemical properties of certain proteins.

Introduction in this chapter will be divided into four parts. First, we will briefly introduce the background of chromatin structure, followed by the major types of CAPs, their crosstalks, and their disease implications. Second, considering we adopted fission yeast as our study model, we will describe its advantages in chromatin research. Next, we will discuss the popular strategies involved in identifying CAPs, focusing on the innovative proteomic strategies. Lastly, we will describe the project objectives.

1.2. Chromatin background

1.2.1. Chromatin architecture

Eukaryotic genomes are packaged into a highly dynamic nucleoprotein complex called chromatin. Histone proteins are the central organizers of this

process. The fundamental building block of chromatin is the nucleosome, which provides the first level of compaction of DNA into the nucleus. Each nucleosome contains a core particle and a linker region that joins adjacent core particles (Figure 1.1). The core particle is composed of a histone octamer formed by two histone H2A/H2B dimers and one histone H3/H4 tetramer. This protein core is wrapped about 1.7 superhelical turns by 147 base pairs of DNA (1-3).

Figure 1.1. A schematic representation of nucleosome core. Nucleosome core particle consists of a histone octamer wrapped by double stranded DNA. The histone octamer comprises two sets of four histone proteins: H2A, H2B, H3, and H4. The unstructured histone tails extend from the nucleosome core and subject to a multitude of post-translational modifications. Histone tails are absent from the crystal structure but have been drawn in for clarity. This figure is adapted from White *et al.* (4).

The four core histones are small and basic proteins composed of an N-terminal tail, a C-terminal tail and a globular domain. The histone tails are subjected to numerous types of post-translational modifications (PTMs), which are proposed to modify the charge of histones and thereby alter DNA accessibility and protein-protein interactions with the nucleosome (5-9). A linker region with variable linker DNA flanks the nucleosome core particle. Linker DNA serves as landing sites for DNA-binding proteins (10, 11). The

linker histone H1 binds to the linker DNA region between nucleosomes and forms the chromatosome. It likely plays a role in nucleosome positioning and higher-order chromatin compaction (12-17).

The nucleosome cores, also known as “beads on a ring”, are packaged onto each other to generate a compact fiber with a diameter of 30 nm (18). The structure of chromatin is well defined, but the exact mechanism of higher-order compaction is still unclear (19, 20). Histone H1 is crucial for compacting nucleosomal DNA, but the precise role for how it pulls nucleosomes together is poorly understood (21). The condensed mitotic heterochromatin and decondensed interphase euchromatin are the two constituents of the 30 nm-fiber (22-24). Heterochromatin is transcriptionally inert and less accessible to transcriptional machinery; whereas euchromatin activates transcription, and comprises the most active portion of the genome within the nucleus (25).

1.2.2. Histone chaperones

Histone chaperones, also known as chromatin assembly factors, are escort proteins to maintain desirable chromatin structure by controlling histone supply and incorporation into chromatin (26). They are essential for maintaining genomic stability and epigenetic memory (26). Chromatin assembly factors form complex with histones and mediate the orderly deposition of histones onto DNA. They help to convey specific histones to the sites where chromatin is being newly synthesized or remodelled.

In vivo studies suggest that nucleosome assembly are conducted via two distinct but functionally overlapping histone deposition pathways; through Chromatin Assembly Factor-1 (CAF-1) and the histone regulatory complex A

(HIRA) (27). CAF-1 depends on DNA replication while HIRA functions independent of DNA synthesis (Figure 1.2) (28, 29).

Figure 1.2. Histone assembly factors CAF-1 and HIRA complex. Chromatin Assembly Factor-1 (CAF-1) is a heterotrimeric complex that participates in DNA synthesis-associated processes, such as DNA replication and DNA repair, through incorporating histone H3.1. Histone regulatory complex A (HIRA) functions in a DNA synthesis-independent manner, depositing the histone variant H3.3. CAF-1 and HIRA are assisted by the histone chaperone anti-silencing factor 1 (ASF1). Dashed red lines in the chromatin indicate newly synthesized DNA. Orange shapes indicate H3–H4 dimers and green shapes indicate H2A–H2B dimers. This figure is adapted from Ramirez-Parra *et al.* (30).

The essential role of CAF-1 in replication-coupled histone deposition was first discovered by replicating and assembling SV40 minichromosomes *de novo* in human nuclei (31), and further confirmed in *Xenopus laevis* egg extracts in assembly during DNA replication (32). CAF-1 is also the main histone chaperone that assists nucleosome assembly coupled to DNA synthesis during DNA repair (33–35). Different from CAF-1, which deposit the major histone H3.1 during DNA replication and possibly DNA repair, HIRA functions to deposit the histone variant H3.3 at any time during the cell cycle or in differentiated cells (34, 36) to mark the transcriptionally active genes (37). HIRA is evolutionarily conserved. Although HIRA is required for H3.3 enrichment at active genes, it is not essential for localization of H3.3 at

telomeres and many transcription factor binding sites (38). In mammalian cells, multiple and distinct factors are responsible for H3.3 localization at specific genomic locations.

Both CAF-1 and HIRA histone chaperones are assisted by anti-silencing factor 1 (ASF1), an H3-H4 histone donor (39). Upon forming the complex with histone H3-H4, ASF1 can enhance histone deposition process (39, 40), and may not directly play a role in histone deposition. ASF1 also contributes to HIRA-dependent gene silencing in budding yeast (41). Therefore, ASF1 is a key chromatin assembly factor in terms of possessing conserved interactions with proteins involved in DNA replication, DNA repair, and chromatin silencing. In human cells, ASF1 consists of two genes, *Asf1a* and *Asf1b*, which are potentially involved in DNA synthesis-dependent and synthesis-independent nucleosome assembly (34). *Asf1a* specifically interacts with HIRA (34, 42), both *Asf1a* and HIRA have been involved in the formation of macroH2A-containing senescence-associated heterochromatic foci (SAHF) (43). By applying immunodepletion strategies targeting ASF1, HIRA, or CAF-1 in *Xenopus*, it is found that ASF1 is not directly involved in *de novo* histone deposition either coupled or uncoupled to DNA synthesis (44).

1.2.3. DNA-binding proteins

DNA-binding proteins are involved in a variety of important DNA-associated transactions, including mitosis, DNA repair and transcription. Most DNA-binding proteins contain DNA-binding domains (DBDs) and thus have a specific or general affinity for either single or double stranded DNA (45). They can incorporate domains such as the zinc finger, the helix-turn-helix, and the

leucine zipper that facilitate binding to nucleic acid. The discovery of many DNA-binding proteins has facilitated the study of cell cycle control, DNA damage, and transcriptional regulation (9, 46); all of which are tightly associated with cell survival and disease development in humans.

1.2.3.1. Cell cycle regulators

The cell cycle has to be carefully controlled to ensure proper cell division. It is an elaborate process set in motion by a set of cell cycle regulators, especially the protein kinases, which are remarkably conserved from yeast to human. They are responsible for triggering the major cell cycle transitions, and preventing uncontrolled cell division. The eukaryotic cell cycle comprises of four sequential phases: duplication of the genome during the DNA synthesis phase (S phase), segregation of complete sets of chromosomes to each of the daughter cells in mitotic phase (M phase); these two phases are separated temporally by gaps known as G1, which connects the completion of M phase to initiation of S phase in the next cycle, and G2, which separates the S and M phases. G1 cells may temporarily or permanently leave the cell cycle and enter a quiescent or senescent phase known as “post-mitotic” phase, or G0. The processes that drive a cell through the cell cycle must be tightly regulated to ensure the events occur in the correct order and take place only once per cell cycle, which is crucial for cell survival.

Cyclin-CDK complex—In fission yeast, the discovery of cell cycle comes off the isolation of temperature sensitive (ts), cell division cycle (*cdc*) mutants, in which the cell population can be arrested with the same morphology upon shifting to the restrictive temperature. Thus, many cell cycle control genes in mammalian cells are called *cdc* genes. The cell cycle control depends on the

activity of cyclin-dependent protein kinases (CDKs). *Cdc2*⁺ is the single CDK to promote cell cycle progression in *S. pombe*. Mutation of this gene arrests the cell cycle in both G1/S and G2/M transition (47). *Cdc2*⁺ is functionally homologous to CDC28 in the budding yeast *Saccharomyces. cerevisiae* (*S. cerevisiae*) and Cdc2 in mammals, highlighting the conserved activity of this cell cycle regulator (48).

Cell cycle checkpoints—The progression through the phases of a cell cycle is monitored and controlled by cell cycle checkpoints. To ensure faithful cell division in eukaryotic cells, cell cycle checkpoints function as sentinels to verify whether each stage of the cell cycle have been accurately completed before progression into the next one. The integrated checkpoint responses to genomic assaults protect the cells from cytotoxic and mutagenic effects caused by chemical and environmental stresses. DNA damage checkpoint, replication checkpoint, and mitotic spindle checkpoint are a set of surveillance mechanism for eukaryotic cells to detect DNA damage, replication arrest, and spindle assembly defects. Cells lacking functional checkpoints display genomic instability due to an inability to properly respond to DNA damage, faulty DNA replication, or aberrant chromosome segregation (49). In mammalian cells, Ataxia Telangiectasia Mutated (ATM) and ATM-Related (ATR) proteins are key factors for maintaining the fidelity of these cell cycle checkpoints, except for mitotic spindle checkpoint (50). ATM and ATR proteins are highly conserved in eukaryotic cells, including budding and fission yeast. I will detail their roles in the following section “DNA damage response factors”.

1.2.3.2.DNA damage response factors

In nature, the genomic DNA is under constant exposure to damaging agents that can compromise its integrity. To maintain genomic integrity, cells have to respond properly to prevent or repair the injury, including cell cycle arrest, stabilization of replication forks, stimulation of DNA repair, and activation of apoptosis (51-53). These are collectively known as DNA damage response (DDR). DDR is one of the most important mechanisms involved in cell cycle (54). The inability to properly react to DNA damage results in genomic instability, and predisposes individuals to cancer (55-57).

DNA damage can be induced by ionizing radiation (IR), ultraviolet light (UV), free radicals, as well as chemicals in the environment and chemicals used in cancer chemotherapy. There are various forms of DNA damage. Firstly, there can be covalent modifications of the four different bases in DNA (A, T, C, G). IR or chemicals can cause single strand breaks (SSBs), or double strand breaks (DSBs). In addition, exposure to chemotherapeutic drugs causes DNA cross-links, which can be formed between bases on the same DNA strand (intrastrand cross-links) or on the opposite strand (interstrand cross-links). All these unwanted modifications and fragmentations result in DNA damage.

DNA damage checkpoint—Given the prevalence of DNA damage and the importance of maintaining replication integrity, there are multiple checkpoints prior to DNA replication and mitosis to monitor the state of the genome. These are termed DNA damage checkpoint. The DNA damage checkpoint recognizes DNA damage, halts cell cycle progression, and provides enough time for the cell to repair the DNA damage and restore the original DNA sequence and structure (58).

In a broader context, DNA damage checkpoint involves a signal transduction pathway (Figure 1.3). Once damage occurs, it will be detected by sensors, which scan the chromatin for DNA abnormalities and convey signals to transducers for cell cycle arrest. The transducers further relay the signals to downstream effectors to either repair the lesions, or target cells for destruction by means of apoptosis if the DNA damage is irreversible. All the checkpoints that assess DNA damage appear to utilize the same sensor-transducer-effector mechanism, and the same components are always shared among these distinct checkpoints. For instance, the protein kinases and phosphatases, which function as DNA damage transducers, are shared by the different checkpoints to varying degrees (50). Furthermore, there is not an absolute differentiation among the various components in a given checkpoint response. For example, DNA damage sensors may also function as signal transducers.

DNA damage checkpoint proteins are discovered by radiation-sensitive mutants whose sensitivity is a result of checkpoint failure. Majority of these proteins are associated with chromatin DNA. In fission yeast, the radiation (Rad) group of checkpoint proteins, including Rad1, Rad9, Rad17, Rad26, and Hus1, act as sensors of DDR. The Rad9-Rad1-Hus1 proteins form a complex (9-1-1) reminiscent of proliferating cell nuclear antigen (PCNA), which is assumed to form a checkpoint sliding clamp (CSC) at or near sites of damage. The CSC may generate a signal to recruit the checkpoint signalling machinery to broken or abnormally structured DNA (59). Rad17, an analogue of replication factor C (RFC), forms a putative checkpoint clamp loading complex (CLC) with RFC subunits, and is essential for the interaction of the 9-1-1 complex proteins with DNA damage (60). In response to DNA damage, Rad3 and Rad26 form a

complex to activate downstream effectors through phosphorylation activity of Rad3 (61). In addition, the Rad3-Rad26 protein complex can bind to sites of DNA damage independently of the 9-1-1 complex (62).

Figure 1.3. The DNA damage checkpoint response. DNA damage is detected by checkpoint sensor proteins and the signal is relayed to downstream effectors, leading to cell cycle arrest and activation of DNA repair. The protein names are based on the fission yeast nomenclature.

At the beginning of checkpoint activation, the DNA damage sensors relay information to the checkpoint kinases. ATM and ATR are thought as the apical checkpoint kinases in all of the DNA damage checkpoints (50, 63, 64). ATM is implicated primarily in response to DSBs induced by IR, while ATR acts as a principal regulator that reacts to UV and stalled replication forks (50, 65-71). They bind to DNA, and interact with many proteins that co-localize at the site of DNA damage (72-75). For example, ATM forms a complex with breast cancer gene 1 (BRCA1), mismatch-repair protein Rad50, and is responsible for the detection and repair of aberrant DNA structures (76). ATR

is also able to bind to Rad17 and BRCA1 (77, 78). These data support an idea that checkpoint protein complexes localize at the sites of DNA damage independently to trigger the checkpoint signalling cascade.

In the G2/M checkpoint, DNA that was damaged in late S or G2 phase, gets repaired before cells enter mitosis. Cells that lack proper G2/M checkpoint control will proceed into mitosis with inappropriate DNA replication, abnormal chromosome segregation, and premature chromosome condensation. These defects will result in cell death. Cdc2 is a highly conserved key player that functions in the G2/M phase transition of the cell cycle. In all eukaryotic cells, progression of G2 phase cells into mitosis requires a phosphorylation cascade that activates the effector kinase downstream of Rad3. In fission yeast, Chk1 and Cds1 are two effector kinases downstream of Rad3. Both of them control the G2/M transition through inhibition of Cdc2 (79-81). They are important for viability in the presence of DNA damaging agents (82). Specifically, Chk1 is preferentially activated in response to DNA damage, while Cds1 is primarily activated by the DNA replication checkpoint (Figure 1.4).

Figure 1.4. Pathway for DNA damage and replication checkpoints in fission yeast. The G2/M DNA damage checkpoint prevents damaged cells from entering mitosis. DNA damage triggers phosphorylation of Chk1, which inactivates Cdc25 and activates Mik1 to increase inhibitory phosphorylation of Tyr15 on Cdc2. The DNA replication checkpoint also controls the G2/M transition through inhibitory phosphorylation of Cdc2. Phosphorylation of Cds1 inactivates Cdc25 and activates Wee1 and Mik1, which phosphorylates Tyr15 of Cdc2 and leads to cell cycle arrest. This figure is adapted from Zhao *et al.* (83).

DNA damage-induced G2 arrest activates Chk1. Deletion of Chk1 completely inactivates the G2/M DNA damage checkpoint (71, 84). Chk1 inactivates Cdc25 tyrosine phosphatase and activates Wee1 and Mik1 tyrosine kinases (85-88), leading to inactivation of Cdc2 via Tyr15 phosphorylation (89-91). This mechanism is highly conserved from fission yeast to human, emphasizing its persistency in tackling DNA damage and defective DNA replication in G2 cells throughout evolution.

DDR and chromatin structure—Any event disrupting chromatin structure may lead to compromised DNA binding and genomic instability. Instead of on the naked DNA, genomic injury occurs in the context of the highly ordered chromatin. Circumstantial evidence indicates that cell cycle checkpoints are activated in response to impaired chromatin structure, highlighting the importance of checkpoint responses in the context of chromatin (92). For example, certain DSB signalling proteins are affected by chromatin structure (93). It is speculated that chromatin structure may act as a selective barrier and functional element to regulate a subset of DDR (94). Given that factors involving in DDR require access to DNA, it is conceivable that histone chaperones associated with active transcription are shared with DDR. But how this process is tightly controlled is circumstantial. Discovery of their interactions may permit understanding of both the transcriptional and the epigenetic program that determine the hierarchy and order of DDR.

1.2.3.3. Transcription factors

Transcription factors are sequence-specific DNA-binding proteins essential for regulation of gene expression. They read and interpret the genetic “blueprint” in the DNA. On binding DNA, they help initiate a program to either

activate or repress the recruitment of RNA polymerase to specific genes, known as transcription (95-98). As such, they are vital for many important cellular processes, ranging from stress response to cell cycle control (99, 100). A transcription factor may contain one or many DBDs, which tend to be well conserved in all living eukaryotic cells.

Transcription factors regulate gene expression in many different ways (101, 102). Firstly, they directly bind to the TATA-binding protein (TBP) which specifically targets a DNA sequence called the TATA box, either alone or with other mediator proteins, and locate the RNA polymerase at the promoter for transcription (103). They can also recruit coactivators or corepressors to the transcription initiation site to up- or down-regulate transcription (104). As a highly dynamic structure, chromatin is maintained by various types of PTMs, which are introduced by chromatin-modifying enzymes (105, 106). Certain transcription factors involve chromatin-modifying enzymes to disrupt DNA-histone contacts, and the altered chromatin structure makes DNA template more or less accessible to the RNA polymerase (107, 108).

In eukaryotic cells, there are sophisticated mechanisms to keep RNA polymerase activity in check. Heterochromatin formation is of key importance to protect genomic integrity through rendering repetitive structures rigid and preventing potentially deleterious transposition events (109). It requires the DNA-based events to recruit sequence-specific DNA-binding factors which act in a parallel mechanism to the RNA interference (RNAi) pathway. In *S. pombe*, RNA polymerase II (Pol II)-based transcription is essential to trigger heterochromatin formation (110). Mutations in RNA Pol II subunits affect RNAi-mediated heterochromatin assembly at fission yeast centromeres (111,

112). Heterochromatin protein 1 (HP1) proteins, the human ortholog of fission yeast Swi6 and Chp2, are fundamental units of heterochromatin packaging involving in transcriptional silencing (113). It has been first identified as being involved in gene repression, termed position-effect variegation (PEV). HP1 is also crucial for the transcriptional regulation of Krüppel-associated box domain zinc-finger proteins (KRAB-ZfPs), which is a large family of transcriptional repressors in mammalian cells (114).

1.2.4. Significance and disease relevance of chromatin-associated proteins

Chromatin is tightly regulated, and CAPs are dynamically organized. Proteins may localize all over chromosomes, or be constrained to specific region, such as the heterochromatin. Moreover, proteins may interact with chromosomes throughout the cell cycle or at specific times of the cell cycle. For example, DNA replication factors are constitutively bound chromatin throughout the cell cycle; while kinetochore components are only loaded onto chromatin during S phase (115-117). These spatiotemporal organizations of chromatin are tightly regulated through coordination of various types of CAPs. The orderly and precise progression of most DNA-related activities such as transcription and replication involves changes of chromatin structure mainly mediated by histone chaperones and chromatin-modifying enzymes.

The study of CAPs has enabled a better understanding of how alteration of chromatin structure can lead to a variety of human diseases, such as cancer and several congenital syndromes (118-120). Importantly, chromatin-modifying enzymes represent a powerful avenue of oncology research (121). Inhibitors of

Histone deacetylase (HDAC) possess direct cytotoxic properties, which can sensitize tumour cells to conventional radiotherapy and chemotherapy (122), offering potential therapeutic strategies for a successful cancer treatment. Thus far, more evidence is emerging that CAPs are implicated in a number of other diseases, especially neurological and autoimmune diseases (123, 124). A striking example is the cell cycle checkpoint protein Chk1 (125). It has been found to be associated with microcephaly disorder, a genetic disease in which affected individuals have a head circumference < 3 standard deviations below the age- and sex-related mean (126). Primary microcephaly (127), Seckel syndrome (128), and Nijmegen breakage syndrome (129) are also postulated to arise through defective regulation of Chk1 (130).

The protein components of chromatin build up the foundation for understanding its higher-order structure and function; and CAPs are tightly associated with the initiation and progression of devastating human diseases. Hence, understanding the underlying mechanisms of various types of CAPs can provide information on how we approach disease intervention and drug development.

1.2.5. Fission yeast as an ideal model for studying chromatin-associated proteins

The fission yeast *S. pombe* is a popular model organism for molecular and cell biology (Figure 1.5A). It is typically three to four micrometers in diameter and seven to 14 micrometers in length. Its genome is approximately 14.1 million base pairs, which is estimated to contain about 5,000 genes. It is

unicellular and contains only three chromosomes. The small genome size and simple manipulation make it an ideal model for chromatin research.

Chromatin organization is highly conserved from yeasts to mammals. They share similar chromosome structure (131), nucleosome core structure (11, 132), and major histone proteins (133). On the other hand, the functional divergence of histone code between yeast and higher eukaryotes will provide further insights into epigenetic regulation during evolution (134).

Figure 1.5. Cellular architecture and morphology of fission yeast. (A) The electron tomogram of a complete yeast cell. It shows plasma membrane, microtubules and light vacuoles (green), nucleus, dark vacuoles and dark vesicles (gold), mitochondria and large dark vesicles (blue) and light vesicles (pink). This figure is adapted from (<http://www.eurekalert.org/multimedia/pub/3409.php?from=91643>). (B) Fluorescence stained image of fission yeast cells. This figure is adapted from (<http://designmatrix.wordpress.com/2010/05/19/nudging-multicellularity-into-existence/>).

Over the last decades, *S. pombe* has become a valuable model in understanding the cell cycle and chromosome biology (135). First, it is well-established for the study of cell-cycle regulation, cytokinesis, and centromere-related events (136-142); second, it is a genetically amenable model to study chromosome segregation (143). As the mechanisms of the DNA damage and replication checkpoint are highly conserved from fission yeast to human (49,

144, 145), it is a useful model system for dissecting the molecular controls over DNA damage and repair (146-150).

S. pombe has advantages over other higher eukaryotic species. It has a short doubling time (DT); a wild-type cell takes about three hours to complete a cell cycle under normal conditions. A normal fission yeast cell has a rod shape, grows by elongation, and divides by medial fission, so that the stage of a cell in the cell cycle can be easily estimated by visualizing the cell morphology (Figure 1.5B). Importantly, its genome can easily be manipulated via homologous recombination, so that the classical genetic tools can be applied to investigate certain mechanisms (131).

A substantial number of fission yeast genes are conserved in higher eukaryotes (151, 152). Given the chromatin structural similarities between fission yeast and human (153, 154), the identification of CAPs in fission yeast might provide mechanistic insights into the underlying mechanisms of various biological processes associated with chromatin. Recently, the fission yeast *S. pombe* is regarded as an excellent model for the identification of cellular mechanisms of sensitivity to anti-tumour drugs. The cloning of human homologs of these proteins could be a useful approach to facilitate the development of novel anti-cancer drugs.

1.3. Strategies to identify chromatin-associated proteins

Many techniques have been established to identify CAPs, which can generally be classified into single-protein strategies and proteomic approaches.

In the section of single protein strategies, I will highlight chromatin fractionation assay using either nuclease digestion or high-salt nuclear extraction methods, both of which aim to determine whether the protein of interest is associated with chromatin. For proteomic approaches, both qualitative and quantitative technologies of identifying CAPs will be elaborated.

1.3.1. Single-protein strategies

Basically, chromatin fractionation assay is the major technique to identify CAPs, through either nuclease digestion or high-salt nuclear extraction. Nuclease digestion is an enzymatic method that digests crude chromatin and releases chromatin-bound proteins; whereas high-salt nuclear extraction dissociates the CAPs from the chromatin context using hypotonic buffer. Whether a particular protein is associated with chromatin is determined by Western blotting (155).

Given that many CAPs bind with DNA, nuclease digestion has been extensively applied to analyze the chromatin binding of proteins in fission yeast (156-159). Different from mammalian and other cultured cells, which can be easily permeabilized with nonionic detergent to extract the soluble nuclear proteins, fission yeast cells possess cell walls that need to be removed by enzymatic digestion. Therefore, our experiment requires zymolyase treatment to partially remove the cell wall, which is also called “spheroplasting”. This method destroys β -glucan but retains α -glucan polymers, so that the cells can withstand detergent extraction to remove soluble proteins without disturbing the chromatin association of chromatin-binding proteins. Although there are dramatic variations among each of the methods (115, 156, 158, 160-163), most

of them are generally achieved by three essential steps: enzymatic digestion with zymolyase, protein extraction with detergent-containing buffer, and DNA digestion with micrococcal nuclease (MNase) or DNase I. By doing this, the chromatin complex can be extracted in their native environment, which facilitates a better way of characterizing and understanding the cellular processes associated with chromatin.

In fission yeast, high-salt nuclear extraction shares the initial two steps with nuclease digestion, while the DNA digestion was displaced by salt extraction using up to 500 mM of NaCl, depending on the protein being studied (155, 164). In contrast to the nuclease digestion methods, the conventional high-salt nuclear extraction disrupt many physiologically relevant protein-protein interactions (165), especially for unstable protein complex. Therefore, this method is more favourable to extract the tightly bound CAPs; for example, the chromo-domain containing proteins which are responsible for gene regulation related to chromatin remodelling and formation of heterochromatin regions (166).

In this study, chromatin fractionation assay using DNase I digestion based on Griffiths *et al.*'s protocol was used (156). Different from MNase, which preferentially cleaves single stranded DNA at (A, T) sequences in chromatin (167), DNase I acts on both single-stranded DNA and double-stranded DNA with less sequence specificity (168). Therefore, using DNase I can systematically enrich CAPs in an unbiased manner.

1.3.2. Proteomic approaches

1.3.2.1. Basics of proteomics

The study of proteins and their interactions has been a laborious and expensive attempt which usually requires the production of specific antibodies to the proteins of interest. Microarray-based mRNA profiling and substantial parallel sequencing technologies suffer from the inability of detecting post-transcriptional regulation (169-171). The development of MS has enabled the successful transition from transcriptomics to “proteomics” (172).

Each proteomic study is composed of three major components: protein preparation, protein separation, and protein identification. Quantitative proteomics requires an additional step to compare the relative protein abundances between two or multiple samples. Typically, protein preparation starts with a raw cell extract, any purification methods can be added to reduce the complexity of the samples, such as traditional biochemical techniques, affinity purification, and Co-immunoprecipitation (Co-IP) (173). Protein separation reflects the resolving power of the MS-based proteomics. The most widely used approaches for separation include one- and two-dimensional electrophoresis (1-DE and 2-DE) (174). 1-DE separates proteins based on their sizes, and 2-DE separates proteins first based on the isoelectric point (pI) followed by separation based on size. The proteins are sliced from the gel and minced into smaller gel pieces for tryptic digestion, and the digested peptides are subjected to MS analysis. Instead of protein separation by gel-based approach, the gel-free method reduces sample complexity at peptide level. After obtaining global tryptic digested peptides from all the proteins, the separation is performed based on their hydrophobicity, charge, molecular weight, or

functionality; peptides collected from this first dimensional separation are then injected into the reverse phase (RP) column linked to a mass spectrometer (175, 176). This is known as offline 2D liquid chromatography (2D-LC), and it can also be performed online. The online method couples two dimensions together in the same column, which is known as multidimensional protein identification technology (MudPIT). After separation, proteins are subsequently identified using mass spectrometer (172).

In nature, CAPs are usually bound by chromatin structures, and not freely accessible in nucleoplasm. They require either nuclease digestion or high salt extraction to be released from chromatin, because only drastic conditions can disrupt the chromatin DNA structures to release nucleosomes and remove DNA-protein interactions. In this study, 2D-LC is not suitable, as the efficiency of obtaining CAPs is extremely low. Furthermore, the presence of highly abundant histones will hinder the detection of low abundance proteins (177).

1.3.2.2. Quantitative proteomics using stable isotope labelling

Quantitative proteomics is the global analysis of the changes in protein abundances in biological samples, through either absolute or relative quantification (178-180). The absolute quantification (AQUA) strategy introduces stable isotope-labelled internal standards, and aims to determine the precise protein levels from cells or tissues (172, 181). Relative quantitative proteomics compare two or more samples using either label-free quantification or stable isotope labelling by providing relative ratios instead of absolute quantitative numbers (179, 182). Compared with label-free quantification (183-185), stable isotope labelling is favourable to obtain more accurate quantification.

Stable isotope labelling aims to distinguish the same peptide from two or more experimental states by generating a pair of “light” and “heavy” versions. The strength of this method is that the labelled species introduce a different mass that can be differentiated for MS quantification without modifying the chemical structure and other properties, which is similar to the analyte of interest. This provides a platform to multiplex various conditions and treatments within the same experiments, minimizing analytical variability and batch effect. Thus far, stable-isotope tags can be introduced into proteins via metabolic labelling using amino acids, heavy salts, and ^{15}N through the growth medium or food (186, 187). The unique advantage of metabolic labelling is that the labelled cells can be mixed at any stage of sample processing step. Samples can be mixed as cells, proteins, or peptides. After the samples are mixed, downstream processing will not introduce further variation on the relative abundance. Therefore, an earlier mixing of samples is preferred.

SILAC is one of the most widely used metabolic labelling methods (Figure 1.6). It incorporates non-radioactive, stable isotope-containing amino acids into newly synthesized proteins through normal cell growth, protein synthesis and turnover. SILAC was first applied to quantify the protein abundance changes during a time course of myoblast differentiation in mouse C2C12 cells (188). It was also extended to other formats, including global protein profiling (189, 190), functional assays in protein-protein interaction (191), identification of proteins enriched in particular cellular compartments (192, 193), and multiplexed analyses with triple encoding SILAC to simultaneously compare three cellular states (194-196). SILAC has also been

demonstrated to study protein turnover (197, 198). In practice, any cell culture system in which the amino-acid sources are defined can be labelled with SILAC.

Figure 1.6. Two phases of SILAC experiment.

(A) Adaptation phase. During the adaptation phase, cells are grown in light and heavy SILAC media until the heavy cells have been fully labelled with the heavy amino acids. MS can distinguish light (black dot) and heavy (red star) peptides by the m/z difference. (B) Experiment phase. In the experiment phase, the two cell populations are subjected to different treatment. The sample is equally mixed, which can be followed by further sample purification and fractionation, and digested to peptides and analyzed by MS for protein identification and quantification. This figure is adapted from Ong *et al.* (199).

SILAC is accurate. Even a small percentage of unlabelled amino acid will be detectable as a population of unlabelled peptides. When proteins or peptides from unlabelled and labelled samples are analyzed by MS, they are separated by a residue-specific mass difference corresponding to the number of stable isotope labels (e.g., 6C of lysine), and the number of amino acid residues being incorporated (e.g., one or two arginines in a peptide). Compared to label-free quantification, SILAC quantification has demonstrated overall greater sensitivity to detect changes in protein expression (200).

An alternative approach of metabolic labelling is ^{15}N labelling. It has been successfully used in both unicellular and complex organisms (201-203). Given that plants and bacteria can synthesize their own amino acids, the SILAC-based incorporation of heavy isotopes would not be complete (204). Therefore, ^{15}N labelling is a good choice for autotrophic organisms (205-207). However, this approach allows only two samples to be compared within a single experiment. Furthermore, ^{15}N labelling introduces “light” and “heavy” peptide pairs with variable mass differences, compromising data analysis.

1.3.2.3. Identifying chromatin-associated proteins with prior knowledge

Single-protein strategies like chromatin fractionation assay require specific antibodies for Western blotting; therefore, these methods are not suitable for high-throughput analysis. Recent advances in proteomic technologies have significantly facilitated studies on chromatin and CAPs (208, 209). However, most of them rely on prior knowledge about chromatin.

For identifying interacting partners of known CAPs, conventional ChIP method is not well-suited to proteomic studies, presumably due to low sample recovery and lack of sample homogeneity (210). An innovative method termed

modified ChIP (mChIP) circumvents these problems by gentle centrifugation and mild sonication, so that the native protein-DNA interaction is largely maintained, and enrichment is significantly improved (211). mChIP was initially designed for purification of histones and their associated protein networks in budding yeast; and used to successfully purify three other non-histone CAPs. This method is able to enlarge ChIP data without additional processing, so that it is flexible for high-throughput screening to establish the network of diverse CAPs. To selectively purify proteins bound with specific DNA regions, a new method known as proteomics of isolated chromatin segments (PICh) was invented, in which chromatin is cross-linked, sheared, and the desired locus is isolated with DNA probe hybridization. This approach has been successfully adopted to purify proteins bound with telomeric regions of chromatin in HeLa cells (212). Both known and novel telomeric factors have been identified, with different set of proteins occupying different type of telomeres. PICh is considered to be a useful tool to characterize chromatin composition. Along with ongoing technological advances of MS, it is conceivable to zoom in genomic loci regulation to single loci. On the other hand, this approach requires huge amount of starting material; furthermore, the analysis of the associated proteins largely relies on the nucleic acid probe that recognize specific genomic loci.

An assay has been developed by Schultz-Norton *et al.* to study chromatin bound protein complexes associated with estrogen receptor alpha (ER α) by reconstituting them *in vitro* prior to MS identification (213). To monitor the ER α -associated protein complex, purified ER α and HeLa nuclear extracts were incubated with DNA oligos containing ER α binding sites. The

unbound materials from the protein complex associated with chromatin were removed by agarose gel electrophoresis. The band corresponding to ER α containing DNA bound complex was excised, and the extracted proteins were analyzed by MS. This method successfully identifies a number of novel proteins that regulate ER α activity and expression of estrogen-responsive genes. Because most of CAPs are low in solubility, this approach is so attractive that it can bypass this problem. Nevertheless, it requires information about DNA binding sites of the protein being studied.

Up till now, most proteomic data were only qualitative in nature. Nevertheless, identification of chromatin-bound protein complexes without appropriate validation can lead to many false positives (214); because it is not easy to distinguish between background contamination and proteins authentically associated with chromatin. To improve the accuracy and reliability of data, quantitative proteomic methodologies are emerging to facilitate the research in epigenetic and chromatin field (215).

A powerful approach by coupling affinity purification with MudPIT has been devised to analyze the chromatin remodelling complex, and normalized spectral abundance factor (NSAF) was introduced to make the results quantitative (216). To study the CCTC-binding factor CTCF in Jurkat cells, the stable isotope labelling method Isotope-coded affinity tag (ICAT) (179) has been used, and a novel role of CTCF in cohesion has been proposed (217). Instead of ICAT, Mittler *et al.* has done similar work using SILAC, and proven that both quantitative proteomic methods are compatible.

SILAC has also facilitated to discover some novel proteins associated with CpG methylation, which offers potential for high-throughput

determination of transcription factor binding profiles. To enrich proteins specifically associated with methylated DNA, the SILAC-labelled nuclear extracts isolated from HeLa S3 cells were subjected to affinity purification (218). The columns were customized with immobilized biotinylated probes containing a CpG island with or without methylation, so that the proteins specifically bind to methylated DNA can be enriched. This method is executable to proteome-wide mapping of various DNA modifications, and provides a basis for understanding single-nucleotide polymorphism (SNP) (219-221). Nevertheless, this kind of work is in need of specialized DNA fragment as bait, which is too laborious to be practical for high-throughput screening.

Collectively, these strategies are useful tools to identify a defined CAPs and protein networks. However, all of them rely on prior knowledge that the protein of interest is associated with chromatin DNA, and a specific antibody is required if tagging of the protein is not possible. Because the use of a given DNA probes or chromatin binding protein in immunoprecipitation enables protein complexes to be purified and characterized, all of these studies focus on proteins targeted to specific protein complex or a given genomic locus.

1.3.2.4. Identifying chromatin-associated proteins without prior knowledge

Recently, only a few studies have been conducted to isolate chromatin and identify all possible associated proteins without prior knowledge, presumably due to the considerable contamination from highly abundant proteins which are not associated with chromatin.

To obtain a global view of chromosomal proteins, Uchiyama *et al.* (177) have performed purification and proteomic analysis of human metaphase chromosomes. This analysis represents the first compositional view of human

metaphase chromosomes and serves as an important reference for future studies on chromosome structure and function. Among the identified proteins, the main components are mitochondrial proteins (38.6%) and nuclear proteins (29.8%), ribosomal proteins (12.7%) and cytoplasmic proteins (11.4%) are also dominant. Chu *et al.* (222) have carried out a proteomic study to identify spermatogenic CAPs that are important for fertility in *Caenorhabditis elegans* (*C. elegans*) using MudPIT. Although a huge number of proteins were copurified with spermatogenic chromatin; by applying filtering criteria, the authors are able to reduce the protein number from 1099 to 132 for subsequent functional studies on spermatogenesis. Most of those removed proteins are highly abundant cytoplasmic and ribosomal proteins. In Tan *et al.*'s study (223), they have developed a protocol for chromatin purification from rice suspension cells and examined proteins copurified with chromatin using 2-DE gel approach. A number of known CAPs have been identified, including core histones and histone chaperones. However, many proteins with known functions unrelated to chromatin were detected, and most of these proteins were also copurified with human chromosome and *C. elegans* chromatin, respectively (177, 222).

Quantitative proteomics has been exploited to identify DNA-binding proteins associated with a particular cellular status. To analyze CAPs upon Myc oncoprotein expression in human B cells, Shiio *et al.* adopted a method by coupling high-salt nuclear extraction with ICAT labelling (219). They identified 64 proteins, including 18 putative transcription factors. They claim this chromatin-ICAT reagent approach will be widely applicable to identify the chromatin dynamic changes under various biological conditions. However, only 64 out of 280 proteins are known to be nuclear, which clearly reveals

contamination by other proteins not related to chromatin, especially the highly abundant cytoplasmic proteins.

To compare different preparation methods for CAPs, HeLa S3 cells were utilized to extract chromatin using a total chromatin extraction, a salt extraction and a total MNase digestion (224). These three distinct chromatin preparations result in the detection of slightly different subsets of non-histone CAPs; with the total chromatin extraction and the MNase digestion containing the highest proportion of CAPs, and the MNase procedure possessing higher specificity for euchromatin- and heterochromatin-related processes (224). By using a NSAF approach (216), they quantified relative abundances of the proteins across the enriched CAPs, and found histone proteins always have the most prevalent hits between all three methods. On the other hand, they also detected a number of non-nuclear proteins due to their tremendous abundance; they are most likely to be contaminants with chromatin enriched fractions.

It is understandable that no single purification method is able to isolate the chromatin without pulling down non-CAPs. Given that the chromatin proteomic study aims to identify CAP candidates rather than a final proof of their association with chromatin, further analysis is required to examine whether these candidates are *bona fide* CAPs or merely possible contamination during the isolation procedures.

In combination with precise and reliable quantification, we are supposed to enrich CAPs without any prior knowledge. Although it is more challenging, it is also more rewarding, because the finding based on this strategy can serve as a platform to analyze diverse classes of CAPs. Furthermore, it will nourish the discovery of novel proteins that interact with chromatin, making the picture

more complete. In this study, we borrow the idea from the experimental design of ChIP-on-chip (225), which is performed to determine the DNA binding sites for a protein of interest by comparing the abundance between genomic DNA and ChIP-enriched DNA. This idea prompted us to test whether the problem of protein contamination from highly abundant non-CAPs can be alleviated by comparing the protein abundance before and after chromatin enrichment.

With the efforts of chromatin proteomics, we are able to better understand the basic cellular processes associated with chromatin. Developing new approaches of identifying CAPs can broaden our knowledge about these processes, which should provide solid basis to understand diverse classes of human pathology.

1.4. Research objectives

1.4.1. Analysis of non-histone chromatin-associated proteins

using SILAC approach

While many non-histone CAPs have been identified through a variety of different approaches, majority of them were based on the previously known proteins to seek their interacting partners in a certain complex, for example, various ChIP methods. Due to the low abundance of CAPs and non-specific DNA binding of positively charged nuclear proteins, rare studies have been done to isolate the *bona fide* non-histone CAPs without prior knowledge. By directly applying the chromatin-enriched samples for mass spectrometric analysis, the highly abundant non-CAPs such as ribosomal proteins will be overestimated, whereas the low abundant true non-histone CAPs will be

underestimated (226). We assume the relative abundance of a non-histone CAP from chromatin enrichment should be definitely higher than that in the whole proteome, and the level of ribosomal proteins would be lower due to relative depletion of non-CAPs after chromatin enrichment. To test this, SILAC-mediated proteomic analysis was involved to estimate the relative abundance of each protein in chromatin-enriched sample and WCE.

In this study, the fission yeast *S. pombe* was used as a model organism to study non-histone CAPs using MS. We applied the chromatin fractionation assay to extract chromatin, and compared the relative abundance of the isolated proteins with SILAC-labelled WCE. The protein ratio of a particular protein between unlabelled chromatin-enriched sample and labelled WCE was utilized to determine whether it is associated with chromatin regardless of its cellular abundance.

The great depletion of highly abundant ribosomal protein in the top ranked proteins based on the SILAC ratio (or top SILAC-ranked proteins) suggested that the SILAC-based approach was effective to remove the high noise background signal. We further tested whether these top SILAC-ranked proteins had greater potential to be associated with chromatin. To this end, we applied several criteria to validate our MS data. First, majority of CAPs are nucleus-localized. Second, CAPs are highly involved in a variety of biological processes associated with chromatin, such as transcription and chromatin remodelling (5). Third, CAPs play a key role in DNA damage and repair (227). Localization and Gene ontology studies suggested that the top SILAC-ranked proteins were dominated by nucleus-localized proteins and proteins with chromatin-related functions. Phenotypic assessment showed that the top

SILAC-ranked nucleus-localized proteins exhibited a higher likelihood of requirement for growth fitness under DNA damage stress. A similar approach of deletion analysis also indicated that proteins responsible for genomic stability maintenance were frequently isolated from the top SILAC-ranked proteins with protein ratio ≥ 2 .

Based on the phenotypic assessment, we selected two of the potential non-histone CAPs from the top SILAC-ranked proteins, Bc947.08c (Hip4) and Bc530.05, for phenotypic and functional characterizations. We found that the mutant deleted for *hip4*⁺ was sensitive to DNA damage and showed elongated cell shape. Our further study focused on whether Hip4 played a role in G2/M DNA damage checkpoint control by epistasis analysis. The mutant deleted for *BC530.05*⁺ was sensitive to cold and spindle damage. Given that Bc530.05 is a novel protein, various biochemical methods were employed to understand its possible functions. These included sensitivity assays to test mutant phenotypes, fluorescence microscopy and FACS analysis to examine the nuclear environment, and Western blotting to check protein upregulation in response to various stresses.

In summary, our work shows that the SILAC-mediated proteomic approach is capable of determining non-histone CAPs without prior knowledge. We have also identified novel candidates of non-histone CAPs, and further discovery of their functions and mechanisms may shed light on their functions in the context of chromatin.

1.4.2. Discovery of novel histone H4-interacting proteins

Histone-interacting proteins are well-established factors that escort histone metabolisms, which are coordinated with key cellular processes such as DNA replication, chromatin assembly and transcription (228-233). Therefore, discovery of histone-interacting proteins may provide mechanistic insight into these multiple chromatin-templated processes.

Because most of these proteins are in low abundance, the extensive PTMs and high abundances of core histones usually mask their identification in MS analysis. To circumvent this problem, we applied the TAP strategy to recover histones and histone-associated proteins in a standard two-step process. The various steps of the TAP method can reduce background contamination caused by abundant cellular proteins, or material leaking from the affinity column. In our phenotypic analysis, histone H4 mutant displayed sensitivity to DNA damaging agent. Therefore, we chose histone H4 as our bait in this pilot study. The strain without TAP tagging was included for removal of proteins with antibody cross-activity. This approach was validated by the identification of all core histones with extremely high confidence. Although the traditional histone-interacting partners did not appear in our list, some novel candidates were proposed to have genuine interaction with histone H4 based on their localizations and functions.

CHAPTER 2. MATERIALS AND METHODS

2.1. Strains

2.1.1. Bacterial strains

The MAX Efficiency[®] DH5A[™] Competent Cells [genotype: F- Φ 80*lacZ* Δ M15 Δ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (*r_K⁻*, *m_K⁺*) *phoA supE44* λ^- *thi-1 gyrA96 relA1*] (Invitrogen, Carlsbad, CA, USA) (234) were used for plasmid transformation.

2.1.2. Fission yeast strains

Fission yeast strains used in this study are listed in Table 2.1 except for Bioneer deletion strains (Bioneer Corporation, Daejeon, South Korea) (Table A.1). General rules of fission yeast nomenclature are elaborated in A.1. The lysine auxotrophic strain (LJY3766) was used in preparation of the SILAC-labelled cells. Hemagglutinin (HA)-tagged strains for western blotting were constructed based on the protocol reported previously (235). The deletion mutants were constructed using a PCR-mediated gene targeting protocol as described previously (236). These methods are described in A.2 and A.3. Primers used for construction and validation of HA-tagged strains and deletion mutant strains are listed in Table A.2.

Table 2.1. Fission yeast strains used in this study.

Strain ^a	Relevant genotype	Source
JHY1014	<i>h⁻ ura4-D18>>ura4⁺ leu1-32</i>	Laboratory stock
LJY95	<i>h⁻ 972</i>	Laboratory stock
LJY98	<i>h⁺ ura4-D18 leu1-32</i>	Laboratory stock
LJY188	<i>h⁻ ura4-D18 leu1-32</i>	Laboratory stock
LJY385	<i>h[?] ura4-D18 nda3Δ-KM311</i>	M. Yanagida (Kyoto University, Japan)
LJY1445	<i>dis1Δ::Kan⁺ GFP-bir1</i>	Laboratory stock
LJY1551	<i>h⁻ ura4-D18 leu1-32 bub1Δ::ura4⁺</i>	Laboratory stock
LJY1669	<i>h⁻ ura4-D18 leu1-32 chk1Δ::ura4⁺</i>	Laboratory stock
LJY1687	<i>h⁻ ura4-D18 leu1-32 cds1Δ::ura4⁺</i>	Laboratory stock
LJY1715	<i>h⁻ ura4-D18 leu1-32 rad3Δ::ura4⁺</i>	Laboratory stock
LJY1791	<i>h⁻ ura4-D18 leu1-32 styl1Δ::ura4⁺</i>	Laboratory stock
LJY2261	<i>h⁻ ura4-D18 leu1-32 pcr1Δ::ura4⁺</i>	Laboratory stock
LJY3236	<i>h⁻ ura4-D18 leu1-32 BC530.05⁺-3HA-6His::ura4⁺</i>	Laboratory stock
LJY3766	<i>h⁻ ura4-D18 lys1-131</i>	Laboratory stock
LJY3998	<i>h⁺ ura4-D18 leu1-32 mad2Δ::ura4⁺</i>	Laboratory stock
LJY4157	<i>h⁻ ura4-D18 leu1-32 hip4⁺-3HA-6His::ura4⁺</i>	This study
LJY4164	<i>h⁻ ura4-D18 leu1-32 rep2Δ::ura4⁺</i>	Laboratory stock
LJY4224	<i>h⁻ ura4-D18 leu1-32 prp16⁺-3HA-6His::ura4⁺</i>	Laboratory stock
LJY4383	<i>h⁻ ura4-D18 leu1-32 hsp16⁺-3HA-6His::ura4⁺</i>	This study
LJY4384	<i>h⁻ ura4-D18 leu1-32 CC18.05c⁺-3HA-6His::ura4⁺</i>	This study
LJY4385	<i>h⁻ ura4-D18 leu1-32 msh3⁺-3HA-6His::ura4⁺</i>	This study
LJY4386	<i>h⁻ ura4-D18 leu1-32 uve1⁺-3HA-6His::ura4⁺</i>	This study
LJY4387	<i>h⁻ ura4-D18 leu1-32 hip4Δ::ura4⁺</i>	This study
LJY4388	<i>h⁺ ura4-D18 leu1-32 hip4Δ::ura4⁺</i>	This study

Table 2.1. Fission yeast strains used in this study (continued).

Strain ^a	Relevant genotype	Source
LJY4429	<i>h⁻ ura4-D18 leu1-32 kap95⁺-3HA-6His::ura4⁺</i>	This study
LJY4430	<i>h⁻ ura4-D18 leu1-32 srp2⁺-3HA-6His::ura4⁺</i>	This study
LJY4431	<i>h⁻ ura4-D18 leu1-32 hip4Δ::kan⁺ rad3Δ::ura4⁺</i>	This study
LJY4432	<i>h⁻ ura4-D18 leu1-32 hip4Δ::kan⁺ cds1Δ::ura4⁺</i>	This study
LJY4433	<i>h⁻ ura4-D18 leu1-32 hip4Δ::kan⁺ chk1Δ::ura4⁺</i>	This study
LJY4434	<i>h⁻ ura4-D18 leu1-32 hip4Δ::kan⁺ rad3Δ::ura4⁺ hip4⁺-3HA-6His::LEU2⁺</i>	This study
LJY4435	<i>h⁻ ura4-D18 leu1-32 hip4Δ::kan⁺ cds1Δ::ura4⁺ hip4⁺-3HA-6His::LEU2⁺</i>	This study
LJY4436	<i>h⁻ ura4-D18 leu1-32 hip4Δ::kan⁺ chk1Δ::ura4⁺ hip4⁺-3HA-6His::LEU2⁺</i>	This study
LJY4437	<i>h⁻ ura4-D18 leu1-32 BC530.05Δ::ura4⁺</i>	This study
LJY4438	<i>h⁺ ura4-D18 leu1-32 BC530.05Δ::ura4⁺</i>	This study
LJY4439	<i>h⁻ ura4-D18 leu1-32 hhf1⁺-TAP::ura4⁺</i>	This study

Note: ^a Bioneer strains used in this study are listed in Table A.1.

2.2. Culture media

The culture media used in this study are listed in Table 2.2. All the bacterial and fission yeast cells were cultured in the liquid broth at indicated temperatures with shaking at 200 rpm or grown on relevant agar medium. LB and SOC medium were used to culture *E. coli* DH5α cells at 37°C (237). Ampicillin was used in the final concentration of 100 µg/ml. The rich medium YES (153) was used to culture fission yeast cells at 30°C. Edinburgh Minimal Medium (EMM2) with appropriate supplements (238, 239) were used in

chromatin fractionation assay and SILAC analysis. The yeast strains used for experiment were freshly revived within a week.

2.3. Plasmids

Plasmids used in this study are listed in Table 2.3. In particular, the pREP1-*hip4*⁺ plasmid was constructed by PCR amplifying the protein coding sequence of *hip4*⁺ (tagged with HA3His6) using oligonucleotide primers *hip4SalI* 5'-GCGCGCGTCGACATGTCGCTTTTGTCTGGCGACCAACGAA TT-3') and *hip4BamHI* (5'-GGGCCCGGATCCTTAATGATGATGATGATG ATGCGCATAGTC-3'), digesting the resulting DNA fragment with *SalI* and *BamHI*, and cloned into pREP1 (Figure A.2) (240) harboring a *LEU2* selectable marker (241). pREP41-*hip4*⁺ plasmid was constructed in exactly the same way, and DNA fragment was cloned into pREP41 (Figure A.2) (242). Restrictive enzyme digestion was performed to ascertain the correct size and orientation of the inserts. DNA sequencing analysis was to ensure the clone is free of mutation. Primers used for DNA sequencing include pREP_F (5'-GAAGAGGAATCCTGGCATAT-3') and pREP_R (5'-CGTAATATGCAG CTTGAATGG-3') (1st BASE). The sequencing results are shown in B.4. The resulting plasmid was cut at *XbaI* site and transformed into the *S. pombe* double mutant strains *hip4Δrad3Δ*, *hip4Δcds1Δ*, and *hip4Δchk1Δ* using the classical lithium acetate method as described before (243). Gene expression of *hip4*⁺ is under the control of the thiamine-regulatable *nmt1* promoter (241), gene expression was induced in the absence of thiamine (Sigma-Aldrich, St. Louis, MO, USA) and repressed in the presence of 15 μM thiamine.

Table 2.2. Culture media used in this study.

Media/ supplements	Preparation ^{a, b, c}	Reference
<i>E. coli</i>		
LB	Bacto-tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g; pH 7.5	(237)
SOC	Bacto-tryptone, 20 g; Difco yeast extract, 5 g; NaCl, 0.5 g; 2.5 ml of 1 M KCl; pH 7.0; add 20 ml of filter-sterilized 1 M glucose and 5 ml of filter-sterilized MgCl ₂ immediately before use	(237)
<i>S. pombe</i>		
YE	Difco yeast extract, 5 g; D-glucose, 30 g	(153)
YES	YE + 75 mg/l adenine, histidine, leucine, and uracil; pH 5.6	
Supplements (stock) ^d	Adenine, 3.75 mg/ml; histidine, 7.5 mg/ml; leucine, 7.5 mg/ml; and uracil, 3.75 mg/ml; filter-sterilized before use	
EMM2	Potassium hydrogen phthalate, 3 g; Na ₂ HPO ₄ , 2.2 g; NH ₄ Cl, 5 g; D-glucose, 20 g; salts (× 50), 20 ml; vitamins (× 1000), 1 ml; minerals (× 10,000), 0.1 ml; appropriate supplements was added as stated after autoclaving; pH 5.6	(238, 239)
salts (× 50)	MgCl ₂ ·6H ₂ O, 52.5 g; CaCl ₂ ·2H ₂ O, 0.735 mg; KCl, 50 g; Na ₂ SO ₄ , 2 g; filter-sterilized and stored at 4°C	
vitamins (× 1000)	Pantothenic acid, 1 g; nicotinic acid, 10 g; inositol, 10 g; biotin, 10 mg ^e ; filter-sterilized and stored at 4°C	
minerals (× 10,000) ^f	Boric acid, 5 g; MnSO ₄ , 4 g; ZnSO ₄ ·7H ₂ O, 4 g; FeCl ₂ ·6H ₂ O, 2 g; molybdic acid, 0.4 g; KI, 1 g; CuSO ₄ ·5H ₂ O, 0.4 g; citric acid, 10 g; filter-sterilized and stored at 4°C	

Note: ^a Prepared in 1 liter; ^b Media without D-glucose were sterilized by autoclaving at 121°C for 20 min, media with D-glucose was autoclaved at 112°C for 15 min to prevent caramelization; ^c Solid media is made by adding 2% Difco Bacto Agar, it is remelted in a microwave oven before using; ^d Supplements were filter-sterilized before being added into the media; ^e Each component is dissolved and autoclaved separately before mixing; ^f After autoclaving, a few drops of preservative was added (1:1:2, chlorobenzene: dichloroethane: chlorobutane).

Table 2.3. Plasmids used in this study.

Plasmid name	Yeast origin	Bacterial (Yeast) markers	Promoter, other features and comments	Source or reference
pBS(SK) [*] - (lp)kan ⁺ (lp)	f1	<i>Kan</i> ⁺	used as a PCR template, followed by transformation for gene deletion	(236)
pBS(SK)- (lp)ura4 ⁺ (lp)	f1	<i>Amp</i> (<i>ura4</i> ⁺)	used as a PCR template, followed by transformation for gene deletion	(236)
pBS(SK)- 3HA6HIS- (lp)ura4 ⁺ (lp)	f1	<i>Amp</i> (<i>ura4</i> ⁺)	used as a PCR template, followed by transformation for C-terminal double tag fusion of proteins by HA3His6 at their normal chromosomal locations	Laboratory stock
pJK210-CTAP	---	<i>Amp</i> (<i>ura4</i> ⁺)	blue–white integrating, no <i>ars1</i> element	(244)
pJK210-CTAP- <i>hhf1</i>	---	<i>Amp</i> (<i>ura4</i> ⁺)		This study
pREP1	<i>ars1</i>	<i>Amp</i> (<i>LEU2</i>)	<i>S. pombe</i> nmt ^{**} 1 full strength; off in EMM2+thiamine, on in EMM2	(240)
pREP1- <i>hip4</i> ⁺	<i>ars1</i>	<i>Amp</i> (<i>LEU2</i>)		This study
pREP41	<i>ars1</i>	<i>Amp</i> (<i>LEU2</i>)	<i>S. pombe</i> nmt1 medium strength (nmt41); off in EMM2+thiamine, on in EMM2	(242)
pREP41- <i>hip4</i> ⁺	<i>ars1</i>	<i>Amp</i> (<i>LEU2</i>)		This study

Note: ^{*} pBluescript II SK, from Strategene, La Jolla, CA, USA; ^{**} no message in thiamine.

2.4. Antibodies

Antibodies used in this study are listed in Table 2.4.

Table 2.4. Antibodies used in this study.

Name	Supplier or source
<i>Primary antibodies</i>	
Anti-HA	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Anti-Histone H4	Upstate, Charlottesville, VA, USA
Anti-TAT1 (β -tubulin)	a gift of K. Gull, University of Oxford, London, UK
Anti-Cdc2 (PSTAIRE)	Cell Signaling Technology, Beverly, MA, USA
Anti-Cdc2-Y15-p ^a	Cell Signaling Technology, Beverly, MA, USA
Anti-Protein A	Sigma-Aldrich, St Louis, MO, USA
Anti-CBP (calmodulin binding peptide)	Santa Cruz Biotechnology, Santa Cruz, CA, USA
<i>Secondary antibodies</i>	
Goat Anti-Mouse IgG (HRP ^b -conjugated)	GE Healthcare, Piscataway, NJ, USA
Goat Anti-Rabbit IgG (HRP-conjugated)	GE Healthcare
Alexa Fluor [®] 488 Goat Anti-Mouse IgG (fluorochrome-conjugated)	Molecular probes, Eugene, Oregon, USA

Note: ^a phospho-specific Cdc2-Y15; ^b the abbreviation for 'horseradish peroxidase'.

2.5. General protein methods

2.5.1. Preparation of cell lysate

To prepare cell lysate, cells were collected at 4,000 rpm for 3 min and quickly chilled in liquid nitrogen, and stored at -80°C before use. Pellets were thawed on ice and washed with 500 µl of pre-chilled Suspension Buffer [62.5 mM Tris-HCl pH 6.8, 4 mM dithiothreitol (DTT), 4 mM PMSF, and protease inhibitor EDTA-free tablets (Roche, Basel, Switzerland)], and spun at 13,000 rpm for 1 min to discard the supernatant. Cells were lysed with acid-washed beads (Sigma-Aldrich) beating in the FastPrep glass 24 Instrument (MP Biomedicals Inc., Solon, OH, USA) in four 30-second bursts. Cell lysis was monitored by observation under light microscope to ensure more than 90% of the cells were broken. The broken cells were then resuspended in 500 µl of pre-chilled Lysis Buffer [62.5 mM Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 4 mM PMSF, and protease inhibitor tablets]. Cell lysates were spun in a micro-centrifuge (Eppendorf, Hamburg, Germany) at 13,200 rpm for 15 min, and clear supernatant was collected by a fresh Eppendorf tube.

2.5.2. Protein quantification

Protein concentration was estimated with a spectrophotometer (Tecan Austria GmbH, Austria) using the Bio-Rad Bradford Assay (the Bio-Rad DC protein assay kit; Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. Bovine serum albumin (BSA) was used as a protein standard (245).

2.5.3. SDS-PAGE

A desired amount of proteins was taken and mixed with equal volume of 2× loading buffer [25% (v/v) glycerol, 4.4% (w/v) SDS (Bio-Rad), 25 µg/ml bromophenol blue, 0.5 M Tris-HCl pH 6.8, 0.2 M DTT] and denatured at 95°C for 10 min. Protein mixtures were separated on 8%, 10% 12% or 14% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) using mini SDS-PAGE (mini PROTEAN[®] 3; Bio-Rad) or large SDS-PAGE (PROTEAN[®] II xi cell system; Bio-Rad) gels according to manufacturer's protocols. The gel was further processed by Western blotting or Coomassie blue staining.

2.5.4. Western blotting

For Western blotting, proteins in gel were electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) for probing with the primary antibody (Table 2.4), followed by the corresponding HRP conjugated anti-mouse or anti-rabbit IgG secondary antibody. The chemiluminescence signal was developed and captured using the ECL protein detection System (GE Healthcare, Hemel Hempstead, UK).

2.5.5. Coomassie blue staining

The gel was rinsed with deionized H₂O for 3× (Milli-Q Millipore, Billerica, MA, USA) (2 min/×), and incubated with Coomassie Blue G250 (Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) for 30 min on a shaker at RT. To destain gel, the staining buffer was discarded, and the gel was placed into deionized H₂O with gentle shaking. Water was changed every 20 min until the desired level of colouration was achieved.

2.5.6. Preparation of tryptic peptides

The Coomassie blue stained bands were excised (~0.5mm width/slice) and destained with 25 mM NH_4HCO_3 50% acetonitrile (Sigma-Aldrich) at 37°C for 30 min. The proteins in gel were further treated in the reducing solution 10 mM DTT 25 mM NH_4HCO_3 , and subsequently in the alkylating solution 55 mM iodoacetamide 25 mM NH_4HCO_3 . The gel was washed in 25 mM NH_4HCO_3 and then in 25 mM NH_4HCO_3 50% acetonitrile and dried in the Speed Vac (Thermo Scientific, Waltham, MA, USA). For protein trypsinization, proteins in gel slices were incubated in 25 mM NH_4HCO_3 solution supplemented with 12.5 ng/μl trypsin (Promega, Madison, WI, USA) for 16-18 h at 37°C with occasional shaking. After digestion, the gel slice was submerged in 5% acetic acid (Sigma-Aldrich) and sonicated for 45 min at 37°C to extract the peptides. The gel slice was re-extracted in 5% formic acid (Sigma-Aldrich) 50% acetonitrile for 45 min at RT. The supernatants were pooled and dried in the Speed Vac. The resulting peptides were resuspended in 0.1% formic acid 2% acetonitrile and centrifuged at 10,000 rpm for 10 min to remove any insoluble matter. Peptides were further cleaned using C18 ZipTip (Millipore) and ready for mass spectrometric analysis.

2.5.7. NanoLC-MS/MS

NanoLC-MS/MS was performed using a nanoflow HPLC system (Eksigent, Dublin, CA, USA) connected to a hybrid LTQ-Orbitrap (Thermo Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source (Thermo Scientific). The peptides were loaded onto a 15 cm analytic column (75 μm inner diameter PicoFrit column) with an integrated tip (New Objective

Inc, Woburn, MA, USA) packed in-house with 4 μm reverse-phase C12 resins (Jupiter Proteo Phenomenex, Torrance, CA, USA). HPLC mobile phase consists of (A) 2% acetonitrile 0.1% formic acid and (B) 98% acetonitrile 0.1% formic acid. Approximately 10 μl peptide solution ($\sim 1\text{ }\mu\text{g}$) was loaded onto a nano trap column (300SB-C18, Agilent) with 100% mobile phase A and washed for 10 min at a flow rate of 20 $\mu\text{l}/\text{min}$. The trap column was then brought in-line with the self-packed nano column using the CN2 nano volume switching valve (VICI Valco Cheminert, Switzerland) and the peptides were eluted by 2-35% mobile phase B over 70 min and 35-90% over 6 min with a constant flow rate of 300 nl/min . Finally the column was washed for 10 min with high concentration of organic solvent (90% mobile phase B) and re-equilibrate for another 15 min with 98% mobile-phase A prior to loading of the next sample. Eluted peptides from HPLC column were directly electrosprayed into the LTQ-Orbitrap mass spectrometer for analysis. The spray voltage was set to 2.0 kV and the temperature of the heated capillary was set to 250°C. The MS instrument was operated in a data-dependent mode by automatically switching between the full survey scans and MS/MS acquisition. High resolution precursor spectra (m/z 300-2,000) were acquired in the Orbitrap with resolution of 60,000 at m/z 400 (after accumulation to a target value of $10\text{E}6$ ions in the linear ion trap). The 5 most intense ions with ion intensity above 1,000 counts and charged state ≥ 2 were sequentially isolated for fragmentation in the linear ion trap using collision induced dissociation (CID; normalized collision energy 35%, activation Q 0.250 and activation time 30 ms) at a target value of 10,000 ions. The dynamic exclusion list was restricted to a maximum retention period of 90 sec and a relative mass window of 10 ppm. The MS and

MS/MS spectra were recorded by the mass spectrometer as raw files using the Xcalibur software 2.0SR2 (Thermo Fisher Scientific).

2.6. Chromatin fractionation assay

Chromatin fractionation assay was performed as described previously with some modifications (156). In brief, ~500 ml (for LC-MS/MS analysis) or ~100 ml (for Western blotting) mid-log-phase cells (~0.8OD₆₀₀) were harvested in 1 mM NaN₃ (Sigma-Aldrich) and were washed sequentially with 25 ml of STOP buffer (0.9% NaCl, 1 mM NaN₃, 50 mM NaF, 10 mM EDTA), 25 ml of double-distilled water (ddH₂O) and 10 ml of 1.2 M sorbitol (Sigma-Aldrich). Cells were resuspended in 1.125 ml of CB1 (50 mM sodium citrate, 40 mM EDTA, and 1.2 M sorbitol), to which 9 mg of Zymolyase 20T (MP Biomedicals Inc) and 2.5 µl of β-mercaptoethanol (β-ME) (Bio-Rad) were added. Protoplasting of cells was monitored every few minutes by removing 9 µl of cell sample and mixing with 1 µl of 10% (w/v) SDS (Protoplasts turned dark when treated with 1% SDS). When cell lysis reached more than 90%, the reaction was stopped by adding an equal volume of ice-cold 1.2 M sorbitol pH 7.5. Protoplasts were harvested by centrifugation at 2,000 rpm for 4 minutes, and washed 3× with 2 ml ice-cold 1.2 M sorbitol. The washed spheroplasts were resuspended in 425 µl of 1.2 M sorbitol, frozen in liquid nitrogen and stored at -80°C for later use. Samples were subsequently thawed on ice and lysed by addition of 50 µl 10× Lysis buffer (500 mM KAc, 20 mM MgCl₂, 200 mM HEPES pH 7.9) supplemented with a complete protease inhibitor cocktail EDTA-free Tablet (Roche) and 20 µl of 25% (v/v) Triton X-100 (TX-100)

(Promega). The sample was incubated on ice for 10 min with occasional mixing. Ten percent of the WCE was preserved as total protein (WCE, or T). The remaining was centrifuged at 12,000 g for 15 min at 4°C. Supernatant containing soluble proteins was transferred to a fresh tube and 10% of the sample was collected (S1). The insoluble pellet was washed twice with 1× lysis buffer without TX-100 to yield the crude chromatin (P1). The resulting chromatin pellet was digested with DNase I (Stratagene, La Jolla, CA, USA) at the concentration of 10 unit/μg DNA in Digestion buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM CaCl₂ and protease inhibitors) at 37°C for 30 min with vigorous shaking. The DNase I-digested chromatin extract (C) was obtained by centrifugation at 14,000 g for 5 min. The pellet (P2) was discarded, which was designated as the insoluble proteins and cell debris. Supernatant and chromatin were then desalted using Zeba spin desalting columns (Thermo Scientific, Bremen, Germany). To ensure DNase I is working, genomic DNA was purified using a standard acid phenol extraction as described previously (246).

2.7. SILAC method

2.7.1. Testing of SILAC incorporation

For testing the incorporation of heavy lysine (¹³C₆-lysine; CLM-2247-0.25, Cambridge Isotope Laboratories, Andover, MA, USA) in various passages, auxotrophic lysine strain (LJY3766) was SILAC-labelled by culturing them in EMM2 in which the natural lysine (¹²C₆-lysine, light lysine) was replaced by heavy isotope labelled counterpart (¹³C₆-lysine, heavy lysine). In brief, 10 ml of overnight cell cultures (passage 0, or p0) in EMM2 was transferred into 90 ml

of the fresh EMM2 supplemented with heavy lysine at the final concentration of 22.5 mg/L and grow for 24 h. The resulting culture (passage 1, or p1) reached the cell density of OD₆₀₀~1.0. The passage was continued in heavy-lysine-containing medium to generate passage 2 (p2) and passage 3 (p3) cultures, and the cell lysates were prepared by beads beating (2.5.1).

2.7.2. Validation of SILAC incorporation

For testing the accuracy of SILAC quantitative analysis, normal (p0) and SILAC-labelled protein lysates (p3) were obtained by beads beating (2.5.1), adjusted to equal concentration, and then mixed with a ratio of 4:1, 2:1, or 1:1 (vol:vol) to generate the pre-mixed protein samples with expected ratio between the light and heavy peptides or proteins.

2.7.3. Preparation of SILAC mixture

Culture p3 was used as the fully SILAC-labelled cells. Equal amounts (150 µg) of WCE from culture p3 and chromatin extract prepared from light lysine-containing medium were adjusted to the same concentration, mixed to the ratio of 1:1.

All protein preparations were fractionated using SDS-PAGE prior to MS analysis to reduce the complexity of protein samples. In case of determining the SILAC incorporation efficiency and the ratio of light and heavy peptides in premixed samples (2.7.1 and 2.7.2), mini SDS-PAGE was applied and one slice of the gel (at the position of ~50 kDa) from each sample was processed in MS analysis. For identification of the putative non-histone CAPs using SILAC (2.7.3), proteins were separated on large SDS-PAGE gels. The

entire sample lane was sliced into 56 pieces and trypsinized individually for MS analysis (Figure 2.1).

Figure 2.1. The schematic view of SILAC labelling and further procedures for analysis of chromatin extract.

The light-lysine ($^{12}\text{C}_6$ -lysine) containing chromatin extract and heavy-lysine ($^{13}\text{C}_6$ -lysine) labelled whole cell extract (WCE) were mixed in equal amounts, and separated by gel electrophoresis. The whole lane was sliced and subjected to in-gel digestion, and the resulting peptides were analyzed using nanoLC-MS/MS.

2.7.4. Data analysis

TPP (Trans-Proteomic Pipeline version 4.2.1, Seattle Proteome Centre, Institute of Systems Biology, Seattle, WA, USA) was used to perform database searching and peptide assignment and validation (Figure 2.2). For this purpose, all RAW spectra files were converted to the mzXML-format. Uninterpreted MS/MS spectra were searched against the *S. pombe* protein databases UniProt

Knowledgebase, including both Swiss-Prot and TrEMBL using SEQUEST algorithm (247). The database includes forward protein (target) and reverse protein (decoy) sequences that were generated by Bioworks 3.3.1 (Thermo Scientific). Search parameters included the requirement of tryptic cleavage on both ends of a peptide (allowing 1 missed cleavage site), minimum peptide length of 7 amino acids, maximal precursor ions mass deviation of 10 ppm, peptide mass tolerance of ± 0.5 Da, static modification on Cys of +57.0215 Da, differential modification on Met of 15.9945 Da, and heavy isotope coupling on Lys of +6.0201 Da. The output was in pepXML-format (248). All assigned peptides were validated using PeptideProphet (249) and the cutoff was set to the probability of 90% or greater. The qualified peptides (probability $\geq 90\%$) were quantified by the XPRESS software (250). The elution profile of the $^{12}\text{C}_6$ -Lys and $^{13}\text{C}_6$ -Lys containing peptides from the qualified peptides were isolated and quantified based on the area of peaks by XPRESS (file 2 in DVD-R). Protein levels were approximated by the median level of unique peptides. A total of 529 proteins with a ratio between light (chromatin extract) and heavy (WCE) protein levels were listed in file 3 in DVD-R. To test whether the non-histone CAP candidates (e.g., nucleus-localized proteins) could be dominated in the most abundant proteins (i.e., based on the absolute abundance) in the chromatin fraction, proteomic analysis of non-histone CAP without SILAC was performed. The complete peptide list is in file 4 in DVD-R. In determination of the absolute abundance, the PeptideProphet-qualified peptides (probability $\geq 90\%$) were quantified by PepQuan within Bioworks 3.3.1 (file 5 in DVD-R) and the abundance of proteins was estimated by the median abundance of the respective unique peptides. A total of 385 proteins whose abundance level was

approximated by the median level of their unique peptides are listed in file 6 in DVD-R.

Figure 2.2. A schematic overview of the Trans-Proteomic Pipeline (TPP) workflow for filtering potential chromatin-associated proteins (CAPs).

All RAW spectra files were converted to the mzXML format. Uninterpreted MS/MS spectra were searched against the *S. pombe* protein databases using SEQUEST algorithm, the search parameters were as follows: the requirement of tryptic cleavage on both ends of a peptide (allowing 1 missed cleavage site), minimum peptide length of 7 amino acids, maximal precursor ions mass deviation of 10 ppm, peptide mass tolerance of ± 0.5 Da, static modification on Cys of +57.0215 Da, differential modification on Met of 15.9945 Da, and heavy isotope coupling on Lys of +6.0201 Da. All assigned peptides were validated using PeptideProphet and the cutoff was set to the probability of 90% or greater. The qualified peptides (probability $\geq 90\%$) were quantified by the XPRESS software within TPP. Protein levels were approximated by the median level of unique peptides, and proteins with a ratio between light and heavy protein levels ≥ 1.5 were selected as potential CAPs.

2.7.5. Statistical analysis

Binomial test was used to test the non-random distribution and elaborated in A.4. In analysis of enrichment or depletion of the nuclear or cytoplasmic proteins, the protein localization information in a genome-wide study by Matsuyama *et al.* (251) was used, in which 4,387 proteins whose subcellular localization has been characterized. Localization of additional 321 proteins is based on the GO terms (i.e., cellular components; <http://www.genedb.org>, or <http://amigo.Geneontology.org>). The subcellular localizations are briefly categorized into three types: the nucleus [it includes the nucleus, nucleolus, spindle pole body (SPB), and nuclear envelope], cytoplasm (it includes cytosol, mitochondrion, Golgi, ER, cytoskeleton, plasma membrane, and other cytoplasmic organelles), or both. Thus, 4,708 proteins (i.e., ~93% of *S. pombe* proteome) have well defined localization types: 763 (16.2%) are

localized at the nucleus, 2180 (46.3%) at the cytoplasm, and 1765 (37.5%) at both the nucleus and cytoplasm. Details of subcellular localization and GO analysis are discussed in A.5.

2.8. Growth assays

The working concentration and stock solution of chemicals used in the growth assays are listed in Table 2.5. For temperature sensitivity testing, the mid-log-phase cells were spotted onto YES agar plates, and incubated at various temperatures as indicated.

Table 2.5. List of chemicals used in growth assays.

Chemicals	Stock Con. [*]	Working Con.	Comments ^{a, b}
Hydroxyurea (HU)	1 M	5 or 8 mM	Dissolved in H ₂ O
Methyl methanesulfonate (MMS)	10% (v/v)	0.005 or 0.0075%	Diluted with H ₂ O
Thiabendazole (TBZ)	10 mg/ml	10 or 15 µg/ml	Dissolved in DMSO ^c
Methyl 2-benzimidazole carbamate (MBC)	10 mg/ml	2.5 or 5 µg/ml	Dissolved in DMSO
4-Nitroquinoline-N-oxide (4-NQO)	0.1 mM	0.2; 0.4 or 0.8 µM	Dissolved in acetone
Camptothecin (CPT)	1 mM	3; 6 or 12 µM	Dissolved in DMSO
Caffeine	75 mM	7.5; 10 or 12.5 mM	Dissolved in H ₂ O
Hydrogen peroxide (H ₂ O ₂)	1 M	0.5; 1 or 2 mM	Diluted with H ₂ O
KCl	1 M	0.1; 0.2 or 0.4 M	Dissolved in H ₂ O

Table 2.6. List of chemicals used in growth assays (continued).

Chemicals	Stock Con. [*]	Working Con.	Comments ^{a, b}
NaCl	1 M	0.025; 0.05 or 0.1 M	Dissolved in H ₂ O
LiCl	1 M	1; 2; or 4 mM	Dissolved in H ₂ O
CaCl ₂	1 M	40; 80 or 160 mM	Dissolved in H ₂ O
EDTA (ethylenediamin-tetraacetic acid)	0.5 M	0.1, 0.2 or 0.4 mM	Diluted with H ₂ O

Note: ^{*} the abbreviation for ‘concentration’; ^a the chemicals dissolved in H₂O were filter sterilized; ^b all the reagents were purchased from Sigma-Aldrich; ^c DMSO, dimethyl sulfoxide.

2.8.1. Mini-culture growth curve assay

To measure cellular growth, an assay based on automatic measurements of optical density (OD) of small (300 µl) liquid cell cultures was used, which allowed us to plot comparable growth curves for each treatment. Briefly, overnight cultures of wild-type cells and mutant cells were inoculated into fresh YES medium to make the OD₆₀₀~0.05. Cells continued growing in the same conditions until they reach an OD₆₀₀~0.1. The cultures were treated with different agents and the 300-µl samples were placed into Honeycomb 2 multiwell plates (Growth Curves USA, Piscataway, NJ, USA), and the OD₆₀₀ of the cells was monitored using the Mini-culture growth curve system (Growth Curves USA). All tests were done in triplicate. Mini-culture growth curve assay settings were as follows: OD was measured at 600 nm, incubation temperature was kept at 30°C, the plates were subjected to continuous shaking and the reading was done every 17 min over a 48-h period (252). T_{50} is the time cells reach the half-maximal-concentration based on the growth curve, and ΔT_{50} is the difference of T_{50} between cultures treated with and without indicated drugs.

For testing the growth fitness of Bioneer strains (Table A.1) in 0.008% MMS, growth fitness score in MMS (GFS_{MMS}) was calculated based on the formula $GFS_{MMS} = 100(\Delta T_{50_mut}/\Delta T_{50_wt})$, in which ΔT_{50} is the difference of T_{50} between cultures supplemented with and without MMS. *mut* stands for mutant strains and *wt* for the wild-type strain (JHY1014). The average GFS_{MMS} of tested strains is listed in file 7 in DVD-R. Unpaired two-sample *t*-test was used to assess the difference of GFS_{MMS} between the SILAC-enriched and depleted proteins with nucleus or cytoplasm or both localizations.

2.8.2. Plating assay

Plating assay was used to carefully analyze cellular sensitivity to various stress factors. Basically, overnight cultures of wild-type cells and haploid deletion strains were inoculated into fresh YES medium to make the $OD_{600} \sim 0.20$. Cells were incubated at 30°C with shaking to generate mid-log-phase cells after growing for 2 generations or 5 h. Log phase growth cells were collected, subjected to ten-fold serial dilutions, and spotted on YES plates containing cytotoxic agents in various concentrations using a 48-pin multiblot replicator (V & P Scientific Inc., San Diego, CA, USA). The growth of each strain at various dilutions (factors of 10^5 , 10^4 , 10^3 and 10^2) was monitored for 4-5 days at 30°C. Mutant phenotypes based on the ability of cells to grow at the highest dilution compared to that of wild-type cells was used to characterize cultures as not sensitive, sensitive, or hypersensitive. In other words, mutants exhibiting growth compatible with that of wild-type cells at the same dilution were designated as “not sensitive” or “+”; mutants displaying growth similar to that of wild-type cells at approximately one dilution factor lower than that of

wild-type cells were designated as “sensitive” or “-”; and mutants exhibiting growth comparable to that of wild-type cells at two (or greater) dilution factors lower than that of wild-type cells were designated as “hypersensitive” or “--”(or “---”). All tests were done in triplicate.

2.9. Microscopy analysis

2.9.1. Indirect immunofluorescence microscopy

For indirect immunofluorescence, 2.5 ml of formaldehyde solution [37% (w/v), BDH AnalaR, Marshelltown, IA, USA] and 100 µl of glutaraldehyde [70% (v/v), Grade I, Sigma-Aldrich] were added into 20 ml of exponentially growing culture ($OD_{600} \sim 0.50$), and incubated at 30°C with shaking of 200 rpm for 30 min. Cells were washed with 10 ml of Phosphate Buffered Saline (PBS) and resuspended in 900 µl of PBS-sorbitol (1.2 M sorbitol made up in PBS). Protoplasting was performed by adding 100 µl of zymolyase 20T into the cell suspension, and the efficiency of protoplasting was monitored every 1-2 min by mixing 9 µl of cell suspension with 1 µl of 10% (w/v) SDS. Once about 50-60% of the cells were protoplasted, 500 µl of PBS plus 1% (v/v) Nonidet P-40 (NP-40) (Sigma-Aldrich) was added and mixed by inverting up and down for 30 s. Following 3× washes with PBS, the cell pellet was resuspended in 1 ml of PBAL [100 mM lysine hydrochloride and 1% (w/v) BSA made up in PBS and filter sterilized] and incubated on a rotator for 1 h. The cells were then incubated with primary antibody against HA (Santa Cruz Biotechnology) overnight at RT. The cells were washed with PBAL for trice, and incubated with fluorochrome-conjugated secondary antibody Alexa Fluor[®] 488 goat anti-

mouse IgG (Molecular probes, Eugene, Oregon, USA) for 1-2 h at RT. Cells were then washed with 400 μ l of PBAL and finally resuspended in 20-30 μ l of PBAL. The cell nuclei was stained in equal volume of 0.4 μ g/ml of 4',6-diamidino-2-phenylindole (DAPI)/Antifade solution (Millipore) for 2 min. Sample slides were visualized using a Zeiss Axiophot 200 inverted microscope equipped with color camera (Zeiss, Wetzlar, Germany), the objective used was Olympus APO 100 $\times$ NA 1.0 (Olympus America Inc., Melville, NY, USA). Images were captured via a Hamamatsu C2400-08 digital camera using MetaMorph image acquisition and analysis software (version.6.3r7; Universal Imaging, Media, PA, USA).

2.9.2. Nuclear and cell wall staining

To stain nuclei, 1 ml of culture samples were fixed by adding one-tenth volume of concentrated formaldehyde [37% (w/v), BDH] and glutaldehyde [70% (v/v), Grade I, Sigma-Aldrich] for 10 min and washed 3 $\times$ with PBS. Nuclear DNA, cell wall and septa were stained with 50 μ g/ml DAPI and 0.5 mg/ml Aniline blue (Sigma-Aldrich), respectively. Cells were visualized as described above.

2.10. Time course study

Overnight cultures at 30°C were inoculated into fresh YES medium, and were refreshed to OD₆₀₀~0.2. After one DT, the cells were subjected to respective stress condition, including 0.1% (v/v) MMS, 15 μ g/ml TBZ, 5 μ g/ml MBC, various concentrations of caffeine ranging from 1 to 10 mM, 0.1 M KCl

and 0.1 M NaCl. Cells were harvested along the time course for cell lysate preparation, SDS-PAGE and Western blotting. For quantifying the bands obtained via Western blotting, ECL images were analyzed using the public domain *ImageJ* program (developed at the National Institutes of Health, Bethesda, MD, USA), using the “Gel Analysis” functions. Result of the analysis is a value for each band which is proportional to the Integrated Density Value (IDV) of that band. The IDV of Cdc2 (PSTAIRE) was used as a loading control to normalize the expression level of the tested proteins.

2.11. Fluorescence-activated cell sorting (FACS)

To measure DNA content, cells ($\sim 10^7$ cells/ml) were fixed with 70% ice-cold ethanol, washed with 50 mM sodium citrate pH 7.0 and digested with 0.1 mg/ml RNase A (QIAGEN, Washington D.C., Metro Area, USA) overnight at RT. DNA was subsequently stained with 2 μ g/ml propidium iodide (PI) (Sigma-Aldrich) for 30 min. In prior to FACS analysis, cells were sonicated for 30s using the Sonifier® 450 digital ultrasonic processor (Branson, Danbury, Connecticut, USA), and transferred to the 5ml polystyrene round bottom tubes (BD Falcon™, BD Biosciences, Franklin Lakes, NJ, USA). Fluorescence intensities of individual cells were measured by flow cytometry using the FL-2 channel in the BD FACScan (BD Biosciences) (253).

2.12. Homology searches and alignments

Position-specific iterated (Psi-Blast) search was performed in Protein Data Bank (PDB, <http://www.rcsb.org/pdb>) to detect potential orthologs of Bc530.05. Alignments presented in Figure 5.2 were made using MUSCLE (<http://www.ebi.ac.uk/Tmuscle/index.html>) (254) and BOX SHADE (http://www.ch.embnet.org/software/BOX_form.html).

2.13. Cloning, expression and purification of Histone H4

2.13.1. Cloning of carboxy-terminal TAP tagging vector pJK210-CTAP-*hhf1*

hhf1⁺ was amplified by PCR using the primers 5'-GCGCGCATCGATAGCTGTTGTGCTACTTTTGC-3' and 5'-GGGCCCAGATCTACCACCGAAACCATAAATGG-3'. Plasmid pJK210-CTAP-*hhf1* was constructed by isolating the 712-bp *Cla*I-*Bgl*II fragments *hhf1*⁺ into the *Bam*HI site of plasmid pJK210-CTAP (Figure 2.3) (244) such that the *hhf1*⁺ gene was in frame with the protein coding sequence of TAP. Restrictive enzyme digestion using *Kpn*I was to ascertain the correct size and orientation of the inserts. DNA sequencing analysis was to ensure the clone is free of mutation. Primers used for DNA sequencing include T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T3 promoter (5'-AATTAACCC TCACTAAAG-3') (1st BASE). The results are shown in B.4.

2.13.2. Transformation of pJK210-CTAP-*hhf1* into fission yeast

The plasmid was digested with fast digest enzyme *HpaI* (Fermentas, Burlington, Canada) for 20 min at 37°C, and then transformed into the *S. pombe* strain LJY188 using the classical lithium acetate method as described before (243).

Figure 2.3. Map of the cloning vector for tandem affinity purification (TAP) tagging in *S. pombe*.

Vector for PCR amplification of sequences for C-TAP tagging. TAP contains two epitope tags of Protein A for immunoglobulin G (IgG) binding, a calmodulin-binding peptide (CBP) for calmodulin binding, which are separated by a tobacco etch virus (TEV) protease cleavage site. Restriction sites are indicated.

2.13.3. Protein expression of pJK210-CTAP-*hhf1*

To check the expression of the tagged gene product, colonies were picked up to re-streak onto freshly prepared EMM2 agar plates lacking uracil

and were incubated at 30°C for two days. Cells were grown to mid-log phase and protein lysates were prepared. About 30 µg of each protein sample was resolved on a 12% SDS-PAGE. Western blot analysis was carried out using anti-Histone H4 (Upstate Biotechnology), anti-Protein A (Sigma-Aldrich) and anti-CBP (Santa Cruz Biotechnology) as primary antibodies. The parental strain was included as a negative control.

2.13.4. Protein preparation by dual-step affinity purification

Purifications of C-TAP-tagged proteins were carried out as previously described (255-257) with a few modifications. Briefly, Six liters of mid-log phase *S. pombe* cells were grown at 30°C and lysed by beads beating in NP-40 buffer (6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 µg/ml leupeptin, 0.1 mM Na₃VO₄) containing EDTA-free protease inhibitor tablet (Roche). The extract was obtained by centrifugation at 14,000 rpm at 4°C for 30 min. After protein extraction, 400 µl of immunoglobulin G (IgG) sepharose beads (Sigma-Aldrich) in NP-40 buffer (1:1 slurry) was added to 300 mg total protein in a 14-ml volume. This slurry was incubated at 4°C, rotating for overnight. After incubation, the lysate and beads were slowly poured onto a pre-equilibrated Poly-Prep Chromatography Column (0.8 x 4 cm) with a funnel (BioRad). The beads were washed three times with 10 ml of IPP150 buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40), and once with 10 ml of freshly prepared tobacco etch virus (TEV) protease cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). After closing bottom of the column with a stopper, the IgG sepharose was resuspended in 1 ml of TEV protease cleavage buffer

containing 600 U of TEV protease (Invitrogen), with top of the column plugged and incubated at 4°C, rotating for overnight. The flow through was drained into a new pre-equilibrated column that was sealed at the bottom. The old column was washed with another 1 ml TEV protease cleavage buffer and combined into the new column. The retrieved proteins were then mixed with 6 ml of 0.1% calmodulin binding buffer (CBB) (10 mM β -ME, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM imidazole, 2 mM CaCl₂, 0.1% NP-40), 6 μ l of 1 M CaCl₂, and 200 μ l of calmodulin beads (Stratagene). This mixture was incubated for 1 h at 4°C, with rotating. After incubation, the column was opened and drained. The beads were then washed twice with 10 ml of 0.1% CBB and then with 10ml of 0.02% CBB (0.1% and 0.02% represent the concentration of NP-40). Finally, the protein complex from calmodulin beads was eluted twice with 500 μ l of calmodulin elution buffer (10 mM β -ME, 10 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM imidazole, 2 mM EGTA, 0.1% NP-40). The two eluates were trichloroacetic acid (TCA)-precipitated by adjusting them to 25% TCA with 100% TCA, placing them on ice for 30 min with periodic vortexing, and spinning at maximum speed for 30 min at 4°C. The TCA pellets were washed twice with ice-cold (-20°C) acetone (MERCK) and spun for 5 min at maximum speed at 4°C. The supernatant were carefully removed and air-dried for 15 min.

2.13.5.Silver staining

The TCA pellet from second eluate was resuspended in 2 $\times$ SDS sampling buffer and resolved on a 14% large SDS-PAGE gel. Silver staining

was carried out using SilverQuestTM Silver Staining Kit (Invitrogen) according to manufacturer's instructions.

2.13.6. Sample preparation and LC-MS/MS analysis

The TCA pellet from the first eluate was subjected to mass spectrometric analysis as detailed previously (257, 258). In brief, gel electrophoresis was utilized to fractionate complex protein mixtures followed by in-gel digestion of the proteins and mass spectrometric identification by TPP as described previously (2.7.4), but without quantification. A total of 22 fractions were obtained. The fractions were combined and proteins were identified using ProteinProphet within TPP. The proteins with two peptide matches with better than 90% probability (FDR < 1%) were considered as validated identifications.

2.13.7. Statistical analysis

The proteins identified from C-TAP-tagged hhf1 are listed in file 8 in DVD-R. In determination of the *bona fide* histone H4-interacting partners, the ribosomal proteins were firstly removed. The Relative Abundance Index (A-index) (a ratio of the total number of MS/MS spectra acquired for a given protein to the number of unique peptide sequences they matched) was calculated for the remaining identified proteins, and those with A-index value less than 1.75 were discarded (file 9 in DVD-R). Proteins present in mock control purification (file 10 in DVD-R) from untagged cells were excluded. The protein list was subsequently filtered by removing the *S. pombe* background proteins [Table S1 of (259)], and the proteins exclusively present in C-TAP-hhf1 were considered to be the true histone H4-interactors (Table 6.1).

CHAPTER 3. IDENTIFICATION OF NON-HISTONE CHROMATIN-ASSOCIATED PROTEINS USING SILAC-MEDIATED PROTEOMIC ANALYSIS

3.1. Introduction

Chromatin is a complex of DNA and proteins, in which the histones H1, H2A, H2B, H3, and H4 are the major protein constituent (1, 260). They are estimated to constitute approximately 70% of chromatin (177). H2A, H2B, H3 and H4 are core histones to form nucleosomes, and the linker histone H1 binds to the linker DNA region between nucleosomes. Chromatin remodelling through PTMs of histone proteins play an important role in modulation of DNA-protein interaction and thus regulate various biological processes such as DNA replication, DNA damage and repair, nucleosome assembly and transcription (5). Many other proteins, such as DNA and RNA polymerases and transcription factors, are also found to be associated with the chromatin temporally or constitutively for their functions (257). Disruption of these proteins leads to a chaotic chromatin environment, and are implicated in a series of epigenetic diseases (261). Since last two decades, chromatin structure and function have attracted remarkable attentions and outstanding progresses (262). However, many questions remain unanswered. For example, which proteins are responsible for chromosome condensation? How chromatin-modifying enzymes are recruited to chromatin? Chromatin dynamics such as

transcriptional control, signal transduction, and DNA damage and repair are with continuously raised concern. Hence, identification of the non-histone CAPs would permit understanding of the molecular mechanisms for chromatin remodelling and regulation of various biological processes, which might pave the road to success for disease treatment in humans.

Fission yeast is a widely used model for the study of chromatin remodelling, DNA replication and repair, and transcription (137, 148, 257, 263). It is also useful for analysis of RNAi directed heterochromatin formation (264, 265). Many non-histone CAPs have been identified by using the high-throughput proteomic analysis of protein complexes purified through ChIP coupled with TAP in which the known CAPs are tagged and used as a bait (258, 266-271). However, it is limited to the identification of the non-histone CAPs that are associated with the complexes containing the previously known CAPs.

Chromatin fractionation assay is a traditional assay for testing whether a protein of interest is associated with the chromatin in fission yeast. With the assistance of Western blotting, components of the origin recognition complex (ORC) such as Orc1, Orc2, and Orc5 are found to be associated with the chromatin throughout the cell cycle (156, 158). On the other hand, the ATR-like kinase Rad3 and the mitotic activator phosphatase Cdc25 are found to be temporally associated with chromatins upon DNA damage (157, 159). However, chromatin is composed of a folded string of DNA and protein, which is surrounded by over thousands of proteins in the nucleus. Therefore, it is unlikely to obtain 100% pure chromatin without pulling down non-CAPs. Consistent with previous studies in human (177), *C. elegans* (222) and plant (223), direct proteomic analysis of chromatin extract in *S. pombe* also reveals

contamination by other proteins in our analysis. Based on the absolute protein abundances, we found that the most abundant proteins identified from chromatin fraction are predominated by the ribosomal proteins. However, they are unlikely to be non-histone CAP candidates because translation does not require physical interaction with the chromatin. Effective depletion of the highly abundant non-CAPs such as ribosomal proteins is essential for *de novo* identification of non-histone CAPs on the basis of protein abundance. Hence, direct proteomic analysis of chromatin fraction is unlikely to be able to determine non-histone CAP candidates without prior knowledge.

In theory, the relative level of a potential CAP in chromatin fraction is higher than that in WCE. On the other hand, the relative abundances of ribosomal proteins in chromatin fraction should not be higher than that in WCE. Recently, SILAC-mediated proteomic analysis has shown to permit the quantitative analysis of the relative protein levels between the SILAC-labelled and unlabelled protein samples (188). This approach would be applicable to compare individual protein levels in WCE and in chromatin fraction, and the protein ratio would help to judge whether the protein is associated with chromatin. By applying the SILAC-mediated proteomic analysis, we show that the highly abundant non-CAP ribosomal proteins are significantly depleted in the top ranked proteins based on the SILAC ratio of chromatin fraction versus WCE. The top SILAC-ranked proteins are enriched for potential CAP candidates such as nucleus-localized proteins; they are also enriched for proteins with CAP-related functions such as chromatin structure organization, DNA replication and repair, and transcription according to GO analysis.

DNA is essential to life, but it is subjected to chemical and environmental assaults, causing DNA damage. Cells have evolved a number of mechanisms to detect and repair the various types of DNA damage. A failure to DNA repair will produce an irreversible mutation, and may lead to cancer. DNA damage can be induced in numerous ways, such as DNA-protein cross-links, DSBs, SSBs, and oxidative lesions. DDR is the defense system to combat these severe biological consequences. It occurs in a chromatin context, and occupies an extensive region of chromatin (272).

Until recently, two genome-wide studies have been conducted to probe gene functions related to DNA damage and repair in the fission yeast *S. pombe* (140, 148). Deshpande *et al.* has used plating method to screen for mutant strains sensitive against various DNA damaging agents (148). To measure the degree of sensitivity, a semi-quantitative score was assigned to each mutant based on the concentration they started to show clear growth inhibition. In contrast to this one-by-one phenotypic screening, Han *et al.* pooled all the deletion strains and applied the barcode sequencing to isolate mutants sensitive to DNA damaging agents (140). With the unique barcode sequence (273), growth fitness of each mutant was calculated based on the PCR product quantity. A growth inhibitory score (GI) was derived to indicate the degree of sensitivity. Both of them show that proteins involved in chromatin architecture maintenance, microtubule-related events and transcription are over-represented in the maintenance of genomic stability.

We believe non-histone CAPs should have a greater propensity to be responsible for DNA damage and repair, and our results agree well with this statement in both liquid and solid media. Phenotypic assessment of mutant

strains in liquid culture shows that the SILAC-enriched but not depleted nucleus-localized proteins exhibit the high likelihood of requirement for growth fitness in presence of the DNA damaging agent MMS, which causes both DSBs and replication errors (274). Furthermore, phenotypic assessment using plating method suggests that more than half of the tested SILAC-enriched proteins are potentially involved in maintaining genomic stability.

Taken together, our results suggest that SILAC-mediated proteomic analysis of chromatin fraction is capable of identifying non-histone CAP candidates without prior knowledge. We propose that our approach is complementary to the ChIP method coupled with TAP-tagging for identification of CAP-interacting partners.

3.2. Results

3.2.1. Cells are fully incorporated with the heavy lysine after nine generations

SILAC is one of the most popular approaches for *in vivo* incorporation of a tag into proteins for relative quantification by MS. In the SILAC proteomic analysis, SILAC-labelled samples are required to be fully incorporated with the heavy stable isotope-coupled lysine (e.g., $^{13}\text{C}_6$ -lysine or heavy lysine) or arginine (e.g., $^{13}\text{C}_6$ -arginine or heavy arginine) or both. Given that there is an extensive conversion of arginine into other amino acids in fission yeast (275), we only labelled heavy lysine to avoid the arginine-conversion problem. To investigate the protein abundances using SILAC method, some pilot

experiments were performed by labelling cells with $^{13}\text{C}_6$ -lysine as common reference for quantitative proteomic analysis.

To determine the labelling efficiency in cells containing $^{13}\text{C}_6$ -lysine, the rate of incorporation with heavy lysine in cells was tested after various numbers of passages in EMM2 supplemented with heavy lysine. In this test, each subculture was maintained for a day (i.e., equivalent to ~3 generations) before subsequent subculturing (Figure 3.1A). The cells were continuously refreshed in $^{13}\text{C}_6$ -lysine containing medium and cells from the third, sixth, and ninth generation were collected at the end of the day. Proteins were extracted, fractionated and digested; the resultant peptide mixtures were analyzed with nanoLC-MS/MS and the relative ratios of light versus heavy peptides/proteins were determined in each of the sample.

The relative level of light versus heavy peptides/proteins was exemplified by the peptide of Enolase Enol (peptide sequence: AVGNVNNIAPVVK) in various subcultures. Cells in the initial culture prior to subculturing in heavy isotope-containing medium (or p0) showed that no heavy peptide was present (Figure 3.1B, see p0). On the other hand, cells in the culture after first passage (i.e., ~3 generations in the $^{13}\text{C}_6$ -lysine containing medium) showed that ~90% of the peptides were labelled with heavy isotope (Figure 3.1B, see p1). In this case, a 6-Dalton (Da) mass difference between “light” and “heavy” peptides may lead to either 2- or 3-Da shift in m/z depending on the peptide charge (i.e., 739.94 Da versus 742.95 Da). We found that cells after the second or the third passage contained hardly any light peptides (Figure 3.1B, see arrows in p2 and p3), indicating the lysine residue

was completely replaced by its heavy counterpart. This result suggested the efficient incorporation of heavy lysine into cellular proteins in fission yeast.

To further test whether the heavy lysine was uniformly incorporated in all peptides/proteins besides the peptide of Enol, the ratios between light and heavy peptide levels in all of the 110 lysine-containing peptide pairs from p1 were calculated. Within TPP, the quantification is performed in two different ways, Xpress Ratio and ASAPRatio. Xpress Ratio is calculated from Single Ion Chromatogram (SIC) of charge state in which peptide has been identified (250); whereas ASAPRatio is performed by reconstructing the SIC after peptide sequence identification and validation are completed (276). Because Xpress Ratio is more suitable for SILAC labelling, we calculated the ratio of each $^{12}\text{C}_6$ -lysine and $^{13}\text{C}_6$ -lysine containing peptide pairs by the Xpress. As the result shown, ~95% of them were found to be below -2 in log2 scale, indicating that the heavy lysine was uniformly incorporated into all the proteins (Figure 3.1C and file 11 in DVD-R). To ascertain proteins were fully incorporated with heavy lysine for SILAC, cell lysates derived from the third passage (i.e., p3, ~9 generations in the heavy lysine-containing medium) were applied as the heavy isotope labelled sample in this analysis.

Figure 3.1. Analysis of heavy lysine incorporation.

(A) A schematic diagram showing the consecutive subculturing in heavy lysine containing medium. (B) MS spectra of the peptide AVGNVNNIAPVVK of Eno1. Spectra of peptides resulted from various passages p0, p1, p2 and p3 are shown. Green and red lines indicate the m/z of light and heavy peptides, respectively. Arrow indicates that the level of light peptide is low or undetected in p2 and p3 lysates. (C) The scatter plot showing the ratio of light and heavy peptide levels. X-axis and Y-axis indicate the abundance of individual peptides and the ratio of light and heavy peptide levels. Each dot represents a peptide.

3.2.2. The observed and expected premix ratio of light versus heavy peptides/proteins are highly correlated in SILAC pre-mixed samples

To assess the accuracy of relative levels between light versus heavy peptides/proteins in our SILAC analysis, pre-mixed protein samples with various ratios between light and heavy proteins were prepared. A slice of SDS-PAGE gel (i.e., containing >100 peptides) from each pre-mixed sample was subjected to the proteomic analysis after in-gel trypsinization. Based on the XPRESS Ratio, the distribution of relative levels of all peptides detected from each pre-mixed sample correlated well with the expected premix ratio, suggesting that our SILAC protocol is adequate for the quantitative proteomic analysis (Figure 3.2A). This is consistent with the notion that SILAC methodology is excellent for quantitative proteomic analysis (188). The correlation became more apparent when the median of ratios between light and heavy peptide levels of all peptides in a given pre-mixed sample was compared to their expected ratio (Figure 3.2B). To estimate the light and heavy protein levels, the median of the unique peptide levels was applied. By using this estimation, the observed relative level between light versus heavy proteins in a given pre-mixed sample also correlated well with the expected relative level in the pre-mixed sample (Figure 3.2C). Hence, the median of unique peptide levels was used to estimate the level of proteins in this study.

Figure 3.2. Observed ratio of the light and heavy peptide levels is correlated with the expected ratio in the pre-mixed samples using SILAC.

(A) The heat map showing the distribution of observed ratios of the light and heavy peptide levels in the premixed samples. X-axis and Y-axis indicate the observed ratio and the expected ratio of light and heavy peptide levels, respectively. Color key is shown at the top. (B) Correlation between the median of the observed ratio and the expected ratio of light and heavy peptides. (C) Correlation between the median of the observed ratio and the expected ratio of light and heavy proteins. The correlation coefficient R is shown.

3.2.3. Chromatin fractionation assay is a valid method to identify chromatin-associated proteins

Chromatin fractionation assay has been used to test a given protein for its association with the chromatin in Western blot analysis (156, 158). To do this, the crude nuclear extract was prepared by removing the soluble cytoplasmic proteins in the supernatant (Figure 3.3A). Spheroplasts initially prepared from cells grown in minimal media were digested with zymolyase, followed by lysis with non-ionic detergent. The cell extracts were further fractionated into supernatant and insoluble nuclear pellet by centrifugation. The resulting insoluble nuclear pellet fraction was resuspended and then subjected to DNase I digestion and followed by centrifugation. Hence, the resulting supernatant should contain CAPs that were released from the DNase I digested crude chromatin, leaving an insoluble pellet of cell debris and nuclear scaffold-

bound proteins. Digestion of genomic DNA was apparent by DNase I (Figure 3.3B). The Coomassie blue-stained SDS-PAGE gel showed that each fraction had extremely different protein patterns (Figure 3.3C). To ensure chromatin-bound proteins were successfully enriched, histone H4 and β -tubulin (TAT1) were used as controls for CAPs and non-CAPs, respectively; herein, the Coomassie blue-stained gel served as a loading control for the Western blotting analysis (Figure 3.3D). As we can see, the amount of H4 protein in the chromatin fraction was much higher than that in the total fraction; and its level was largely reduced and became invisible in the cytosolic fraction. On the other hand, the level of β -tubulin in the enriched chromatin was extensively decreased, indicating that the cytoplasmic proteins were depleted in the chromatin fraction; as expected, β -tubulin was found to be enriched in the cytosolic fraction. It was obvious that histone H4 was chromatin bound, and β -tubulin was not chromatin bound.

Figure 3.3. The procedure and validation of chromatin fractionation assay. (A) Schematic outline of the experimental protocol for chromatin fractionation assay. (B) DNA was purified by hot phenol extraction and ethanol precipitation from whole cell extract (T), soluble proteins (S1), crude chromatin (P1), chromatin extract (C) and insoluble proteins and cell debris (P2), and subjected to electrophoresis in a 0.8% agarose gel containing ethidium bromide (EB). The gel image shows the presence or absence of genomic DNA (gDNA) in these samples. (C) The protein content of each fraction as indicated in (A) were examined by 12% SDS-PAGE followed by Coomassie blue staining. (D) To analyze the proteins by Western blotting, histone H4 and β -tubulin (TAT1) were used as controls for chromatin-bound and non-chromatin bound proteins, and the amount in T, S1 and C from this assay was determined by immunoblotting with anti-Histone H4 polyclonal antibody or anti-tubulin monoclonal antibody, respectively. Coomassie blue-stained SDS-PAGE was served as a loading control for the Western blotting analysis.

3.2.4. SILAC analysis of proteins from chromatin preparation

Due to the limited depletion, highly abundant cytoplasmic proteins may still be remained in high abundance in the chromatin fraction after depletion. On the other hand, low abundant CAPs could still be remained in low abundance in the chromatin fraction after enrichment. Therefore, we judged that the relative abundances of the non-CAPs in chromatin fraction would be lower than that in WCE, although the absolute abundance of many ribosomal proteins still remained higher than that of non-histone CAP candidates (e.g., the nucleus-localized proteins). In other words, the relative abundances of non-histone CAP candidates in chromatin fraction should be higher than that in WCE.

To identify proteins whose relative abundance in chromatin fraction is higher than that in WCE, equal amounts of protein samples from chromatin fraction and SILAC-labelled WCE were mixed and subjected to nanoLC-MS/MS analysis. As a result, a total of 29493 spectra were obtained (file 2 in DVD-R). 6567 out of them (~22.3%) are either $^{12}\text{C}_6$ -lysine or $^{13}\text{C}_6$ -lysine containing peptides, the remaining are arginine-terminated tryptic peptides. This is because trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine (K) or arginine (R). A significantly higher number of arginine-terminated peptides were observed, because the ionization efficiency of arginine-containing peptides is higher than that of lysine-containing peptides (277). Based on the high quality peptides selected through the TPP, we identified a total of 529 proteins whose ratio between levels in chromatin extract and in WCE was determined (file 3 in DVD-R).

3.2.4.1. The top ~10% proteins ranked by SILAC ratio are depleted of highly abundant ribosomal proteins

In this analysis, protein localization was based on the study of *S. pombe* ORFeome by Matsuyama *et al.* (251), and gene ontology cellular component terms (www.geneontology.org). For direct analysis of chromatin extract without SILAC labelling, we found that the highly abundant ribosomal proteins were predominated in the top ~10% ranked proteins based on the level of abundance (abundance ranked proteins) compared to the background level (i.e., 51.4% versus 14.9%; p-value = 2.21e-07) in chromatin extract (Figure B.1 and file 6 in DVD-R). This result suggests that the highly abundant ribosomal proteins are not adequately depleted in chromatin extract.

Of the 529 proteins identified from our SILAC experiment, 507 proteins have defined localization. We found that the occurrence of ribosomal proteins in the top ~10% (or top 50) SILAC-ratio ranked proteins was ~35% lower than the background level (6% versus 9.5%), and was significantly lower than occurrence in the top 10% abundance ranked proteins (p-value = 9.32e-12), suggesting the effective depletion of the highly abundant non-CAPs (Figure 3.4). On the other hand, the occurrence of ribosomal proteins in the second top 10% SILAC-ratio ranked proteins was not reduced compared to the background level (i.e., 14% versus 9.5%). This result suggests that the enrichment for non-histone CAP candidates in the second top 10% SILAC-ratio ranked proteins was not very effective. Hence, only the top 10% SILAC-ratio ranked proteins (i.e., top SILAC-ranked proteins) were considered to efficiently enrich for non-histone CAP candidates and were further analyzed in this study.

Figure 3.4. The ribosomal proteins are depleted in the top 10% SILAC-ranked proteins. X-axis indicates the percentile ranks by ratio of light and heavy protein levels. Y-axis indicates the occurrence of ribosomal proteins at various levels of SILAC-ranked proteins.

3.2.4.2. The top SILAC-ranked proteins are enriched for nucleus-localized proteins

Non-histone CAPs would have nucleus-localization because they needed to interact with chromatin for function. We therefore wanted to examine if the nucleus-localized proteins were enriched in the top SILAC-ranked proteins. All proteins were characterized into 3 categories: the nucleus-localized, the dual-localized, and the cytoplasm-localized proteins. To this end, we found that the occurrence of nucleus-localized proteins in the top SILAC-ranked proteins was significantly higher than the background level (i.e., 24% versus 9.47%; $p\text{-value} = 2.0\text{e-}03$) (Figure 3.5A). This result supports our conclusion that the top SILAC-ranked proteins are enriched for non-histone CAP candidates (148, 155, 160), suitable for non-histone CAP discovery without prior knowledge. On the other hand, no apparent enrichment was observed for the cytoplasm-localized or dual-localized proteins. Interestingly, in the non-SILAC proteomic analysis of chromatin extract, nucleus-localized proteins

were not overrepresented in the top most abundant proteins (Figure B.1 & file 6 in DVD-R).

Next, we wanted to know if the CAP-related functions such as regulation of chromatin structure and DNA replication and repair would be enriched in the top SILAC-ranked proteins. Gene ontology analysis (i.e., biological process terms) showed that the occurrence of functions such as transcription, chromatin modification, DNA replication and repair in the top SILAC-ranked proteins was higher than the background levels (Figure 3.5B). The enrichment for CAP-related functions was statistically significant (p-value < 0.05) when the total number of occurrence was greater than 15. These results were consistent with a notion that the top SILAC-ranked proteins were enriched for non-histone CAP candidates.

Although proteins involved in translation were not completely eliminated from the top 10% SILAC-enriched proteins, the frequency of occurrence was significantly lower than that of the top 10% most abundant proteins in the non-SILAC analysis (i.e., 15.1% versus 65.8%; p-value = 9.4×10^{-14}). We found proteins involved in proteolysis and mRNA splicing were also present but not statistically significant.

Figure 3.5. The nucleus-localized proteins are enriched in the top 10% SILAC-ranked proteins. (A) Display is identical to Figure 3.4. Levels of nucleus-localized (nuc), dual-localized (nuc; cyt), and cytoplasm-localized (cyt) proteins are shown. (B) Gene ontology analysis of the top 10% SILAC-ranked proteins.

3.2.4.3. The top SILAC-ranked nucleus-localized proteins are associated with chromatin

Chromatin is “floating” in the nuclear solution that contains over thousands of proteins; therefore, it would be unlikely that all nucleus-localized proteins were associated with the chromatin. We thus wanted to test if the non-top SILAC-ranked nucleus-localized proteins were not associated with the chromatin. For this reason, the randomly selected three top SILAC-ranked proteins Msh3, Prp16 and Cc18.05c (e.g., their SILAC ratios were 5.86, 4.72, and 4.19 in log₂ scale, respectively) and two other SILAC-depleted proteins Srp2 and Kap95 (e.g., their SILAC ratios are -0.46 and -1.30 in log₂ scale) were tagged with HA-epitope (Figure B.2), and subjected to chromatin fractionation assay. The analysis indicated that the top-ranked or most enriched nucleus-localized proteins in SILAC analysis were the true non-histone CAPs (Figure 3.6A). On the other hand, both the non-top ranked nucleus-localized proteins were detected in the soluble fraction but hardly in chromatin extract,

supporting their independent relationship with the chromatin (Figure 3.6B). To ascertain that the subcellular localization of the HA epitope-tagged protein used in the chromatin fractionation assay would not be altered by the epitope, we performed the indirect immunofluorescence microscopic analysis (Figure 3.6C). Clearly, the HA-tagged proteins remained to be nuclear (251, 278). Hence, we concluded that only the top SILAC-ranked nucleus-localized proteins in the SILAC analysis were likely to be the true non-histone CAPs.

We next tested if the top SILAC-ranked dual-localized proteins would also be associated with the chromatin. To this end, the three random selected dual-localized proteins Uve1, Hsp16, and Bc530.05 (e.g., their SILAC ratios were 4.83, 4.12, and 3.9 in log₂ scale, respectively) were subjected to chromatin fractionation assay. The analysis indicated that all three proteins exhibited apparent enrichment in the chromatin extract when compared to WCE (Figure 3.7). On the other hand, the presence in the soluble fraction was detected in two out of three dual-localized proteins (i.e., Uve1 and Hsp16), consistent with their subcellular localization. This result suggested that most of the top SILAC-ranked proteins were true non-histone CAP candidates.

Figure 3.6. The chromatin association of the SILAC-enriched and SILAC-depleted nucleus-localized proteins.

(A) SILAC-enriched nucleus localized proteins are associated with the chromatin. Chromatin fractionation assay was used to confirm the chromatin association of the top-ranked nucleus-localized proteins in the SILAC analysis. Histone H4 (H4) and β -tubulin (TAT1) were used as positive loading controls to ensure the assay was valid. Tested proteins and their respective SILAC ratio in parentheses are indicated. Loading quantity of each protein sample including whole cell extract (T), supernatant 1 (S1) and chromatin (C) is indicated at the bottom of each immunoblotting image. 1, 0.25, 0.25 indicates the ratio of protein loading amount is 4:1:1. (B) SILAC-depleted nucleus-localized proteins are not associated with the chromatin. The methods and display are identical to that in (A). (C) Subcellular localization of the HA-tagged Srp2. DAPI staining and indirect immunofluorescence microscopy were performed to ensure the localization of Srp2 was not changed upon HA tagging.

Figure 3.7. The chromatin association of the SILAC-enriched dual-localized proteins. SILAC-enriched dual-localized (localized to both nucleus and cytoplasm) proteins are association with the chromatin. Chromatin fractionation assay was used to confirm the chromatin association of the top-ranked dual-localized proteins in the SILAC analysis. Histone H4 (H4) and β -tubulin (TAT1) were used as positive loading controls to ensure the assay was valid. Tested proteins and their respective SILAC ratio in parentheses are indicated. Loading quantity of each protein sample including whole cell extract (T), supernatant 1 (S1) and chromatin (C) is indicated at the bottom of each immunoblotting image. 1, 0.25, 0.25 indicates the ratio of protein loading amount is 4:1:1.

3.2.4.4. SILAC-enriched nucleus-localized proteins exhibit the high likelihood of requirement for growth fitness under DNA damage stress

Of the 507 proteins with defined localization, 413 were found to be either SILAC enriched (i.e., $\log_2^{\text{SILAC ratio}} > 0.585$) or depleted (i.e., $\log_2^{\text{SILAC ratio}} < -0.585$). Non-histone CAPs likely to have functions such as chromatin remodelling, replication, DNA damage and repair, and transcription that required physical interaction with the chromatin. Here, we wanted to test if the SILAC-enriched proteins were more likely to play a role in DNA damage and repair than the SILAC-depleted ones. For this reason, we obtained 188 (~45.5%) *S. pombe* strains bearing a deletion allele encoding one of the 413 SILAC-enriched or depleted proteins from the Bioneer deletion strain collection. Mini-culture growth curve assay (252) was applied for determining the growth fitness in DNA damaging agent MMS. Two-fold serial dilution cultures of wild-type cells were used to ensure the system was working properly (Figure B.3).

By using gene deletion mutant strains, functional requirement for DNA damage and repair could be readily assessed by determining their growth fitness in presence of MMS. Cellular response to MMS was followed kinetically from early log phase (i.e., $OD_{600} = 0.1$), and level of growth fitness was estimated by GFS_{MMS} . The higher the GFS_{MMS} is, the more likely the protein is required in DNA damage and repair. To this end, we found that the median GFS_{MMS} of strains bearing a deletion allele of the SILAC-enriched nucleus-localized proteins was significantly higher than that of the SILAC-depleted nucleus-localized proteins (1.2 versus 0.79; p-value < 0.05; file 7 in DVD-R), supporting the idea that the SILAC-enriched nucleus-localized proteins are *bona fide* non-histone CAP candidates (Figure 3.8A). On the other hand, the median GFS_{MMS} of the SILAC-enriched cytoplasm-localized or dual-localized proteins showed no apparent differences from that of the SILAC-depleted proteins (i.e., 0.93 versus 0.98 or 1.05 versus 1.02) (Figure 3.8B, C). This result was consistent with the observation that the cytoplasm-localized or dual-localized proteins were not overrepresented in the top SILAC-ranked proteins (Figure 3.5A). Hence, we concluded that the SILAC-enriched nucleus-localized proteins are most likely to play a role in growth fitness under the MMS stress, consistent with the function of CAPs.

Figure 3.8. SILAC-enriched nucleus-localized proteins exhibit the high likelihood of requirement for growth fitness in MMS.

The boxplots show the level of growth fitness in mutant strains containing a deletion allele of the SILAC-depleted or enriched proteins. Nucleus-localized (Nuc), dual-localized (Dual), or cytoplasm-localized (Cyto) proteins are shown in (A), (B), or (C).

3.2.5. Phenotypic analysis of SILAC-enriched proteins

responsible for maintaining genomic stability

To carefully characterize the phenotypes of the SILAC-enriched proteins, we chose the deletion mutants lacking the proteins enriched by at least two fold ($\text{Log}_2^{\text{SILAC ratio}} \geq 1.0$, 30 stains) from our SILAC analysis, and performed the plating assay by treating the mutant cells with DNA replication inhibitor HU and DNA alkylating agent MMS. The ability of these mutants to grow on plates supplemented with HU and MMS is shown in Figure 3.9. Their growth defects to these stresses were subjected to gradations, a summary of sensitivity measurement obtained from the assay is compiled in Table 3.1.

Figure 3.9. Phenotypic assessment of the deletion mutants lacking one of the SILAC-enriched proteins ($\text{Log}_2^{\text{SILAC ratio}} \geq 1.0$).

The 30 viable haploid h^+ mutant strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, approximately 5 μl of the cells were spotted onto YES agar plates supplemented with various dosages of HU (DNA replication inhibitor) and MMS (DNA alkylating mutagen) as indicated (at the bottom of the figure) and incubated at 30°C for 4-5 days. The cell densities from left to right are 10^5 , 10^4 , 10^3 and 10^2 , labelled as 1, 2, 3, and 4, accordingly. Each mutant is represented by the name of the deleted gene, unless only a systematic name is available. The ratio in log2 scale from the SILAC analysis is displayed on the right hand side of corresponding mutant. A mutant was described as “hypersensitive” when its growth under stress was similar to that of wild-type cells at ≥ 2 dilution factors lower. Mutant growth phenotypes under HU and MMS are individually summarized in Table 3.1. Abbreviations: *wt*, wild-type cells; HU, hydroxyurea; MMS, methyl methanesulfonate; R, the ratio in log2 scale from the SILAC analysis. Due to space limit, the systematic name was simplified, e.g., *SPAC17H9.10cΔ* was written as *C17H9.10cΔ*; and R was displayed with only one decimal place.

Table 3.1. Summary of growth phenotypes of the deletion mutants lacking one of the SILAC-enriched proteins.

Systematic name ^a	Gene symbol ^b	Ratio (log2) ^c	Growth with indicated stress			
			HU ^d (mM)		MMS ^e (%v/v)	
			5	8	0.005	0.01
SPAC17H9.10c ^f	<i>ddb1</i>	6.10	---	---	--	---
SPAC8F11.03	<i>msh3</i>	5.86	+	+	+	+
SPBC947.08c	<i>hip4*</i>	4.90	-	--	+	+
SPBC19C7.09c	<i>uve1</i>	4.83	+	+	+	+
SPCC1672.06c	<i>asp1</i>	4.44	-	--	+	-
SPAC13A11.06		4.34	-	-	-	-
SPBC3E7.02c	<i>hsp16</i>	4.12	+	+	+	-
SPBC530.05		3.90	+	+	+	+
SPBC3D6.02	<i>but2</i>	3.79	+	+	+	+
SPAC23D3.10c	<i>eng2</i>	3.56	+	-	+	+
SPAC1F3.09	<i>mug161</i>	3.54	+	+	+	+
SPAC27F1.06c		3.26	+	+	+	+
SPCC1223.02	<i>nmt1</i>	2.94	-	-	+	+
SPBC1685.02c	<i>rps1202</i>	2.93	+	-	+	+
SPCC736.13		2.45	+	+	+	+
SPAC3H1.07	<i>aru1</i>	2.18	+	-	-	-
SPCC1450.12		2.00	+	+	+	+
SPAC22A12.16		1.90	+	+	+	+
SPBC8D2.03c	<i>hhf2</i>	1.86	+	-	+	+
SPCC364.06	<i>nap1</i>	1.77	-	--	+	+
SPAC23H4.09	<i>cdb4</i>	1.68	+	-	+	+

Table 3.1. Summary of growth phenotypes of the deletion mutants lacking one of the SILAC-enriched proteins (continued).

Systematic name ^a	Gene symbol ^b	Ratio (log2) ^c	Growth with indicated stress			
			HU ^d (mM)		MMS ^e (%v/v)	
			5	8	0.005	0.01
SPBC1D7.04	<i>mlo3</i>	1.68	+	-	+	--
SPAC977.14c		1.61	-	--	+	+
SPAC9G1.05 ^g		1.58	-	---	+	-
SPBC16D10.08c	<i>hsp104</i>	1.57	+	+	-	-
SPBC646.13	<i>sds23</i>	1.44	+	-	+	-
SPBC216.05 ^{f,g}	<i>rad3</i>	1.42	---	---	----	---
SPCC1281.07c		1.32	+	+	+	+
SPAC26F1.05	<i>mug106</i>	1.28	+	+	+	+
SPAC57A7.04c	<i>pabp</i>	1.04	+	+	+	+

Note: ^a systematic name is used for proteins; ^b gene symbol is used for gene; ^c ratio of light and heavy protein levels in log2 scale; ^d hydroxyurea; ^e methyl methanesulfonate; ^f sensitivity to HU was detected in both SILAC and genome-wide analysis for corresponding deletion mutant; ^g sensitivity to MMS was detected in both SILAC and genome-wide analysis for corresponding deletion mutant. +, wild-type growth (not sensitive); -, growth at 1 dilution factor less than that of wild-type cells (sensitive); --&---, ≥ 2 dilution factors less than that of wild-type cells (hypersensitive). * gene symbol was assigned after this experiment.

To determine the sensitivity of deletion mutant strains to HU and MMS, we supplemented YES medium with two different concentrations of each drug, and monitored the cell's ability to form colonies at lower dilutions. As in our assay, 18 out of 30 mutant strains displayed sensitivity or hypersensitivity to at least one of the stress conditions (Figure 3.10, left panel). We compared our results with a genome-wide screening of fission yeast mutants sensitive against various DNA damaging agents (148). By screening a total number of 2662 mutants (i.e., 65% of the non-essential *S. pombe* genes), they have isolated 90

strains to be sensitive to either HU, or MMS, or both (Figure 3.10, right panel). If we only take the eight hypersensitive mutants (i.e., *rad3Δ*, *ddb1Δ*, *AC9G1.05Δ*, *asp1Δ*, *nap1Δ*, *BC947.08cΔ*, *AC977.14cΔ*, and *mlo3Δ*) into account, the occurrence of sensitivity in our analysis was still significantly higher than the background level (i.e., 26.7% versus 3.4%; p-value = 5.336e-06). Similarly, the occurrence of HU-hypersensitivity and dual-hypersensitivity (i.e., hypersensitivity to both HU and MMS) was extraordinarily higher than that in the genome-wide studies (Figure 3.11) (140, 148). Therefore, it may be conceivable that there was a greater chance of obtaining candidates related to DNA damage and repair from the SILAC-enriched proteins. Because there were only 6 candidates displaying exclusive hypersensitivity to MMS in the genome-wide study, it is reasonable that we were unable to isolate single mutant who was exclusively hypersensitive to MMS.

Figure 3.10. Venn diagram showing the occurrence of HU and MMS sensitivity in the SILAC analysis and in the genome-wide study.

The number of mutants displaying sensitivity to HU, MMS, or both is indicated. The total number of tested strains in SILAC analysis (left panel) or the genome-wide study (right panel) (148) is in the parentheses.

Figure 3.11. The frequency of obtaining candidates related to DNA damage and repair from the SILAC-enriched proteins is much higher than from the two genome-wide studies. The bar chart shows the frequency of deletion mutants that display hypersensitivity to HU only (HU-HS) or to both HU and MMS (Dual-HS) in SILAC analysis and the two genome-wide studies (140, 148).

We realized that majority of MMS-sensitive mutants were also found to be HU-sensitive, *hsp16Δ* and *hsp104Δ* were the only mutants exclusively sensitive to MMS (279). As a potent inhibitor of the enzyme ribonucleotide reductase (RNR), HU not only inhibits DNA replication, but also induces DNA damage (280), both of which activate the DNA integrity cell cycle checkpoints (150, 281). Similar situations were also observed in the study by Bimbo *et al.* (235), in which the HU-hypersensitive mutants also exhibited hypersensitivity to MMS.

In addition, we have only one mutant from each category in common with Deshpande *et al.*'s study (Table 3.1) (148). This may suggest a novel discovery of proteins involving in DNA damage and repair from SILAC analysis. Alternatively, the condition of large scale screening procedure was less sensitive than our smaller scale experiment.

Defective growth observed under a particular stress condition may imply that the mutant is potentially involved in the cellular response given to that stress. Our studies agreed that *rad3Δ* is a hypersensitive strain to both HU and MMS, consistent with the notion that Rad3 is involved in cell cycle arrest following DNA damage, as well as inhibition of DNA replication (282-284). Ddb1 is a UV-damaged DNA-binding protein implicated in nucleotide excision repair (NER) (285). Although *ddb1Δ* was not revealed as an HU-hypersensitive mutant in their screening, we confidently believed it is. Given that Ddb1 is required for degradation of the RNR inhibitor protein Spd1 (285, 286), we propose it is also responsible for degradation of HU. Worth mentioning, *AC9G1.05Δ* only showed hypersensitivity to higher concentration of MMS, but could not form colonies when subjected to 8 mM of HU. It was noted that the observed sensitivity from their study was generally lower than reported in the literature (148), which could explain why *AC9G1.05Δ* was not identified to be MMS-sensitive. Nevertheless, another genome-wide screening by barcode sequencing further supported our result that *AC9G1.05Δ* was sensitive to both HU and MMS (140).

Comparing with *AC9G1.05Δ*, *asp1Δ*, *nap1Δ*, *BC947.08cΔ* and *AC977.14cΔ* exhibited slightly weaker hypersensitivity to HU, whereas *asp1Δ* is the only one that showed sensitivity to MMS (Table 3.1). Ac9g1.05 and Asp1 are two related proteins associated with cortical actin cytoskeleton (287, 288). Studies in *Dictyostelium* mutant *AC9G1.05Δ* suggested that the protein is required for precise chromosome segregation and genomic stability maintenance (289-291). It is not clear how chromosome segregation is linked with DNA damage (or replication block) agents. Given that both mitotic

spindles and chromosomal DNA are in the nucleus and responsible for stabilizing genomic integrity, the stress factors may indicate an indirect effect to perturb the cell growth (235). Previous report also indicated that mutations in regulatory proteins of the actin network might be one consequence of genetic instability of malignant tumour cells (289). The mild variation of HU-sensitivity between these two mutants might reflect their importance in maintaining genomic stability. In *S. pombe*, the chromatin assembly factor Nap1 has been implicated in activation of DNA replication checkpoint (292), so that deletion of this protein leads to cell cycle arrest in the presence of HU. Bc947.08c is a putative protein, but it contains a region sharing low similarity with Hpc2 in *S. cerevisiae*. It is named Hip4 after being discovered as the fourth HIRA protein, which is responsible for mediating replication-independent nucleosome assembly (293). In contrast to the Bioneer mutant strain, our newly constructed mutant *hip4* Δ were found to be sensitive to both HU and MMS (Figure 4.1). Although the biological role of Ac977.14c in *S. pombe* is unknown yet, the aldo-keto reductase family members are found highly involved in DNA replication in human (294).

Different from the other 7 hypersensitive mutants, *mlo3* Δ lacks of a protein encoding an RNA annealing factor required for nuclear mRNA export (295). Its sensitivity to DNA damaging agents has been demonstrated by the alternative genome-wide screening (140). Mlo3 interacts with many CAPs for their functions, and its over-expression results in a complete failure of chromosome segregation, leading to abnormal mitosis. It has been demonstrated that both DNA and RNA could be the targets for most of deleterious DNA damaging agents, including alkylating agents, reactive oxygen

species (ROS) and UV-radiations (296). This indicates that not only DNA-binding proteins, but also RNA-binding proteins are highly associated with chromatin (264).

Ac13a11.06 shares a sequence homology with the budding yeast pyruvate decarboxylases THI3, which is responsible for thiamine biosynthesis (297). This protein binds to purine nucleotide for gene regulation (298). Herein, *AC13A11.06Δ* exhibited mild sensitivity to both HU and MMS. It is plausible that genes regulated by this protein are involved in maintaining genomic stability. Another thiamine synthase mutant *nmt1Δ* showed similar mild sensitivity to HU but MMS. *aru1Δ* resembled the behavior of *AC13A11.06Δ*, but to a lesser extent. Aru1 codes for a protein responsible for arginine catabolism (299). Recent study has suggested a chromatin-linked role of lysine synthase in DNA damage (300). The nuclear localization of Aru1 plus the extensive arginine modification on chromatin might be an interesting clue to seek a role independent of its catalytic activity. Nevertheless, whether these proteins are involved in DNA replication or DNA repair requires further clarification.

Sensitivity to MMS was detected in both *hsp104Δ* and *hsp16Δ*. When cells are exposed to DNA damage stresses, heat shock proteins (HSPs) are capable of stimulating DNA repair enzymes for their functions (301-303). Because Sds23 is required for normal DNA replication through phosphorylation by Cdc2 (304), it should be undeniable that disruption of this protein could lead to sensitivity to HU and MMS. Interestingly, Sds23 is also a potential regulator of M phase progression in *S. pombe* (305), further supporting its role in maintaining genomic stability.

Different from *hip4Δ*, in which the HU sensitivity was observable in both low and high concentrations; *hhf2Δ*, *cdb4Δ*, *eng2Δ* and *rps1202Δ* gradually manifested sensitivity when subjected to higher dosage (i.e., 10 mM).

As the core component of nucleosome, Hhf2 maintains the chromatin structure, responsible for DNA replication and repair and chromosomal stability (306-308). Although *hhf2Δ* did not show sensitivity to MMS in our study, the alternative screening via barcode sequencing isolated it as MMS-sensitive mutant (140). It is highly likely that the curved DNA-binding protein Cdb4 (309) is involved in DNA replication, as many reports have implicated the role of the curved DNA in DNA replication, recombination and transcription (310, 311). We are not sure why the cell wall mutant *eng2Δ* was sensitive to HU (312), but previous study reported that cell wall synthesis is one of the component for initiation of DNA replication (313). It is difficult to judge the role of Rps1202 in DNA replication, as this protein is more likely to be involved in translational activities (137).

According to the functions of the deleted gene products which showed HU and/or MMS sensitivity, 12 of them are responsible for chromatin-related process (Figure 3.12). The functions of the other six proteins sound irrelevant, which are amino acid metabolism, oxidation-reduction and cell wall catabolism.

On the contrary, the remaining mutant strains displayed a growth profile reminiscent of wild-type strain, suggesting that they are dispensable in DNA replication and repair. This does not mean they are trivial, as they could be responsible for other crucial biological activities, such as chromosome segregation and transcription. Indeed, we also found a few mutants (*BC530.05Δ* and *uve1Δ*) that were hypersensitive to the microtubule depolarizing drug TBZ

(data not shown), indicating the potential role of the corresponding proteins in mitosis. Besides, a handful of mutual-sensitive strains exhibited growth defects in response to TBZ as well, including *ddb1* Δ , *asp1* Δ , *aru1* Δ and *hsp104* Δ . Therefore, deletion of these genes may lead to a general perturbation of genomic integrity.

Figure 3.12. Pie chart of function categories of the SILAC-enriched proteins whose deletions lead to sensitivity to HU, or MMS, or both. Majority of these proteins have functions related to chromatin. The number for each category is indicated.

Taken together, we showed that the SILAC-enriched proteins ($\log_2^{\text{SILAC}} \text{ratio} \geq 1.0$) were extensively involved in DNA replication and repair. Our results could be a basis for the further characterization of their exact roles in maintaining genomic stability.

3.3. Discussion

In this study, we show that the top SILAC-ranked proteins are significantly depleted of highly abundant cytoplasm-localized proteins such as ribosomal subunits, implying that it is possible to identify non-histone CAPs *de*

novo in SILAC-mediated proteomic analysis of chromatin extract. Consistent with this, the top SILAC-ranked proteins are found to enrich for nucleus-localized proteins, the potential candidates associated with chromatin (177, 222). The top SILAC-ranked proteins are also found to enrich for chromatin template functions, especially transcription, chromatin modification, DNA replication and repair. Besides, the chromatin association of a few selected top SILAC-ranked proteins were validated by chromatin fractionation assay. Furthermore, SILAC-enriched nucleus-localized proteins appear to be more likely to play a role in growth fitness under DNA damage stress than do the SILAC-depleted nucleus-localized ones. Based on the plating assay results, some of them are responsible for maintaining genomic stability. Hence, SILAC-mediated proteomic analysis allows identification of non-histone CAPs without prior knowledge.

We show, in this study, that Psm3, Cbh2, and Ac27f1.06c that are involved in chromosome organization and chromatin remodelling; Ddb1, Msh3, Spp1, and Uve1 that are involved in DNA replication and repair, and Eri1, Hip4, Rpb1, and Bc530.05 that are involved in transcription are found in the top SILAC-ranked proteins (see file 3 in DVD-R). These proteins represent a small subset of non-histone CAPs that are relatively soluble, abundant, and constitutively associated with the chromatin. Deep analysis of peptides in chromatin extract using SILAC-mediated proteomics should allow identifying more proteins with CAP-related functions without prior knowledge. Due to the inaccessibility of non-histone CAPs, DNase I was utilized to separate chromatin from the cell extract. It is known that most transcription factor binding events

take place in a DNase I accessible chromatin regions; this is consistent with our result that transcription factor is the most enriched category.

We also find Prp16, Smb1, and Mug161 that are involved in mRNA splicing and Ubr1, Bp8b7.11, and Rpt2 that are involved in proteolysis in the top SILAC-ranked proteins. In fact, it is not unusual to find components of the proteolysis machinery that are associated with the chromatin. For instance, the ubiquitin E3 ligase Pcu4 is found to be associated with the RNA-induced transcriptional silencing (RITS) complex involved in heterochromatin assembly (314); and the E3 ligase Ubr1 is associated with the Set1 complex involved in histone H3 methylation (315, 316). On the other hand, proteins responsible for mRNA splicing have been found to be assembled into the co-transcriptional spliceosome on chromosome (317). Hence, our analysis identifies the *bona fide* non-histone CAPs that play a role in proteolysis and mRNA splicing.

Not all nucleus-localized proteins are candidate of non-histone CAPs. For example, proteins involved in nuclear transport do not require physical interaction with the chromatin for function. In this study, we find that some nucleus-localized proteins are depleted in SILAC analysis. By using the chromatin fractionation assay, we confirm that the nucleus-localized karyopherin Kap95 (251) and mRNA export factor Srp2 (278) are indeed not associated with chromatin. Therefore, SILAC-depleted nucleus-localized proteins are truly unassociated with chromatin based on our analysis. We claim they are unlikely to be false negatives.

Among the nucleus-localized proteins, the SILAC-enriched ones are shown to exhibit higher likelihood of requirement for growth fitness under MMS stress than do the SILAC-depleted ones. This difference is not apparent

among the dual-localized or cytoplasm-localized proteins. It implies that most proteins involved in DNA damage and repair are likely to be exclusively localized in the nucleus.

Although the dual-localized proteins are not overrepresented in the top 10% most enriched proteins, three SILAC-enriched dual localized proteins are found to be truly associated with chromatin by Western blotting. Therefore, the dual-localized proteins are complex: they may or may not be non-histone CAPs. It is understandable that the cytoplasm-localized proteins cannot be non-histone CAP candidates, as they have to be localized in nucleus in association with chromatin. That is why they are not overrepresented in the top SILAC-ranked proteins in chromatin extract.

We also show proteins involved in translation are significantly reduced in the top 10% SILAC-enriched proteins in chromatin extract. However, it was not completely depleted. This is probably that highly abundant nucleolus-localized proteins are hard to be removed from chromatin (318). Consistent with this, majority of the proteins identified in a proteomic analysis of the chromatin-associated complex using TAP-tagging are ribosomal proteins (259, 266). Hence, based on the absolute abundance, it is hard to separate the non-histone CAP candidates from the highly abundant ribosomal proteins (see file 6 in DVD-R).

Determining the effect of gene deletions is a fundamental approach to understanding gene functions. Comparing with the genome-wide analysis (148), we find that there is an over four-fold increased chance of obtaining proteins potentially mediating DNA replication and repair through the deletion analysis of the SILAC-enriched proteins [$\log_2^{\text{SILAC Ratio}} \geq 1.0$]. The protein functions

such as DNA replication and repair (Rad3, Ddb1, Sds23 and Cdb4), chromatin assembly (Hip4, Nap1 and Hhf2), cell division (Ac9g1.05, Asp1 and mlo3), and stress response (Hsp16 and Hsp104) are frequently observed, indicating our assay is valid. It is unlikely that proteins involved in amino acid metabolism (Ac13a11.06, aru1) and oxidation-reduction process (Ac977.14c) are associated with chromatin. Nevertheless, the sensitivity might indicate a role independent of their enzymatic activity. For example, recent study has suggested a chromatin-linked role of lysine synthase in DNA damage (300).

We have only three mutants in common (i.e., *rad3Δ*, *ddb1Δ* and *AC9G1.05Δ*) with the genome-wide screening (148). All of them are extremely hypersensitive (i.e., “---”) to DNA damaging agents. In our case, majority of the mutants only display sensitivity to higher concentration of the drugs; it is understandable that they are not isolated from the relatively insensitive large scale study. Different degree of sensitivity might reflect the hierarchy of the proteins in maintaining genomic stability, or due to their contribution to retain an integrated chromatin environment. Unravelling the functions of these proteins might provide mechanistic insights into the mechanism of maintaining the genomic stability in human.

Proper preparation of chromatin fraction is crucial for achieving high-quality results. Many proteins, including DNA and RNA polymerases, chromatin remodelers, and transcription factors, are tightly bound by chromatin structure. They are transiently expressed, or in low abundance, or difficult to extract from nucleus. Furthermore, no single purification method is perfectly fit for chromatin extraction. Chromatin fractionation assay results in a fraction of enriched chromatin-associated components which are present in the insoluble

nuclear portion, but not the soluble cytoplasmic portion of the cell. In fission yeast, the first critical challenge of this assay is the preparation of spheroplasts. If zymolyase treatment is incomplete, many cells will fail to be lysed properly and nuclei will remain within cell debris; so that the chromatin will not be possible to be solubilised later. If the zymolyase treatment is excessive, many cells will lyse before addition of detergent and nuclear morphology will be affected, which will lead to leakage of loosely bound non-histone CAPs. Therefore, the timing has to be controlled tightly by frequent microscopic observation of the spheroplasted cells. The rule of thumb is to stop the zymolyase treatment until about 90% of cells were lysed. To assess the quality of spheroplasting, we routinely conducted DAPI staining of the lysed cells and observed the nuclear morphology under microscope (Figure B.4A). In a good preparation of spheroplasts, most of the nuclei should be intact and out of the cell debris, although a few of them may remain associated with each other. If the chromatin is underdigested, the nuclei will still be inside the cell remnant and remain intact; if the chromatin is overdigested, the nuclei will be swollen, or appear as threads of DNA. Western blotting and MS-based proteomics suggest that prolonged enzymatic digestion will also cause protein degradation (Figure B.4B&C). The spheroplasts from enzymatic digestion were lysed by addition of 1% TX-100. This amount of detergent is sufficient to lyse the nuclear membrane without significantly disrupting the chromatin, provided that spheroplasting has been well controlled. The washing after detergent lysis is crucial to remove residual cytoplasmic proteins. The second challenge is the activity of endogenous nucleases. Under-digestion of the cells leads to poor release of proteins from chromatin, and majority of the CAPs still remain bound

with chromatin DNA. Over-digestion of the cells leads to the activation of endogenous nucleases and DNA degradation. Given that the efficiency of DNase I can vary greatly among different samples, titration of DNase I is important before carrying out this reaction.

It is understandable that ribosomal proteins are still highly abundant in the second top 10% SILAC-ratio ranked proteins. This is mainly due to its absolute high abundance in the whole proteome. Furthermore, ribosomal proteins are imported into the nucleus and assembled into subunits in the nucleolus; although the contributions are trivial. To alleviate the severe contamination from ribosomal proteins, we should carefully assess the protein pattern of chromatin extract by running SDS-PAGE and coomassie blue staining. By comparing the protein bands with those extracted from ribosome isolation, we should be able to check whether the ribosomal proteins are successfully removed from the chromatin extract; and decide whether we should proceed with downstream proteomic analysis, which is much more time-consuming and labor-intensive. On the other hand, ribosomal proteins can be partially removed through differential centrifugation, and sedimentation by sucrose cushion is most commonly used to separate chromatin-enriched fraction from the nucleolus.

We noted that chromo-domain or bromo-domain containing proteins are rarely identified in our SILAC analysis. This is because they are highly insoluble to be extracted and require high salt concentration (211), which is more favourable to disrupt the chromatin DNA structures (158). Nevertheless, previous study designed to profile Pol II-interacting proteins has failed to identify any known transcription or elongation factors, which illustrates the

limitations of the high salt extraction in characterizing chromatin-related protein complexes (319). Modification of chromatin extract preparation and improvement of SILAC proteomic analysis should allow identification of the chromo-domain and bromo-domain containing proteins in future studies. Nuclease digestion is routinely used to assess chromatin binding, while low salt might be too mild to dissociate the protein complexes which are tightly bound with chromatin. We propose that by combining DNase I digestion with high salt extraction in chromatin fraction preparation, less soluble non-histone CAPs could also be obtained without disrupting lower affinity protein-protein interaction. Alternatively, these proteins could have escaped the detection in LC-MS/MS due to their relatively low abundance and high level of interference from the cytoplasm-localized abundant proteins. This can be improved by better chromatographic separation of the trypsinized peptides using a longer column or Ultra performance liquid chromatography (UPLC) separation before mass spectrometric analysis. Nevertheless, some CAPs are extremely insoluble; neither DNase I treatment nor high salt extraction can release them into soluble fraction. For example, Mcm10, an essential CAP involved in the initiation of DNA replication in both fission yeast (320) and budding yeast (321), which is unable to be identified using chromatin fractionation assay.

We realize that the number of quantifiable peptides is relatively low in our SILAC analysis. To circumvent the arginine conversion problem, we omitted arginine labelling. Arginine has three protons whereas lysine has only one, and thus, arginine gains much better ionization than lysine in the mass spectrometer (277). Although the recently reported method solves the problem of arg-pro interconversion (275), making the genetically engineered

auxotrophic strain is a daunting task. With the development of quantitative proteomic pipeline (322), ^{15}N labelling becomes realizable. According to our preliminary data using ^{15}N labelling (see Figure B.5, Figure B.6 and Table B.1), six generations is sufficient to incorporate ^{15}N into the whole proteome, which is compatible with SILAC labelling. This is further validated by mixing the “light” and “heavy” protein samples into various ratios. And the high correlation between the expected and the observed ratios in pre-mixed samples emphasized the high accuracy and precision of our analysis. As a result, this approach is entirely applicable to the fission yeast *S. pombe*, which has never been reported before. Because ^{15}N labelling is performed on the autotrophic strain, there is no need to genetically modify the strain, and the cells can grow dramatically faster than the auxotrophic ones. Furthermore, ^{15}N labelling is much more cost-effective than SILAC labelling (201).

Underdigested fission yeast cell wall results in different protein identifications from overdigested ones (Figure B.4C), so that hampers accurate estimation of protein abundance between chromatin fraction and WCE. Given that fission yeast cells have strong cell walls, it is difficult to get even spheroplasting in different samples. To circumvent this problem, we should switch to other organisms in which spheroplasting is not required for chromatin extraction. Recently, MS-based quantitative proteomics has provided a high throughput approach to measure the subcellular distribution of human proteome (323). This spatial proteomic approach has been applied to measure the relative distribution of proteome between cytoplasm, nucleus, and nucleolus in human colon carcinoma cell line HCT116 using SILAC. From the identified 2,000 proteins, they demonstrate that only a small subset of proteins are equally

distributed between two or more compartments, and majority of them are predominantly localized to specific subcellular localizations (323). On the one hand, this study supports the idea that quantitative proteomics are more suitable for identifying proteins associated with a particular subcellular compartment. On the other hand, it indicates that mammalian cell lines could be better models to study non-histone CAPs. Knowing that human cell lines are better choice for chromatin analysis, it is worth of performing chromatin fraction assay on mammalian cells, and the results should be more reproducible than using fission yeast. Recently, a similar study coupling chromatin extraction to MS-based proteomics has been conducted in HeLa S3 cells (224), and it provides a valuable source to evaluate the quality and fidelity of chromatin fractionation assay. By incorporating quantitative approach like SILAC-based chromatin proteomics into this study, we should be able to identify more non-histone CAPs with fewer improbable CAP contaminations. Furthermore, the identified proteins would be more vital to human health.

In summary, we show that the SILAC-mediated proteomic approach is capable of determining non-histone CAPs without prior knowledge. We propose this method is complementary to the proteomic analysis of protein complexes purified via ChIP with TAP-tagged CAPs for identification of CAP-interacting partners. Generally speaking, this study has provided a platform for selecting non-histone CAPs that potentially contribute to maintaining genomic stability and chromatin organization. In-depth structural and functional characterization of these novel non-histone CAPs would broaden our knowledge about chromatin. They may even turn out to be important therapeutic drug targets for devastating human diseases in future.

CHAPTER 4. PHENOTYPIC ANALYSIS OF HIRA PROTEIN HIP4

4.1. Introduction

HIRA proteins are evolutionarily conserved histone chaperones that function in multi-subunit complexes to mediate replication-independent nucleosome assembly and regulate chromatin structure (29, 324, 325). Hip4 is a new subunit of HIRA complex in *S. pombe* (326). Besides Hip1, Slm9, and Hip3, Hip4 is recently found to be the forth HIRA protein, and named after HIRA interacting protein 4 (326). Hip4 contains a conserved Hpc2-related domain (HRD), which is a characteristic of the budding yeast and human HIRA proteins, Hpc2 and UBN1. This domain is responsible for mediating the interaction with the other members of the protein complex (34).

The importance for HIRA in modulating chromatin structure is well studied by its requirement in maintaining heterochromatin silencing. In *S. cerevisiae*, HIRA genes are required for position-dependent gene silencing in the absence of the histone chaperone CAF-1 (327). Roles for HIRA proteins in the maintenance of heterochromatin have also been reported in *S. pombe* (139, 293). Hip1 and Slm9 are responsible for maintaining the integrity of heterochromatin at centromeres (328). Mutation of either protein alleviates the silencing of marker genes located in centromeric outer repeats and leads to increased levels of chromosome missegregation. Hip4 is also responsible for the integrity of heterochromatin at multiple loci (293). Recent finding suggests

that loss of Hip4 function alleviated the heterochromatin silencing of reporter genes located in the mating-type (MAT) locus and centromeres. Furthermore, it is required for the distinct form of silencing that represses the expression of Tf2 long terminal repeat (LTR) retrotransposons (293).

The actions of HIRA complex are important for DNA damage resistance. It is reported that HIRA is important in regulating G₂/M transition of mitotic cell cycle (328, 329). For example, deletion of Hip1 and Slm9 results in cell cycle delay at G₂/M transition, in which the best-characterized cell cycle checkpoint is the DNA damage checkpoint. The G₂/M DNA damage checkpoint inhibits mitosis through phosphorylation of a conserved tyrosine residue Tyr15 on Cdc2, which is largely conserved from fission yeast to human (150, 330-332). With normal feedback control, the level of Tyr15 phosphorylation decreases in mitosis; once DNA damage is encountered, Cdc2 will be phosphorylated at Tyr15, resulting in inhibition of its activity, and thus, halt of entry into mitosis (333, 334). The level of Tyr15 phosphorylation is upregulated by the Wee1 and Mik1 protein kinases and downregulated by the Cdc25 protein phosphatase (48, 335). The checkpoint kinase Rad3 activates Chk1, which further activates Wee1 and Mik1 tyrosine kinases and inactivates Cdc25 tyrosine phosphatase (85-88), leading to inactivation of Cdc2 via Tyr15 phosphorylation (89-91). Mutants defective in phosphorylation of Cdc2 at Tyr15 are sensitive to both MMS and UV irradiation and enter lethal premature mitosis with damaged DNA (89-91). It is known that deletion of *S. cerevisiae* HIRA subunits exhibit an increased rate of chromosome missegregation and chromosome loss, and render cells more susceptible to MMS (327). Therefore,

HIRA proteins are indispensable for maintenance of genomic integrity and regulation of cell cycle (336).

The mechanism of HIRA proteins in modification of chromatin structures is critical for maintaining genomic stability (337). In eukaryotic cells, the genome integrity is continuously challenged by DNA damaging agents, and the chromatin structure is disrupted upon DNA damage and needs to be restored following DNA repair. HIRA proteins have been implicated in DNA repair-based mechanism, presumably through rearranging chromatin structure by histone replacement (338). As such, HIRA proteins may have biphasic functions to act both as cell cycle regulators and as nucleosome assembly factors to fight against DNA damage and restore normal chromatin structure.

Hip4 has been ranked as the 14th most enriched protein in our SILAC-mediated proteomic analysis (file 3 in DVD-R, its SILAC ratio was 4.90 in log₂ scale). The Bioneer deletion strain lacking Hip4 showed sensitivity to DNA replication inhibitor HU (see Figure 3.9), indicating a protective role of this protein in DNA replication. However, the phenotype of the Bioneer deletion strain was not reproducible in our newly constructed mutants, which showed hypersensitivity to DNA damaging agent MMS, but only mild sensitivity to HU. In this study, we report the phenotypic characterization of Hip4, suggesting it is important for maintaining genomic stability. The elongated cell shape and growth inhibition against microtubule inhibitors indicate its requirement in chromosome segregation. Genetic analysis suggests that Hip4 is epistatic to the DNA damage checkpoint kinase Rad3. Given that HRD-containing proteins are highly conserved throughout evolution (339, 340) and responsible for

nucleosome assembly, further characterization of Hip4 will provide mechanistic insights into the organization of chromatin architecture in eukaryotic cells.

4.2. Results

4.2.1. *hip4*⁺ is required for DNA damage resistance

Our previous data suggested that *hip4*⁺ deletion strain in Bioneer collection was sensitive to the RNR inhibitor HU, and showed resistance to DNA methylating agent MMS (see Figure 3.9). To confirm these results, the deletion mutant was newly constructed in both mating types (*h*⁻ and *h*⁺), and the plating assay was repeated. *rad3Δ*, *cds1Δ*, and *chk1Δ* were included as positive controls to validate this assay, as all of them are hypersensitive to HU. By contrast, *cds1Δ* cells are not sensitive to DNA damage including MMS (341). Rep2 is a transcriptional activator that regulates transcription in G1/S phase of the cell cycle (342), and its deletion mutant shows intermediate sensitivity to HU and MMS (140, 343). As shown in Figure 4.1A, *hip4Δ* and *rep2Δ* had similar sensitivity to HU, but *hip4Δ* was more susceptible to MMS than *rep2Δ*. In other words, *hip4*⁺-null mutant was quite sensitive to MMS, but exhibited a relatively mild increase in sensitivity to HU. This might suggest that *hip4Δ* is important for protection against DNA damaging agents rather than DNA replication stress. The discrepancy with previous results indicated that the Bioneer mutant strains may have other background mutations. Moreover, *hip4Δ* cells turned out to be extremely sensitive to the UV-mimetic agent 4-NQO (Figure 4.1B). Further testing also discovered its hypersensitivity to the DNA enzyme topoisomerase I (topo I) inhibitor CPT (Figure 4.1C). Growth assay in

liquid culture agreed very well with plating assay (Table 4.1). The results indicated that *hip4*⁺-null strain did not affect growth rate in HU. On the contrary, *hip4*Δ had reduced growth, took a significantly longer time to reach stationary phase in MMS and 4-NQO compared to the wild-type cells. Taken together, these results indicated that loss of *hip4*⁺ compromised growth in the presence of DNA damaging agents. We hypothesize that Hip4 may be required for proper functioning of the G2-phase DNA damage checkpoint caused by genomic insults in the absence of *hip4*⁺ (150).

Figure 4.1. *hip4Δ* cells are sensitive to DNA damaging agents. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with the indicated genotoxic agent for growing 4-5 days at 30°C. The cell densities from left to right are 10^5 , 10^4 , 10^3 and 10^2 , labelled as 1, 2, 3, and 4, accordingly. (A) The indicated strains were treated with HU and MMS. (B) The indicated strains were treated with 4-NQO as described. (C) The indicated strains were treated with CPT as described. *wt*, wild-type; HU, hydroxyurea; MMS, methyl methanesulfonate; 4-NQO, 4-Nitroquinoline 1-oxide; CPT, camptothecin.

Table 4.1. Comparison of the cell growth between wild-type and *hip4Δ* cells in liquid culture containing the indicated genotoxic agents.

Treatment condition	ΔT_{50} of growth curve ^a (hr)		p-value ^b
	<i>wt</i>	<i>hip4Δ</i>	
5 mM HU	2.40±0.33	2.11±0.08	0.270 (>0.05)
0.005% (v/v) MMS	1.92±0.33	3.17±0.14	0.012
0.2 μM 4-NQO	2.79±0.30	4.95±0.46	0.004

Note: ^a the time at the half-maximal cell concentration based on the growth curve using the Mini-growth culture system. The ΔT_{50} was calculated based on the triplicates, and was shown in terms of average±standard deviation (SD). ^b p-value for differences between *wt* and *hip4Δ* cells with various treatment conditions. *wt*, wild-type; HU, hydroxyurea; MMS, methyl methanesulfonate; 4-NQO, 4-Nitroquinoline 1-oxide.

4.2.2. Disruption of *hip4*⁺ results in growth defects and sensitivity to spindle damage

Given that *hip4Δ* had universal defects to the mutagenic stresses, further characterizations were done to test whether loss of *Hip4*⁺ would lead to any detectable growth defects. Although the impaired cell growth of *hip4Δ* at 30°C was not obvious in serial dilution spot assays (Figure 4.2A), we observed a distinctive slower growth rate of the mutant than that of wild-type via growth assay in liquid culture [7.79 h versus 9.66 h for mean value of T_{50} between *wt* and *hip4Δ*, p-value = 0.005466 (< 0.01)] (Figure 4.2B). Besides, *hip4Δ* cells were temperature-sensitive, having extremely low viability at both warm (i.e., 36°C) and cold conditions (i.e., 20°C, 18°C and 15°C) (Figure 4.2A). Its viability was also extensively decreased following transition from 30°C into 36°C (Figure 4.2C). The temperature-sensitive phenotype of *hip4Δ* might be an indication of cell cycle delay (328, 329), which suggested a possible defect in cell cycle progression.

To further characterize the function of *hip4*⁺, the mutant cells were examined under microscope. As expected, *hip4Δ* cells revealed an elongated phenotype in exponential phase (10.08 ± 2.30 μm versus 14.88 ± 3.37 μm; p-value = 5.04×10^{-24}) (Figure 4.3), which indicated a severe G2 cell cycle delay (139, 329). The mutant also manifested cell elongation after reaching stationary phase, implicating an aberrant cell division (data not shown). The slow growth and elongated cell shape suggested a possible role of *hip4*⁺ in chromosome segregation.

Figure 4.2. *hip4Δ* cells display growth defects. (A) The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar plates for growing 4 days at 30 and 36°C; or 7 days at 20, 18 and 15°C. The cell densities from left to right are 10^5 , 10^4 , 10^3 and 10^2 , labelled as 1, 2, 3, and 4, accordingly. (B) Comparison of the cell growth between wild-type (*wt*) and *hip4Δ* cells in liquid culture at 30°C using Mini-growth culture system. The liquid culture was incubated at 30°C with continuous shaking, and the OD₆₀₀ was automatically recorded every 17 min during a 48 h period. The growth curve is the average value of triplicates, *T*₅₀ (the time at the half-maximal cell concentration) of *wt* and *hip4Δ* cells are indicated. (C) Comparison of the cell growth between *wt* and *hip4Δ* cells in liquid culture after transition from 30°C to 36°C (left). The cell growth at 30°C (right) was shown for comparison. Growth was monitored by measuring OD₆₀₀ every 30 min for a 5-h period.

Figure 4.3. *hip4Δ* cells display elongated cell morphology. Comparison of the cell morphology between *wt* and *hip4Δ* in mid-log phase. Cells were grown at 30°C for 5 h to mid-log phase. Cell length was calculated from 100 cells, and is indicated in terms of mean±standard deviation (SD) at the bottom of the micrograph. Bar, 10μm.

We therefore investigated its sensitivity to spindle damage by using the microtubule-depolymerizing drug TBZ and the microtubule inhibitor MBC. The two mitotic spindle checkpoint mutants, *mad2Δ* and *bub1Δ*, were included as positive controls. The *hip4Δ* cells displayed remarkable hypersensitivity to both drugs, and the defect was even more severe than the spindle checkpoint mutant *mad2Δ* (Figure 4.4). These results implied that *hip4⁺* was required for accurate chromosome segregation. The phenotypes of elongated cell shape and aberrant chromosome segregation in *hip4⁺*-deficient cells are shared by all other HIRA complex members (139, 328).

Figure 4.4. *hip4Δ* cells are hypersensitive to spindle damaging agents. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar plates supplemented with DMSO as control, or with the indicated genotoxic agent for growing 4-5 days at 30°C. The cell densities from left to right are

10⁵, 10⁴, 10³ and 10², labelled as 1, 2, 3, and 4, accordingly. TBZ, thiabendazole; MBC, methyl benzimidazol-2-yl carbamate.

4.2.3. Hip4 is localized to the nucleus and associated with chromatin

Given that *Hip4*⁺ was found to be responsible for DNA damage resistance and chromosome segregation, these functions might require Hip4 protein to be preferentially localized to the nucleus and interact with chromatin. The endogenous *hip4*⁺ was tagged with HA, and the indirect immunofluorescence microscopy validated that Hip4 was located in the nucleus (Figure 4.5A) (251). This result also ascertained that the subcellular localization of Hip4 was not altered by the HA epitopes. Western blotting further confirmed its protein expression (Figure 4.5B). Interestingly, there were two visible bands, indicating possible modification of this protein, probably phosphorylation. We performed immunoprecipitation assays of the HA-tagged fusion protein using anti-HA resin or Ni-NTA agarose (data not shown). Both methods were able to precipitate these two bands, suggesting that both bands should belong to Hip4. After ensuring that the strain was fully functional, we wanted to test whether this nucleus-localized protein is associated with chromatin or not. By using the chromatin fractionation assay, majority of Hip4 was found in the chromatin fraction, just similar to histone H4, but opposite to β -tubulin (TAT1) (Figure 4.5C). The minute amount of Hip4 in the soluble fraction may suggest its minor partition in cytoplasm, although we did not observe corresponding pattern in the microscopic analysis. In short, Hip4 is predominantly localized to the nucleus and associated with chromatin.

Figure 4.5. Hip4 is localized to the nucleus and associated with chromatin. (A) Hip4 is localized to the nucleus. The endogenous *hip4*⁺ was tagged with HA, and the cells were subjected to indirect immunofluorescence microscopy, the nuclear DNA was stained with DAPI. The superimposed signal indicates the protein is localized to the nucleus. (B) Western blotting was performed to confirm the expression of Hip4 using anti-HA antibody. “+”, Hip4 tagged with HA; “-”, wild-type without tagging. (C) Chromatin fractionation assay was conducted to ensure the chromatin association of Hip4. Equal volume of T (whole cell extract), S1 (Supernatant 1) and five volumes of C (Chromatin) was loaded onto the SDS-PAGE for detection of Hip4, histone H4 and β -tubulin (TAT1) by Western blotting. The ratio of protein loading amount is 4:1:1.

4.2.4. Hip4 is not responsible for G2/M DNA damage checkpoint

In eukaryotic cells, the G2/M DNA damage checkpoint prevents the initiation of mitosis until the lesion is repaired. Key regulator of this transition in fission yeast is the conserved cyclinB/Cdc2 complex (135, 344). Upon DNA damage, activation of this complex and entry into mitosis is inhibited by Wee1 kinase, which negatively regulates Cdc2 protein kinase by phosphorylation of its Tyr15 residue. In fission yeast, Tyr15 is the single detectable

phosphorylation site in Cdc2 (79). Previous results showed that *hip4Δ* cells were highly vulnerable to DNA damage. Therefore, we were interested to know whether Hip4 plays a protective role in the G₂/M DNA damage checkpoint. We hypothesized that DNA damage in *hip4*⁺-null mutants resulted in dysfunctional DNA damage checkpoint, so that the mitotic entry was not blocked by inhibitory phosphorylation of Cdc2. In other words, the amount of phosphorylated Cdc2 would not change.

To address this question, both WT and *hip4Δ* were treated with 0.01% (v/v) MMS for four hours, cell lysates were extracted, and the protein level of phosphorylated Cdc2 was accessed in one-hour interval. We observed an elevated protein level of Cdc2-Y15-p in wild-type cells (Figure 4.6, left panel). This is because the G₂/M DNA damage checkpoint remains intact and functional in normal cells. Similar to the wild-type cells, Cdc2-Y15-p was continuously accumulated in *hip4Δ* along MMS treatment (Figure 4.6, right panel). This result indicated that Hip4 might play a dispensable role in Tyr15 phosphorylation of Cdc2. The protein accumulation suggested that cells lacking Hip4 could still properly recognize and arrest in response to DNA damage.

In eukaryotic cells, DNA replication strictly relies on an intact DNA template, and this process is especially vulnerable to the lesions such as UV radiation, which may lead to mutations or even cell death (345). To avoid these detrimental consequences, cells have evolved to rapidly respond to the presence of DNA lesions and adjust the replication problems; and eventually determine to resume the replication or undergo programmed cell death (148). To check whether Hip4 plays a role in the slowing of replication in response to DNA damage, we also examined the requirement of Hip4 in the fission yeast S-phase

DNA damage checkpoint. Both wild-type and *hip4Δ* cells were treated with 0.01% MMS, and the progress of replication was monitored by flow cytometry. The *hip4Δ* culture had only 2C peak at first 30 min, it started to have less than 2C DNA content after 1 h, and lasted in the following 3 h (Figure 4.7). Similar results could be observed in the MMS-treated wild-type cells, indicating that both cells have not finished replication by the time they divide. This result may suggest that the mutant cells were capable of slowing down replication in response to DNA damage, and the S-phase DNA damage checkpoint was unaffected in the absence of Hip4.

Figure 4.6. Comparison of the phosphorylated Cdc2-Tyr15 protein level between wild-type and *hip4Δ* cells.

The cells were treated with 0.01% MMS for 4 h, and samples were collected at indicated times. Total cell lysates were prepared, and equal amount of proteins were loaded in each lane. Protein levels were analyzed by Western blotting using a polyclonal phospho-specific anti-Tyr15 antibody (Cdc2-Y15-p) and a polyclonal Cdc2 antibody (PSTAIRE), and quantified using *ImageJ* software.

Figure 4.7. FACS analysis of S-phase DNA damage checkpoint in *hip4Δ*. Both wild-type and mutant cells were grown to mid-log phase and treated with 0.01% (v/v) MMS. Cells were collected at the time indicated and subjected to ethanol fixation and DNA content measurement. 1C, 1C DNA content; 2C, 2C DNA content.

In *S. pombe*, DNA damage initiates the activation of a signal transduction cascade mediated by Rad3. The downstream effector kinase Chk1 and Cds1 are activated by the phosphorylation activity of Rad3 to govern the G2/M transition in response to DNA damage (60, 62, 144). To investigate the genetic interactions of *hip4*⁺ with *rad3*⁺, *cds1*⁺ and *chk1*⁺, we constructed the double deletion mutants *hip4Δrad3Δ*, *hip4Δcds1Δ*, and *hip4Δchk1Δ*. Because *cds1Δ* is not sensitive to MMS, the hypersensitivity of *hip4Δcds1Δ* to MMS was solely due to deletion of *hip4*⁺. The examination of *hip4Δrad3Δ* and *hip4Δchk1Δ* suggested that they had growth defects that were not obviously more severe than those of the parental strains under MMS stress (Figure 4.8).

This might implicate that mutations in *hip4*⁺, *rad3*⁺, and *chk1*⁺ are epistatic, and Hip4 may function in the same pathway with Rad3 and Chk1.

Figure 4.8. Phenotypic analysis of double deletion mutants in response to DNA damaging agent MMS.

The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions from left to right (the cell densities from left to right are 10^5 , 10^4 , 10^3 and 10^2 , labelled as 1, 2, 3, and 4, accordingly), and spotted onto YES agar plates supplemented without or with various concentrations of MMS for growing 5 days at 30°C. MMS, methyl methanesulfonate.

To check whether overexpression of *hip4*⁺ can rescue the MMS-induced growth defects, we introduced two different plasmids ectopically expressing *hip4*⁺ (pREP1-*hip4*⁺ and pREP41-*hip4*⁺) into those double deletion mutants (see Materials and method). RE digestion suggested that orientation of the inserts was correct (Figure 4.9). The coding sequences were verified by sequencing (B.4). Furthermore, the introduction of the plasmids rescued the elongated cell phenotype of *hip4*⁺ null mutant cells, suggesting the plasmids are functional (Figure 4.10, left panels). A slightly milder compensatory effect was observed in *nmt41-hip4*⁺-containing *hip4*Δ, which is coherent with the varied strength of the promoters (241). On the contrary, the long cell phenotype was similar to that in *hip4*Δ if the promoters were turned off (Figure 4.10, right panels).

Figure 4.9. Validation of the expression vectors pREP1-*hip4*⁺ and pREP41-*hip4*⁺. RE digestion of pREP1-*hip4*⁺ (A) and pREP41-*hip4*⁺ (B) using two pairs of restriction enzymes *Clal/BamHI* (upper panels) and *SalI/BamHI* (bottom panels). The numbers (lane 1-10 of left panels or lane 1-3 of right panels) indicate the bacterial colonies with successful ligation of pREP1-*hip4*⁺ or pREP41-*hip4*⁺. The undigested vectors pREP1 and pREP41 (lane U), the digested empty vectors pREP1 and pREP41 (lane E), and purified PCR product (lane P) are indicated. 1 kb ladder (M) was used for all the electrophoresis, 3 kb, 1 kb and 500 bp are indicated.

We then introduced these plasmids into the double deletion strains and conducted the sensitivity assay to MMS. According to the results, the growth defects of *hip4Δrad3Δ* in MMS could not be compensated by overexpressing *hip4*⁺ (Figure 4.11). This result might suggest that the Hip4 functions independent of Rad3. Surprisingly, *hip4Δrad3Δ* containing pREP1-*hip4*⁺ had better growth in response to 0.00125% MMS when the promoter was turned off. Similar phenomenon was observed by *nmt41* promoter-induced overexpression of *hip4*⁺ in the presence of 0.0025% MMS. This might be due to the presence of thiamine, which is able to promote cell proliferation. Furthermore, there was no difference of cellular resistance to MMS in *hip4Δcds1Δ* before and after the promoter was induced.

Figure 4.10. Functional examination of the expression vectors pREP1-*hip4*⁺ and pREP41-*hip4*⁺. Microscopic analysis of phenotype rescue by introducing pREP1-*hip4*⁺ and pREP41-*hip4*⁺ into *hip4*⁺-null mutant. The plasmids were cut at *Xba*I site, and transformed into *hip4*⁺-null mutant using lithium acetate method. Cells containing the corresponding constructs were grown to mid-log phase and shifted into thiamine-free media (left panels) or media containing 15 μ M thiamine (THM) (right panels) to either induce or repress *nmt1-hip4*⁺ (or *nmt41-hip4*⁺). The *hip4* Δ cells are able to recover from the elongated cell phenotype by stimulating the *nmt* promoters. Bar, 10 μ m.

Therefore, although Hip4 was required for DNA damage resistance, its deletion did not impede the functioning of G2/M DNA damage checkpoint. Based on our results, Hip4 was doubtful to interact with the cell cycle checkpoint kinases, and it might protect cells from DNA damage through the mechanism independent of cell cycle control.

Figure 4.11. Genetic interactions between *hip4*⁺ and DNA damage checkpoint kinases. Double mutants were constructed and sensitivity assay by sequential spots were performed. Equal numbers of exponentially growing cells were diluted ten-fold and spotted onto YES and YES containing MMS for for growing 4-5-days at 30°C.

4.3. Discussion

In this study, we have characterized a novel CAP Hip4 responsible for DNA damage resistance and chromosome segregation. Loss of Hip4 leads to increased sensitivity to genotoxic agents, suggesting that Hip4 is required for safeguarding the genomic integrity. Our results further suggest that Hip4 is not required for G2/M DNA damage checkpoint responses.

We show that Hip4 plays an important role in protecting cells against DSBs and UV-induced DNA lesions (274, 346). Because *hip4*Δ cells display universal defects to various kinds of DNA damaging agents, we believe that the absence of *hip4*⁺ leads to global perturbation of chromatin structure (327). This

is consistent with the fact that HIRA is essential to protect chromatin from being attacked (326). We also show that lack of *hip4*⁺ weakly affects DNA replication. It is not surprising that most of DNA damage responsive genes have auxiliary contribution to DNA replication (148). This is because induction of DNA damage and inhibition of DNA replication trigger the same transduction signalling pathway in *S. pombe* (347), and they are highly interdependent to maintain genome integrity in *S. cerevisiae* and mammalian cells (348, 349).

We report that cells deleted for *hip4*⁺ are cold and temperature sensitive. This phenotype is indicative of G2 cell cycle delay (328, 329), a delay at the G2/M transition. The elongated cell morphology of *hip4*Δ is similar to the mutations in other HIRA proteins (*hip1*Δ, *slm9*Δ and *hip3*Δ), suggesting they act together to maintain normal cell cycle progression. Human cells have a single HIRA protein (350), whereas both budding and fission yeast cells recruit four related proteins that function in concert (328, 329, 351). They should not simply represent gene duplication, and may have additional functions independent of HIRA complex. For example, Slm9 does not precisely co-fractionate with Hip3 by gel filtration chromatography, and Hip1 plays a role in sexual development that are independent of Hip3 and Slm9 (328).

Our data suggest that Hip4 does not affect cell cycle through Cdc2 phosphorylation. In wild-type cells, DNA damage causes cell cycle arrest through inhibitory phosphorylation of Cdc2 until the lesion is repaired (150). Since Hip4 deletion did not affect Cdc2 phosphorylation, the G2/M DNA damage checkpoint control should remained intact upon MMS-induced DSBs in the *hip4*⁺ disruptant. It is known that deletion of other HIRA members, *hip1*⁺, *slm9*⁺, and *hip3*⁺ are synthetically lethal with a temperature-sensitive allele of

cdc25, and one of the component Slm9 is partially regulated by the Wee1 kinase (328, 329). Previous genetic studies also showed that inactivation of Wee1 can fully suppress the cell elongation phenotype caused by the *slm9* mutation (329). Given that HIRA proteins share many similar functions with each other, we should check whether Hip4 is the cell cycle-regulated protein using the above criteria.

The position of Hip4 in DNA damage signalling transduction is helpful to determine its precise role in regulating G2/M transition during cell cycle progression. We found that Hip4 functions in concert with the essential DNA damage checkpoint kinases Rad3; however, overexpression of *hip4*⁺ cannot rescue the growth defect of the double deletion mutant *hip4Δrad3Δ*. Therefore, Hip4 may not be directly involved in early transduction during DNA damage. It is interesting to test whether other HIRA proteins share also similar phenotypes.

We show that *hip4Δ* is slow-growing, and sensitive to spindle poisons, suggesting that Hip4 is necessary for accurate chromosome segregation. Hip4 is required for heterochromatin silencing. Because heterochromatin is important in maintaining the integrity of chromosome, the slow growth and distinctive defects in chromosome segregation could be explained by a loss of centromeric heterochromatin structure. Therefore, our results are consistent with the fact that packaging of the centromeric repetitive structures into silent chromatin facilitates and reinforces the kinetochore assembly (352).

We realize that the deletion strain constructed by us displays different phenotypes comparing with *hip4Δ* from the Bioneer deletion strains. The contradiction might be due to the length of the tag sequences for homologous recombination; the longer the nucleotide sequence, the less the chance of

generating false positives. Given that the Bioneer deletion strains are constructed based on 20 bp unique tag sequence, whereas we use 80 bp sequence homology for introducing deletions, we rely on *hip4Δ* generated by ourselves. Our *hip4Δ* strain phenotypes have also been verified by Anderson *et al.*, including temperature sensitivity, elongated cell morphology and hypersensitivity to the spindle poisons (293).

We detect two bands from immunoblotting of Hip4, suggesting certain PTM takes place on this protein. It has been reported that human HIRA is an *in vivo* substrate of a cyclin-cdk2 kinase (353). Therefore, it is worth of testing whether Hip4 can also be phosphorylated by Cdc2. If so, this result may also provide a clue how this protein controls the cell cycle.

To our knowledge, the HRD-containing proteins are highly conserved throughout evolution, and their role for nucleosome assembly is important in various organisms (339, 340). Thus, further characterization of Hip4 will provide mechanistic insights into the organization of chromatin architecture in eukaryotic cells. On the other hand, although these HIRA proteins are well conserved, they maintain genome integrity and cellular homeostasis by various means (336, 354). For instance, human HRD-containing protein UBN1 plays an important role in tumour suppression and tissue aging (339). It contributes to the formation of SAHF, which represses expression of proliferation-promoting genes and symbolizes cellular senescence. UBN1 binds to proliferation-promoting genes and associates with histone methyltransferase activity that methylates lysine 9 of histone H3. In budding yeast, Hpc2 is required for the repression of histone gene transcription outside of S phase; when DNA replication forks are stalled by HU, or if cells are arrested in G1 by the mating

pheromone alpha-factor (355, 356). Hpc2 is essential to mediate the recruitment of the other HIRA members to the histone gene promoter (357). Future studies are required to address the roles of HIRA complex in regulating histone gene levels and stoichiometry. Given that Hip4 is evident in maintenance of genome integrity, further studies are required to elucidate the exact mechanism by which Hip4 carries out this function. Furthermore, it will be interesting to speculate on how these histone chaperones may be modified and utilized as therapeutic targets in the future.

In *S. cerevisiae*, HIRA are replication-independent histone assembly factors, the activity of which is assisted and activated by the histone chaperone ASF1 (324), the counterpart of Cia1 in *S. pombe* (358). Recent study suggests that ASF1 is required for restoration of chromatin structure following DSB repair (359). However, little is known about how the chromatin structure is reassembled after DNA repair. Given that the HIRA interacting partner ASF1 does not play a direct role in histone deposition (44), we propose that Hip4 is likely to coordinate with other HIRA proteins to remove or replace nucleosomes following DNA repair. If this hypothesis holds true by future evidence, the sensitivity of *hip4*⁺ disruptant to DNA damage could also be explained by its responsibility for restoring the chromatin structure and organization after DNA damage.

CHAPTER 5. PHENOTYPIC ANALYSIS OF ZINC FINGER PROTEIN BC530.05

5.1. Introduction

In eukaryotic cells, zinc finger proteins are one of the largest families involving in transcriptional regulation. Each zinc finger protein harbours a common motif, known as the zinc finger. This motif was first identified in the *Xenopus* transcription factor TFIIIA (360), and it is named after the discovery of the “finger-like” shape (361). Zinc binding stabilizes this domain to maintain proper protein structure and function (362, 363). Majority of zinc finger proteins bind to DNA, thereby playing important roles in transcription (362). They are also involved in many other physiological functions, such as chromatin remodelling, lipid binding, and zinc sensing (362). Based on the conserved consensus amino acid sequences, zinc finger proteins are classified into three major classes (Figure 5.1). Class I represents Cys(2)His(2) proteins and is often referred to as the classical zinc finger (363); class II encompasses the Cys4 zinc finger (362), and class III contains the Zn(II)2Cys6 binuclear cluster DNA binding motif (364). Each class has distinct structural and functional properties.

Class III Zn(II)2Cys6 zinc finger proteins (or zinc cluster proteins) contain a DBD known as zinc binuclear cluster, in which two Zn atoms are bound by six cysteine residues. Zinc cluster proteins are involved in a plethora of cellular processes, such as amino acid metabolism, meiosis, mitosis, and

chromatin remodelling (365). In particular, a large proportion of zinc cluster proteins have been implicated in stress response and multi-drug resistance (MDR), a condition enabling cells to become resistant to a variety of structurally and functionally different cytotoxic compounds (366, 367). They function as transcriptional activators or repressors or both activators and repressors by binding to target genes as homodimers, heterodimers or monomers (365, 368). This type of transcriptional regulator has only been identified in fungi, and is best characterized in *S. cerevisiae* (369-371). In *S. pombe*, a total of 30 members have been isolated based on the well-conserved consensus amino acid sequence of Zn(II)2Cys6 motif. However, most of these putative zinc cluster proteins are functionally unknown.

Figure 5.1. Cartoon representation of three major classes of eukaryotic zinc finger proteins. Left, Cys(2)His(2) zinc finger motif consists of an α helix and an antiparallel β sheet; the zinc ion (green) is coordinated by two histidine residues and two cysteine residues (adapted from pymol.org). Middle, Cys4-type Zn ribbon with the Zn atom shown in gray is coordinated by four cysteine residues (adapted from Meades *et al.*) (372). Right, Zn(II)2Cys6 fungal-type zinc finger motif contains a binuclear zinc cluster in which two zinc ions are bound by six cysteine residues (adapted from Wikipedia open source).

In *S. cerevisiae*, the most well-studied zinc cluster protein is Gal4, which is involved in activation of genes that encode enzymes for galactose metabolism (373, 374). The *S. cerevisiae* sequencing project has allowed the identification of additional 54 members within the Gal4 family (371). A systematic deletion analysis has revealed phenotypes for a number of yeast

strains carrying deletion of genes encoding zinc cluster proteins (369). Interestingly, almost 20% of the mutant strains display sensitivity to caffeine.

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) is an analogue of purine, which has been involved in a series of cellular processes in eukaryotic cells, ranging from yeast to mammalian cells. It affects a diverse array of biological processes through interfering with DNA damage and repair and recombination pathways, delaying cell cycle progression, and modulating intracellular homeostasis. However, how caffeine triggers these cellular responses is still unclear. In *S. pombe*, caffeine has been shown to inhibit DNA repair mechanism (375, 376), and to interfere with both meiotic and UV-induced mitotic recombination (377, 378). All these phenotypes arise because caffeine disrupts genomic integrity. Recent study has reported that genes mutated in caffeine-sensitive clones are involved in a number of cellular roles including the H₂O₂-induced Pap1 and Sty1 stress pathways, the cell integrity and calcineurin pathways, cell morphology and chromatin remodelling (379). Both Pap1 and Sty1 pathways are responsible for cellular tolerance of caffeine (380). Pap1 is an activator protein-1 (AP-1)-like transcription factor belonging to the basic leucine zipper (bZIP) family. In *S. pombe*, a series of caffeine-resistant mutants have been isolated and characterized to share similar pleiotropic effects, including UV sensitivity and cell cycle delay (381). These mutated genes are termed *caf* genes. They are functionally-related, and converged to the same pathway that is connected to Pap1 and other proteins essential for normal tolerance of caffeine (379). The Sty1 pathway is activated in response to a whole variety of stress signals, including high osmolarity, oxidative stress, and heat shock (382). Components centred on the Mitogen-

activated protein (MAP) kinase Sty1 have been demonstrated to be sensitive to caffeine (379).

In *S. pombe*, mutation in components of calcineurin pathway leads to caffeine sensitivity. Calcineurin is a highly conserved protein phosphatase requiring for Ca^{2+} /calmodulin-dependent signalling ranging from yeast to human (383-386). It activates the T cells of the immune system and can be specifically blocked by the immunosuppressant drugs, such as cyclosporin A (CsA) and tacrolimus (FK506) (386). Calcineurin-regulated signalling pathways are involved in vital cellular events including cell polarity control, ion fluxes and chloride ion (Cl^-) homeostasis (387). The multicopy suppressors of calcineurin deletion have demonstrated a cross-talk between Mitogen-activated protein (MAP) kinase and calcineurin signalling pathways (388-391). *S. pombe* has been an excellent model to study the calcineurin-regulated signalling pathways. Previous genetic analysis has shown that disruption of the calcineurin gene (*ppb1*⁺) results in a drastic chloride ion (Cl^-)-sensitive growth defect (392). This defect is suppressed by a high copy number of a novel gene *pmp1*⁺, which regulates Pmk1 MAP kinase by direct dephosphorylation. Thus, calcineurin and the Pmk1 MAP kinase pathway may play antagonistic functional roles in the Cl^- homeostasis. Transcription factors are important calcineurin targets. In fission yeast, it is hypothesized that the Pmk1 MAP kinase substrate linking calcineurin and the Pmk1 MAP kinase pathway might be a transcription factor, which is dephosphorylated by calcineurin phosphatase and involved in the regulation of Cl^- transport. In budding yeast, mutants of calcineurin exhibit altered growth in the presence of several ions (393-395).

The effect of calcineurin on Ca^{2+} homeostasis is complex and probably involves regulation of multiple targets (396).

From our SILAC analysis, a novel zinc cluster protein Bc530.05 was significantly enriched in the chromatin fraction ($\text{Log}_2^{\text{SILAC ratio}} = 3.9$). In this study, we show that the *BC530.05*⁺-null mutant cells are hypersensitive to cold condition and microtubule inhibitors, but do not display aberrant nuclear or septa formation at low temperature. We also report the activity of this potential non-histone CAP is essential for cellular resistance to caffeine. Furthermore, Bc530.05 plays a protective role against CPT-induced DNA damage and cation toxicity. Thus, Bc530.05 is implicated in multiple stress responses and important for maintaining genomic and cellular integrity. We propose this protein may act as a transcriptional regulator of the calcineurin pathway. Whether it functions by regulating the expression of genes responsible for maintaining Cl^- homeostasis is unclear.

5.2. Results

5.2.1. *BC530.05*⁺ encodes a zinc finger fungal specific DNA

binding protein

BC530.05⁺ encodes a 762-amino acid protein with a calculated molecular mass of 85.1 kDa. A motif search revealed it contains a zinc finger motif of ACDMCRKRKIRCDGKQPACSNCVSHGIPC, known as the Zn(II)₂Cys₆ fungal-type DBD signature (Figure 5.1, right panel). This domain forms a binuclear Zn cluster, in which two Zn atoms are bound by six cysteine residues (397, 398). As a novel protein, Bc530.05 was predicted to be a

transcription factor based on its sequence. Zinc finger proteins typically function as interaction modules that bind to DNA, RNA, proteins, or small molecules. In our previous analysis, the gene containing the HA-tagged allele as a sole copy in the genome was constructed, and the protein was found to be associated with chromatin based on the chromatin fractionation assay (Figure 3.7). This result further confirmed that Bc530.05 binds to DNA.

Until now, no ortholog of Bc530.05 has been discovered from *S. cerevisiae*. Our previous result showed that Bioneer mutant strain lacking Bc530.05 lost viability at low temperatures (data not shown). This is similar to *STB5* in *S. cerevisiae* (399), which lacks a Zn(II)₂Cys₆ domain-containing transcription factor Stb5 (400, 401). The sequence alignment showed a 45% sequence similarity between these two proteins (Figure 5.2A). Stb5 is involved in regulating MDR and oxidative stress response (399-401). Therefore, we were interested to test whether Bc530.05 is responsible for any related functions in *S. pombe*.

A few proteins were found to share limited sequence similarity with Bc530.05, but mostly restricted to the zinc finger region (Figure 5.2B). The closest member is Gal4 (398, 402). Other zinc cluster proteins include Leu3 (403), and Ppr1 (404) of *S. cerevisiae*. These proteins are mainly responsible for amino acid metabolism. Other fungi also contain proteins with sequence homology within this region (sequences not shown).

A

SPBC530.05	1	MENLKSASPEEDSPRHGDNMGKPKRIPR..ACDMCRKRKIRCDGKOPACSNVCVSHGIFCV
scSTB5	1	MDGENFAHQGGRSQRTTELYSCARCRKLKKKCGKQIPTCANCNDKNGAHC
SPBC530.05	59	ETARPKRRT.....GQORQMYIKSLVSRLEQMESTLRSVIPNYD
scSTB5	51	YPGRAPRRTKKELADAMLRGEYVPVKRNKKVCKSPSTKSMFNSSSPLSAN.GAITPGFS
SPBC530.05	97	Q.QPDIIIFPSSTPFNYHDLNCTKGE.....TSDDSDDDIAFTNEKMGTL
scSTB5	110	PVENDDAHKMKQLKPSDPIINLVMGASPNSSSEGVSSLSVLTSLNDNSNPFSSHTSSNENSM
SPBC530.05	141	VTT...PIGSOK....YFGSSSTFS.....IICHAAKFASGVESDKVLEH
scSTB5	170	IPSRSLPASVQOQSSTTSFGGYNTPSPLISSHV PANAAVPLQNNNRNTSNGDNGSNVNH
SPBC530.05	179	LSMAKSGCLFDPDESFDGSKAELPSEIANI.....
scSTB5	230	DNNNGSTNTPQLSLTPYANNSAPNGKFDSPVVDASSIEFETMSCCFKGGRTTSWVREDGS
SPBC530.05	210	YIDAYFKSYNPLFFVFTRENFYQKFGSPNCKKPDGSDIDLVNYASYVV
scSTB5	290	FKSIDRSLLDRFAAYFKHNHRLFFEMIDKIAELNDAATITDERLYDNKNYPDSFVFKVY
SPBC530.05	258	VLSLGLCLA.....IADTFEQVSRANALFKNTLGISIEVTKDM.SFETLVFNFLTSTVY
scSTB5	350	MIMAIGCTTLQIRAGMVSQDEECLSEHLAFLAMKKFRSVIILQDIETVRCL...LLLGNTYS
SPBC530.05	310	CAVSKPNAVWLVNVCVVVRVAQTLGTHRN...SAMWSIGKEDAEKARLEWYIYLDRVSS
scSTB5	407	FFEPKGSSTWITISCIIMRLTIGLGNRELTAKKLKMSALEAEARYRVEWSACFERLVC
SPBC530.05	367	MMTGKPVAFQDDDIQDMVPFYSIVCYGLKEPEGDPITGTFNFLEAEVOLTRIVGQVLREL
scSTB5	467	TSLGRISGIDDEITVPLP.RALV.....DERDDLEMTKLM...ISLRKMGGRIVKCV
SPBC530.05	427	YVSGMKSNSS...QVMEKILEFDLLNNWYNLSLPDCMQPR.....NRFKIPKF
scSTB5	517	HVSAGRQKLTIECKQEIISGLRKELDEIISRESERRKLKSQMDQVERENNSTTNVISE
SPBC530.05	473	CSSNLILTSAIYYSCLITLHRHSITKNLQVNCVHRGTGSITDSQALCIAARSITNIF
scSTB5	577	HSSEIWL..AMRYSQLOIILYRPSALMPKPPID.....SLSTLGEFCLOANKHTYTY
SPBC530.05	531	VESVDLOPLIMKIIMYHAFTSSITIFISILKRPLASICSEDINCLISVKNRLISFETHGF
scSTB5	628	KKR..LLPL.....NWITLERTL.....TICNTIYCLCQWS.....
SPBC530.05	591	VRLNVMDALESMTSTAQAAMQAKQIAINFSSNLATNEDVTNSGMPDIADVSLKSQSHV
scSTB5	658	IDLIESKLEIQQCV.....
SPBC530.05	651	PFRTSSNHSDTSVKSNPSFSSIFDNGYLNLSLNSILOMHQNLQSSNTNDQYKFDVQEN
scSTB5	672	..EILRHFGERWIFAMRCADVFN.....ISNTILDI.....
SPBC530.05	711	ELHANITFVLDQTMSPFPKQDLNLNFAANVYNPNVFDMDGLDCSEFYNGNL
scSTB5	702	SLSHGKVENMDQLTREL.....FGASDSYQ.DILDENNVDVSNVVDKLV

B

scLEU3	1	MEGRSDFVATSSQSGSEMSESHSETRNRTGMNARKRKFAVECRQOKSKCDAHERAPEPCTKC
scPPR1	1	..MKQKKFNSKKSNTDLKRGDSPNIGI..SKSRTACKRCRLKKIKCDQEEFSS...CKRC
scGAL4	1	..MVTDOGSRHISIQSKOPAYVNKQPKRQ..QRSSVACLSCRKRIKCEGNNP...COKC
SPBC530.05	1	MENLKSASPEEDSPRHGDNMGKP..KRIPRACDMCRKRKIRCDGKOPA...CSNC
scLEU3	61	AKKNVPCH..LKRDFRRTYKRA.RNEAIEKRFEKLT...RTLTLNLTSDIIL.....
scPPR1	55	AKLEVPCVSLDPATGKDVPRS.YVFFLEDRTAVMM...RVLEHYGV.DFTKIRGNIPATS
scGAL4	54	VTSNAICEYLEPSKIVVSTK.YLQOLOKDLNDKTEENNRLKALLLREVSVRGKDNSDD
SPBC530.05	51	VSHGIPCVETARPKRRTGQORQMYIKSLVSRLEQME...STLRSVI.....

Figure 5.2. Sequence alignment of Bc530.05 with zinc finger fungal specific proteins. (A) Alignments between *S. pombe* Bc530.05 and *S. cerevisiae* Stb5. The complete amino acid sequences are shown. The zinc finger protein sequences were aligned using MUSCLE and BOXSHADE. Black boxes indicate identical amino acids, grey boxes indicate similar amino acids. The representative structure of Zn(II)2Cys6 DNA-binding domain signature [GASTPV]-C-x(2)-C-[RKHSTACW]-x(2)-[RKHQ]-x(2)-C-x(5,12)-C-x(2)-C-x(6,8)-C is underlined. (B) Alignments between *S. pombe* Bc530.05, and *S. cerevisiae* Gal4, Leu3 and Ppr1. Only the zinc finger regions are shown. The alignment was performed similarly as that in (A).

5.2.2. *BC530.05*⁺ is required for normal resistance to microtubule inhibitors

To confirm the phenotype of the Bioneer mutant strain, the deletion mutant of *BC530.05*⁺ containing the *ura4*⁺ marker (denoted as *BC530.05*Δ) was created. As shown in Figure 5.3A, *BC530.05*Δ cells lost viability as the temperature decreased, whereas their growth at warm condition (i.e., 36°C) was unaffected. An equivalent strain with *kan*⁺ marker behaved similarly (data not shown). This result suggested *BC530.05*Δ was a cold sensitive (cs) mutant (405, 406), which blocks sister chromatid separation in mitosis.

Because an initial sign of the chromosome segregation is the dissolution of the cytoplasmic microtubules, we wanted to examine whether this mutant encounter growth defects in the presence of spindle damaging agents. Consistent with our previous data, *BC530.05*Δ was hypersensitive to the spindle damaging agent TBZ; similarly, the mutant cells were unable to proliferate in the presence of another tubulin assembly inhibitor MBC (Figure 5.3B). Nevertheless, the sensitivity was much milder compared to spindle checkpoint mutants *bub1*Δ and *mad2*Δ, suggesting that *BC530.05*⁺ was less important in cellular tolerance to spindle damage. Given that MBC is more potent than TBZ (407), 5 μg/ml of MBC can cause more severe growth defect than 15 μg/ml of TBZ.

To test whether *BC530.05*⁺ can be activated upon TBZ treatment, the cells of the HA-tagged strain were treated with TBZ and collected over a 2-hr time course. The total lysate was prepared and the protein expression was monitored by Western blotting with anti-HA antibody. The protein levels were

normalized to the amount of PSTAIRE, which served as loading control. Upon TBZ treatment for the first 10 min, the level of HA-tagged protein increased by more than two-fold, but did not change within next 20 min (Figure 5.3C, left panel, lane 1-3). The elevated level of Bc530.05 continued to be observed during the following 1.5 h (lane 4 and 5). This might indicate that the protein was upregulated by TBZ. On the other hand, the protein levels were relatively stable in HA-tagged cells following MBC treatment (Figure 5.3C, right panel). The protein amount increased to ~1.7 fold after 10 min, but returned to normal level for 1 h (lane 6-9). The protein level became higher again in the following one-hour incubation (lane 10). Therefore, MBC caused no more than two-fold changes in the protein expression during the entire time course. This further suggested that the protein could only be accumulated by TBZ, presumably due to the different properties of these two drugs.

One possible variation between TBZ and MBC treatment could reside in their different mechanisms to depolymerize microtubules in *S. pombe* (407). Unlike TBZ, which depolarizes the actin cytoskeleton through affecting non-tubulin targets (407); MBC causes the depolarization by disrupting microtubules directly (408-410). Moreover, TBZ affects cell polarity (411), whereas MBC does not. Therefore, we speculated that Bc530.05 was involved in organization of the actin cytoskeleton in a tubulin-independent manner. Alternatively, it might be responsible for the establishment or maintenance of cell polarity. Nevertheless, although both TBZ and MBC are benzimidazole derivatives and are similar in structure (412), they could be utilized for different purposes.

Figure 5.3. *BC530.05*⁺ is required for normal tolerance to spindle damaging agent TBZ and MBC, and the protein is activated by TBZ.

(A) Confirmation of temperature sensitivity in *BC530.05Δ*. *BC530.05Δ* was constructed, the indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions and spotted onto YES agar plates and incubated at 30, 15, 20, 25 and 36°C. The cell densities from left to right are 10⁵, 10⁴, 10³ and 10². (B) *BC530.05Δ* is sensitive to TBZ and MBC. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with the indicated genotoxic agent for growing 4-5 days at 30°C. (C) Bc530.05 protein level increases following TBZ treatment, but remains constant following MBC treatment. The cells of the HA-tagged Bc530.05 were treated with 15 μg/ml of TBZ or 5 μg/ml of MBC over a 2-h time course, and samples were collected at indicated times. Total cell lysate was prepared, separated on 10% SDS-PAGE, and subjected to Western blotting with antibody against HA and PSTAIRE. The amounts of Bc530.05-HA were quantified relative to corresponding protein levels of PSTAIRE (Relative AMT) using *ImageJ*. Abbreviations: *wt*, wild-type; TBZ, thiabendazole; MBC, Methyl 2-benzimidazolecarbamate; Relative AMT, relative amount.

5.2.3. *BC530.05*Δ displays normal cell division and septa formation at restrictive temperature

It is known that the *cs* mutants typically show irregular chromosome movements during mitosis at restrictive temperature (406, 413, 414). To determine whether *BC530.05*Δ can have similar phenotypes, the wild-type and mutant cells were grown at permissive temperature (i.e., 30°C) to mid-log phase and shifted to restrictive temperature (i.e., 20°C) for 12 hr, and the morphology of nucleus and septum in the arrested cell was examined by fluorescence microscopy. The two classical *cs* mutants, *dis1*Δ and *nda3*Δ, were included for comparison.

Both wild-type and the *BC530.05*⁺-null mutant cells behaved normally at permissive temperature, displaying symmetric nuclear division and organized nuclear shape. These phenotypes remained unchanged in *BC530.05*Δ cells after shifting to low temperature (Figure 5.4). They neither exhibited condensed chromosomes in mononucleate cells like *dis1*Δ, nor displayed elongated nuclei or U-shaped chromatin regions as in *nda3*Δ (white arrowheads) (405, 406). On the other hand, these aberrant nuclear formations started emerging in *dis1*Δ and *nda3*Δ at relatively low frequency at permissive temperature, and became aggravated after shifting to restrictive temperature. Furthermore, the septa were sharp and thin in wild-type and *BC530.05*Δ cells; whereas the cells stained with Aniline blue in *nda3*Δ exhibited condensed and threadlike septa. Therefore, inactivation of *BC530.05*⁺ did not cause aberrant nuclear division or cell plate formation at restrictive temperature. Moreover, *BC530.05*Δ is unlikely to be a nuclear division arrest mutant, which normally accommodates a high percentage of elongated cells with a single nucleus at restrictive temperature.

Figure 5.4. Microscopic analysis of *BC530.05Δ* at restrictive temperature. *BC530.05Δ* displays normal cell division and septa formation at restrictive temperature. Fluorescence micrographs of wild-type and mutant strains following shift to 20°C. Cells were grown at 30°C to mid-log phase (upper panel) and shifted to 20°C for 12 h (bottom panel), cells were collected and stained with DAPI and Aniline blue as described in Materials and Methods. Arrows indicate the condensed nuclei in *dis1Δ*, and U-shaped chromatin regions in *nda3Δ*.

5.2.4. *BC530.05⁺* is required for normal tolerance to caffeine

In *S. pombe*, it is interesting to know that some cs mutants are sensitive to caffeine at permissive temperature (i.e., 30°C) (142). Therefore, we were curious to test this possibility in our *BC530.05⁺*-null mutant. As shown in Figure 5.5A, *BC530.05Δ* displayed impaired growth on caffeine-containing agar medium. The growth deficiency of *dis1Δ* in the presence of 10-12.5 mM caffeine validated previous data that all the *dis* mutants are supersensitive to caffeine. Interestingly, our mutant exhibited greater sensitivity to caffeine toxicity than *dis1Δ*. However, *nda3Δ* also showed stronger sensitivity than wild-type cells when the drug concentration was increased to 12.5 mM, which contradicted previous study that *nda3* mutants were resistant to caffeine toxicity (142, 406). Such discrepancy could be due to the different strains being used. Instead of using deletion mutant, Toda *et al.* utilized allele-specific mutant for their studies (406). To validate our experiment, another two mutants, *sty1Δ* and

pcr1 Δ were also included. It has been discovered that the stress-activated MAP kinase Sty1 is sensitive to caffeine, while the downstream bZIP transcription factor Pcr1 is dispensable for the cellular response to caffeine (379, 415). Therefore, our data was in line with previous results. Furthermore, the growth assay in liquid culture showed that the deletion strain took much longer to reach the same cell density than the wild-type cells (i.e., 12.042 versus 14.875 h in ΔT_{50}) after shifting to 10 mM caffeine (Figure 5.5B). The growth retardation of *BC530.05* Δ cells to caffeine was also reflected by the maximum OD₆₀₀ they could reach after 48 h (i.e., 0.353 versus 0.642 in ΔOD_{max}).

We were interested to know whether low concentration of caffeine would affect nuclear organization in the mutant cells. Similar to wild-type cells, *BC530.05* Δ did not show any alteration of nuclear division after shifting asynchronous cells to 5 mM caffeine for 1 h (Figure 5.6A). Consistent with this, FACS analysis did not show any difference in the 1C and 2C DNA contents within the first two hours (Figure 5.6B). However, we observed a slowdown of DNA replication in *BC530.05* Δ cells after 4-h exposure to caffeine, reflecting the growth defects of the mutant cells. This slowing could be due to the physical blocking of replication imposed by caffeine. DAPI staining showed that the size of the nuclei in *BC530.05* Δ cells from this stage were significantly reduced compared to that in wild-type cells (Figure 5.6C). Moreover, there was an elongated cell accommodating four nuclei without cell division (white arrowhead). This indicated that continuous treatment of caffeine may lead to retarded chromosome segregation. Nevertheless, the frequency of such abnormal tetranucleated cells was rare, and no 4C peak could be observed in our FACS data.

Figure 5.5. *BC530.05Δ* cells are sensitive to caffeine treatment.

(A) The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with the various concentrations of caffeine for growing 4-5 days at 30°C. The cell densities from left to right are 10^5 , 10^4 , 10^3 and 10^2 , labelled as 1, 2, 3, and 4, accordingly. (B) Both *wt* and *BC530.05Δ* were grown to mid-log phase in YES medium, half cells were shifted to fresh YES medium, the other half were shifted to YES medium containing 10mM caffeine. Mini-culture growth curve assay was applied to monitor the cell growth in YES (solid line curve, control) or YES containing 10mM caffeine (dotted line curve, treatment) for 48 h. X-axis and Y-axis indicate the time point and the cell density. The three numbers on the top of the plot represents ΔT_{50} (the difference of the time at the half-maximal cell concentration between treatment and control), $\Delta slope$ (the ratio of curve slope between treatment and control), and ΔOD_{max} (the difference of the maximum OD_{600} between treatment and control), respectively.

Figure 5.6. Nuclear organization in *BC530.05Δ* upon caffeine treatment. (A) Fluorescence micrographs of wild-type and mutant strains before and after caffeine treatment. Cells were grown in YES medium at 30°C to mid-log phase ($OD_{600} \sim 0.4$) and shifted to fresh YES medium containing 5 mM caffeine and incubated for 1 h, cells were collected and stained with DAPI as described in Materials and Methods. (B) FACS analysis of wild-type and mutant strains following caffeine treatment. Cells incubated with 5 mM caffeine were collected as the time indicated and subjected to ethanol fixation and DNA content measurement. (C) Comparison of nuclear morphology between wild-type and mutant strains after 4-h incubation in 5 mM caffeine. The method is the same as described in (A). Bar, 10μm.

To examine whether caffeine triggers the upregulation of Bc530.05, the HA-tagged strain was exposed to various drug doses for 5 min, ranging from 1 to 7.5 mM, neither protein level increase nor modification patterns was observed by any tested dosage of caffeine (Figure 5.7A). This is different from Sty1, whose phosphorylation can be induced by caffeine (379). We further treated cells with 7.5 and 10 mM caffeine over a 2-h time course. As we can see, the protein levels were not altered following treatment of 7.5 mM caffeine. By increasing the drug dosage, a distinct protein level increase of Bc530.05 can be observed within the first 20-min exposure to caffeine (Figure 5.7B, bottom panel). There was ~3 Fold (> 2 fold) increase of protein expression from 0 to 20 min; however, no obvious protein accumulation was detected upon continuous incubation in 10 mM caffeine, and the protein level fluctuated randomly.

Figure 5.7. The protein level change of Bc530.05 following caffeine treatment. (A) The cells of the HA-tagged Bc530.05 were pulse treated with various caffeine concentrations as indicated for 5 min. (B) The cells of the HA-tagged Bc530.05 were treated with 7.5 mM (upper panel) or 10 mM (bottom panel) caffeine for 2 h, and samples were collected at the indicated times. For samples from both (A) and (B), total cell lysates were prepared, equal amounts of protein were loaded and separated on 10% SDS-PAGE, and subjected to Western blotting with antibody against HA and PSTAIRES. The amounts of Bc530.05-HA were quantified relative to corresponding protein levels of PSTAIRES (Relative AMT) using *ImageJ*. AMT, amount.

Caffeine exerts a genotoxic effect mainly through inhibition of the MAP kinase pathway and the cyclic AMP (cAMP) phosphodiesterase (416). Through the inhibition of the cAMP phosphodiesterase, caffeine increases the intracellular concentration of cAMP (417). Hence, we examined whether cAMP

directly affects the cell growth in *BC530.05Δ*. The real-time growth of both mutant and wild-type cells in liquid culture was slowed down by 100 and 200 mM cAMP (Figure 5.8). The mutant and wild-type cells displayed similar growth rates in increasing cAMP concentrations, suggesting the defects have nothing to do with this second messenger system.

Figure 5.8. The caffeine sensitivity of *BC530.05Δ* is not due to accumulation of cAMP. Growth curves of wild-type and *BC530.05Δ* cells in the presence or absence of cAMP. Mid-log phase cells at an OD_{600} of 0.1 were either treated or not treated with the indicated concentrations of cAMP. Mini-culture growth curve assay was performed to monitor the cell growth at 30°C by measuring OD_{600} every 17 min during a 48-h period.

Collectively, Bc530.05 was required for normal tolerance to caffeine at permissive temperature, and upregulation of its protein level was apparent in 10 mM caffeine during the first 20 min. Furthermore, the induced caffeine sensitivity was independent of the cAMP pathway. Bc530.05 has never been identified in previous screens conducted to isolate mutants sensitive to caffeine.

5.2.5. Deletion of *BC530.05*⁺ results in hypersensitivity to CPT

It is known that caffeine disrupts genomic integrity through various mechanisms (375, 376, 378). Because deletion of *BC530.05*⁺ caused hypersensitivity to caffeine, we wanted to investigate whether *BC530.05*⁺ plays a role in maintaining genomic integrity. To examine which pathway it may be involved in, we conducted the sensitivity assays to test the vulnerability of *BC530.05*⁺-null mutant against various DNA mutagens.

Our results showed that deletion of *BC530.05*⁺ did not affect cell growth in HU, MMS, or 4-NQO (Figure 5.9A), but resulted in dramatic hypersensitivity to the Topo I inhibitor CPT (Figure 5.9B); suggesting that Bc530.05 maintains DNA integrity in a restrictive manner. This drug specificity might be related with the mechanism of action of these four drugs. While HU, MMS and 4-NQO all cause an arrest of replication fork progression, CPT frequently induces DNA breakage at replication forks and causes SSBs (418-420). Topo I is an isomerase that regulates unwinding of DNA (421). It plays a critical role in the removal of superhelical torsions, and inhibition of this enzyme interferes with DNA replication and transcription (421-424). Therefore, Bc530.05 may be responsible for maintaining genomic integrity through targeting and protecting Topo I from being attacked. Given that Bc530.05 is a putative transcription factor, CPT-induced DNA damage in *BC530.05*Δ may also interfere with the transcriptional activities that are essential for cell growth (423).

Figure 5.9. The sensitivity of *BC530.05Δ* to DNA damaging agents. *BC530.05Δ* only displayed hypersensitivity to CPT. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with the indicated genotoxic agent for growing 4-5 days at 30°C. (A) The indicated strains were treated with HU, MMS and 4-NQO as described. (B) The indicated strains were treated with CPT as described. Abbreviations: *wt*, wild-type; HU, hydroxyurea; MMS, methyl methanesulfonate; 4-NQO, 4-Nitroquinoline 1-oxide; CPT, camptothecin.

5.2.6. *BC530.05*⁺ is required for normal resistance to toxic cations

In *S. pombe*, a subset of genes deleted in caffeine-sensitive mutants are required to respond to cation toxicity (425). For example, the key member of stress-activated protein kinases/Jun N-terminal kinases (SAPKs/JNKs) Sty1 is essential for cellular resistance to certain cations, such as, Na⁺, K⁺, Ca²⁺ and Li⁺, concentrations of which are normally kept relatively low in cytoplasm (426). Therefore, the similar requirement of both Bc530.05 and Sty1 in caffeine resistance prompted us to check if Bc530.05 is also needed for survival in toxic concentrations of cations.

We put *BC530.05*⁺-null mutant through osmotic stress using various salts as osmolytes. Except for KCl, we noticed that *BC530.05*Δ exhibited general defects to all of them, but to a lesser extent compared to *sty1*Δ (Figure 5.10). On the other hand, wild-type cells displayed normal growth even at highest concentrations of these toxic cations. About 0.1 M NaCl was able to cause an apparent growth defect in the deletion mutant, suggesting that Bc530.05 may be responsible for cell survival under Na⁺ stress. The same concentration of KCl also significantly inhibited the growth of *BC530.05*Δ cells, whereas only a mild sensitivity was observed in *sty1*Δ. Interestingly, *BC530.05*Δ cells became no more sensitive as the concentration increased, and turned out to be more resistant than *sty1*Δ in the presence of 0.4 M KCl. As we did not observe similar trend in the other circumstances, the deficiency should be specifically attributed to K⁺ instead of Cl⁻ or osmolarity. One possible explanation for this unique feature is that *BC530.05*Δ cells cannot sustain high concentration of K⁺, which rendered the cells less sensitive. We did not observe

similar phenomenon in *sty1Δ*. Previous study suggested that 0.1 M KCl does not inhibit the growth of *sty1Δ* cells, and a *sty1* mutant with point mutation had growth defect in 0.4 M KCl (426). These data are consistent with ours, suggesting the phenotype observed in *BC530.05Δ* cells is not random or due to experimental errors.

It is known that caffeine affects Ca^{2+} homeostasis in both *S. cerevisiae* (427) and *S. pombe* (379). Because *BC530.05Δ* cells exhibited retarded growth in both NaCl and KCl, we wanted to test whether CaCl_2 can also cause its growth deficiency. Addition of 160 mM CaCl_2 to the growth medium interfered with the growth of *BC530.05Δ* (Figure 5.10). This result may indicate that *Bc530.05* is important in the cellular resistance to Ca^{2+} , although not as critical as *Sty1*. On the other hand, *BC530.05⁺*-null mutant displayed much better growth in 80 mM CaCl_2 (i.e., 160 mM Cl^-) than in 0.1 M NaCl and KCl. These results further confirmed the sensitivity of *BC530.05Δ* in response to NaCl and KCl was due to cation toxicity rather than high anion concentration.

Further examination demonstrated that deletion of *BC530.05⁺* led to hypersensitivity to Li^+ , indicating that *BC530.05Δ* cells may have deficient lithium efflux activity. Previous study has shown that tolerance to high sodium and lithium concentrations requires the functioning of the Na^+/H^+ antiporter *sod2* (428, 429). This might further suggest that *Bc530.05* is responsible for regulating the antiporter-dependent export of Na^+ and Li^+ . Because the assay was conducted at extremely low concentrations, the observed growth inhibition could not be due to toxicity of Cl^- or high osmolarity. Therefore, *Bc530.05* may play a positive role in maintaining Li^+ homeostasis.

Figure 5.10. *BC530.05⁺* is required for cellular resistance to the toxic cations. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with various concentrations of toxic cations for growing 4-5 days at 30°C. *sty1Δ* and *pcr1Δ* were included as controls.

We then checked whether cells can induce upregulation of Bc530.05 in response to Na⁺ and K⁺ stress. Unfortunately, addition of 0.1 M NaCl or KCl to the growth medium did not bring about any protein accumulation (Figure 5.11), probably due to mild osmostress. Hence, although Bc530.05 was found to be responsible for cellular survival under Na⁺ and K⁺, neither of them could elicit

upregulation/accumulation of this protein. Therefore, K^+ and Na^+ are not activating stimuli for the Bc530.05-mediated pathway. Alternatively, it is also possible that such low osmstress is not sufficient to launch stimulation of protein expression.

Figure 5.11. Bc530.05 is not upregulated in response to toxic cation. Toxic cations cannot cause protein accumulation of Bc530.05. The cells of the HA-tagged Bc530.05 grown to mid-log phase at 30°C in YES medium was treated with 0.1 M NaCl (upper panel) or 0.1 M KCl (bottom panel) for 2 h, and samples were harvested at the indicated times. Total cell lysates were prepared, equal amounts of protein were loaded and separated on 10% SDS-PAGE, and subjected to Western blotting with antibody against HA and PSTAIRE. The amounts of Bc530.05-HA were quantified relative to corresponding protein levels of PSTAIRE (Relative AMT) using *ImageJ*. AMT, amount.

Taken together, these results suggested that Bc530.05 plays a protective role against cation toxicity. Although the basal level of Bc530.05 is required for cation tolerance, it is not responsible for inducing upregulation of protein expression when exposed to these cations.

5.2.7. Bc530.05 is not involved in oxidative stress pathways

It is frequently observed that some caffeine-sensitive mutants also display defects to oxidative stresses including H_2O_2 (430, 431); for example, *sty1* Δ . Unlike *sty1* Δ , which is sensitive to 2 mM H_2O_2 , *BC530.05* Δ did not confer sensitivity to H_2O_2 at all concentration tested (Figure 5.12). Thus, Bc530.05 is not involved in the oxidative stress response.

Figure 5.12. Bc530.05 is not involved in oxidative stress pathway. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with the various concentrations of H_2O_2 . H_2O_2 , hydrogen peroxide.

5.2.8. Bc530.05 might be involved in calcineurin-mediated signalling pathway

It is found that majority of the mutants in the cell integrity pathway, whose central component is the MAP kinase Pmk1 (432, 433), exhibit sensitivity to caffeine, but not to H_2O_2 (379). For example, the components of the calcineurin pathway. Because calcineurin is a major player in Ca^{2+} -dependent signal transduction pathways in eukaryotic cells (387), we wanted to check whether Bc530.05 is responsible for regulating Ca^{2+} homeostasis. As the result shown, *BC530.05* Δ cells were sensitive to 0.4 mM EDTA, which is a chelating agent for Ca^{2+} (Figure 5.13). Since EDTA binds to Ca^{2+} and

diminishes its activity, this result suggested that deletion of *BC530.05*⁺ may inhibit extracellular Ca²⁺ uptake and lead to disturbance of Ca²⁺ homeostasis. Therefore, this protein is likely to be located along the calcineurin-mediated signalling cascade.

Figure 5.13. Bc530.05 is responsible for regulating Ca²⁺ homeostasis. The indicated strains were grown to mid-log phase in YES medium, subjected to ten-fold serial dilutions, and spotted onto YES agar and YES agar supplemented with the various concentrations of EDTA for growing 4-5 days at 30°C. EDTA, ethylenediaminetetraacetic acid.

5.3. Discussion

From our SILAC analysis, we isolated a putative transcription factor possessing Zn(II)2Cys6 domain. Except for the zinc finger domain, BLAST searches of *S. pombe* genome did not reveal any homologs, suggesting that it is not simply due to gene duplication. Although Gal4 in *S. cerevisiae* shares highest sequence similarity with Bc530.05, we did not find any common phenotypes between their deletion mutants. In this study, we have characterized a series of phenotypes of the *BC530.05*⁺-null mutant, including sensitivity to low temperature, caffeine, spindle and DNA damaging agents, and toxic cations. Furthermore, we found Bc530.05 is unlikely to be involved in the H₂O₂-induced oxidative stress pathways.

We found that Bc530.05 contributes to cellular resistance to caffeine. As a stimulant drug, excess caffeine is cytotoxic to all eukaryotic cell types.

Caffeine can interfere with multiple cellular activities and induce a number of cytotoxic effects, such as general fitness impairment, protein traffic impairment, cell cycle arrest, DNA damage and cell wall damage (375-378, 434-436). A genome-wide screening of caffeine sensitivity has been conducted in an *S. pombe* deletion collection (379). The isolated genes are mapped to miscellaneous pathways, such as the H₂O₂-induced Pap1 and Sty1 pathways, DNA damage and repair, and the integrity and calcineurin pathways. Therefore, the pleotropic effects triggered by caffeine toxicity can either be direct or indirect. We showed that Bc530.05 is not significantly activated (or upregulated) by caffeine. In fission yeast, both the Pap1 and Sty1 pathways are essential for normal tolerance to caffeine. However, only the Sty1 pathway can be activated by this drug, in the manner of protein accumulation and translocation from cytoplasm to nucleus (379). Given that Sty1 governs the global stress response pathway; it is inducible by any type of environmental stress that compromises cell viability. It is known that some mutants lacking components of Pap1 and Sty1 pathways also display defects in the presence of H₂O₂, implicating a connection between caffeine tolerance and oxidative stress. However, this connection is not present in our mutant, which might implicate an independent role for Bc530.05 in either Pap1 or Sty1 pathway. This result further suggests that the toxic effects of caffeine are not mediated through direct generation of reactive oxygen species (ROS) (437). Furthermore, the caffeine sensitivity of *BC530.05Δ* is not caused by the inactivation of cAMP phosphodiesterase. cAMP phosphodiesterase has been regarded as the best known protein target inactivated by caffeine (438). Earlier data suggested that caffeine toxicity was mediated through the inhibition of cAMP phosphodiesterase (142). Therefore,

Bc530.05 is not responsible for maintaining normal activity of cAMP phosphodiesterase. In fact, previous systematic analysis did not detect single caffeine-sensitive mutant in this pathway (379), which further reflects the diversity of cellular functions influenced by caffeine.

We also showed that the deletion mutants of Bc530.05 are hypersensitive to CPT, supporting a specific role of Bc530.05 in maintaining DNA integrity. Proteins responsible for DNA replication, recombination and repair have been reported to be essential for normal tolerance to caffeine (375, 376, 378), which emphasizes intimate relationship between DNA integrity and caffeine toxicity. Different DNA damaging agents attack DNA with different mechanisms of action. HU specifically targets RNR to inhibit DNA replication (439), while MMS and 4-NQO target DNA strand and form bulky adducts (440, 441). The resistance of *BC530.05Δ* to these DNA damaging agents suggests this protein may function independently from the DNA damage checkpoint kinases, which are responsible for sensing DNA damage along the signal transduction cascade. CPT is a specific inhibitor of Topo I (418). It locks Topo I covalently onto the DNA strand, which slows down the dissociation of Topo I and DNA, and induces DNA strand break strictly within the replication fork region. Therefore, CPT damages DNA by replication fork breakage, whereas the other genotoxic agents cause replication fork arrest without targeting the replication fork. The mechanism of CPT might provide a clue that *BC530.05Δ* is defective in maintaining the structure of the replicative intermediates. As a transcription factor, Bc530.05 may play a role in interacting with Topo I (encoded by *topI*⁺) and protecting it from being attacked by CPT. However, this hypothesis should be confirmed by testing the genetic interaction between

BC530.05⁺ and *top1*⁺. Previous study has discovered a DNA repair enzyme Pnk1 that is sensitive to CPT, but not to MMS and 4-NQO (420). Hence, Bc530.05 may alternatively act as a transcriptional activator to stimulate activities of the DNA repair enzymes after DNA damage. Further analysis is required to determine its precise role in protecting the cells against CPT toxicity. Examining the recovery of DNA replication after CPT-induced replication fork breakage will be valuable to dissect its role in stabilizing replication fork integrity. It may help to answer whether Bc530.05 confers activity through preventing fork collapse or enhancing the fork regeneration. We should also investigate the combinatorial defects of CPT and caffeine in *BC530.05Δ* and check whether there is a direct link between these two types of toxicity.

We found that cells lacking Bc530.05 are sensitive to a series of alkali cations. Alkali cations, such as K⁺, Na⁺, Ca²⁺, and Li⁺, are vital elements that play diverse roles in all living cells. However, excessive concentration of these cations may lead to severe cellular toxicity. To avoid toxicity, many proteins are required to maintain the cations over a limited range of concentrations (442-445). Some metabolic enzymes cannot tolerate Na⁺, but can withstand K⁺. Therefore, the concentration of Na⁺ is kept relatively low in many cell types, whereas a much higher concentration of K⁺ is sustainable. The uniform growth defects of *BC530.05Δ* in response to various concentrations of KCl may indicate that lack of Bc530.05 is able to trigger the permeabilization of plasma membrane, which may lead to collapse of normal K⁺ homeostasis in low osmostress (446). We realized the behaviour of *BC530.05Δ* cells to cation toxicity resembles that of *atf1Δ* cells (426). The deleted gene encodes the transcription factor Atf1, which is phosphorylated by Sty1 upon stress to

regulate expression of many stress resistance genes (447, 448). Atf1 is also involved in the cell integrity signalling regulated by Pmk1 MAP kinase, and its deletion leads to hypersensitivity to cell wall damaging agent (432, 433, 449). To test whether Bc530.05 has similar functions with Atf1, we may first need to examine whether Bc530.05 is responsible for cell wall synthesis.

Deletion of *BC530.05*⁺ leads to cellular disturbance of Ca²⁺ homeostasis. In fission yeast, proteins required for caffeine but oxidative stress tolerance have been extensively identified from calcineurin pathway (379). One of the most established functions of calcineurin is to regulate gene expression in response to Ca²⁺ stimuli (392). The sensitivity to Ca²⁺-chelator EDTA in *BC530.05*Δ implicates that Bc530.05 is likely to play a role in calcineurin signalling. Whether *BC530.05*⁺ is responsible for regulating genes in this pathway is still elusive and requires further investigation. To our knowledge, the components of calcineurin pathway are responsible for maintaining Cl⁻ homeostasis, and hypersensitivity to Cl⁻ caused by their mutation is due to loss of calcineurin phosphatase activity (392). Based on our results, *BC530.05*⁺-disruptant shows universal sensitivity to all the basic salts salts, and the sensitivity may partially contributed by the anion ion Cl⁻. This possibility requires further verification by a classical sensitivity test, which differentiates the sensitivity caused by Cl⁻ from high osmolarity (450). We should also test whether the mutant confers hypersensitivity to the most promising calcineurin inhibitors CsA and FK506. There has been a well-established genetic approach to identify regulators of calcineurin (392). This approach may facilitate dissecting the precise role for Bc530.05 in calcineurin signalling, provided that *BC530.05*Δ cells are sensitive to Cl⁻. For instance, suppressors of calcineurin

deficiency (e.g., Pmp1) have been identified; the overexpression of these suppressor genes in *BC530.05Δ* can be used to test if they can rescue the sensitivity to Cl^- .

There is a coincidence that both *BC530.05⁺*- and *S. cerevisiae STB5⁺*-null mutant show cold and caffeine sensitivity (369). Recent discovery has identified Stb5 to be a transcriptional activator that binds to the HACT Sin3, the component of Sin3-Rpd3 HDAC complex (400). Coincidentally, the fission yeast ortholog of Sin3 is also essential for caffeine tolerance. As a chromatin remodeler, it may have functions indirectly related to the cellular response to caffeine, such as affecting pathways required to counteract the effects of caffeine, or required to facilitate its degradation. Nevertheless, we yet have evidence to show that Bc530.05 physically interact with any member in HDAC complex in *S. pombe*. The stress responses of strains lacking genes encoding Zn(II)2Cys6 motif-containing proteins are diverse in fission yeast. For example, *moc3⁺*-null mutant is sensitive to salt stress and DNA damaging agents (451), whereas the *grt1⁺* deletion mutant does not exhibit any sensitivity to either caffeine or DNA damaging agents (452). Recently, autoregulation and cross-regulation of the expression of zinc cluster proteins are becoming a common theme for unravelling their distinct and overlapping functions. Mechanisms such as phosphorylation have been frequently observed to regulate activity of zinc cluster proteins. Therefore, deep analysis of the mechanism of Bc530.05 may help us understand diverse phenotypes of its deletion mutant in *S. pombe*.

CHAPTER 6. IDENTIFICATION OF HISTONE H4 INTERACTING PARTNERS USING TANDEM AFFINITY PURIFICATION

6.1. Introduction

Histones are the primary proteins to mediate the folding of DNA into chromatin (1). They are essential for viability as they package DNA into the nucleus and regulate access to the genetic information embedded in DNA. Most of the epigenetic information associated with chromatin is encoded by core histones (3). Histones and histone-interacting partners are involved in a diverse array of cellular processes, such as DNA replication, DNA repair, and transcriptional silencing (6, 229, 453-458).

Histones are very basic proteins. The tight association of histones with DNA makes the extraction of histones difficult. Histones are solubilized in low concentration of HCl or H₂SO₄, conditions under which nucleic acids and other nucleoid proteins will precipitate (459). Thus far, most of the histone extraction work has been based on solubility of histones in acids (460, 461). Although acid extraction is a classical and well established protocol for the study of histones, it is not well-suited for fission yeast, as it may result in a histone preparation containing huge amounts of carbohydrates and polyphenols, which readily crosslink and oxidize the histone proteins. Importantly, this method is unable to discover histone-interacting proteins because only basic proteins can be

extracted. To examine the protein-protein interactions for histones, a method called “Far-Western blotting” was invented (462-465). “Far-Western blotting” is based on Western blotting, and employs non-antibody proteins to detect interactions with histones immobilized on membranes. However, this technique is time-consuming, because both chromatography and Western blotting require post experimental readings which have to be prepared separately.

Nowadays, a method termed TAP has been successfully applied to discover thousands of novel proteins and their associated functions. It is a specially-designed and well-suited strategy for the identification of protein interactions (255). TAP tag contains two different affinity tags, the IgG-binding units of protein A from *Staphylococcus aureus* and the CBP, which are added consecutively to a protein of interest (Figure 6.1). The two tags are connected by placing a TEV protease cleavage sequence. Extracts prepared from cells or organisms containing a tandem affinity tag are used to recover the target protein and associated interacting partners. Different from previous methods, TAP involves a two-step purification process. After an initial affinity purification step with IgG sepharose, the bound complex is released by incubating with TEV protease. A second affinity step is performed by incubating this first eluate with calmodulin affinity resin in the presence of Ca^{2+} . Finally, the bound proteins are released into solution with EGTA, and the eluted product is then resolved by SDS-PAGE and directly subjected to tryptic digestion followed by nanoLC–MS/MS. TAP synergizes several advantages of the standard biochemical purification and the Co-IP strategies. Similar to classic biochemical purification, successive purification in TAP allows the recovery of less abundant proteins, and the use of a constant peptide tag avoids time-consuming

optimization for each new target. As in Co-IP, the TAP strategy facilitates selective and efficient recovery of the *in vivo*-associated protein complexes, and the multiple processing steps can reduce background contamination from abundant cellular proteins or material leaking from the affinity column. In this way, the TAP method allows recovery of low abundant proteins from extensively purified complexes.

Along with the development of MS technology, TAP has been successfully applied in high-throughput model (269, 271, 466). As an effective and comprehensive strategy to identify protein components from large molecular complexes, TAP coupled to MS has gained great popularity in chromatin research (467). CAF-1 is involved in *de novo* deposition of histones H3 and H4 at the DNA replication fork (468). TAP of CAF-1-interacting proteins using 400 mM NaCl in human cells revealed that CAF-1 forms protein complex with DNA-dependent protein kinase KU70/80 complex, which is consistent with the role for CAF-1 in DNA damage and repair (469). TAP has also been employed to investigate the interaction between chromatin-modifying enzymes and heterochromatic targeting molecules (470), shedding light on the relationship between chromatin organization and gene expression. A modified TAP, termed DEF-TAP (differential elution fractionation after TAP) has been used to analyze the interacting partners of histone H4 in HeLa cells (471). By incorporating a last purification step of $6 \times \text{His-Ni}^{2+}$, the protein complex can withstand a variety of harsh washing conditions, so that the coverage of the analyzed protein mixture is significantly improved. As a result, they were able to identify some important histone chaperones. Because histone proteins are the key components of chromatin, investigation of these proteins may facilitate our

better understanding of chromatin organization. It will also be interesting to figure out what kinds of proteins have physiological interactions with histones and whether they regulate chromatin through orchestrating the histone function machinery. Given that MS is a powerful technique for the characterization of protein interactions, the proteomic analysis of histone-associated proteins is expected to facilitate the discovery of new epigenetic markers.

Histone H4 is the most highly conserved histone, and makes extensive contacts with the other three core histones in the nucleosome core particle (472). It has no known protein sequence variants, but variants with identical sequence are found to be expressed in a cell cycle-independent manner as opposed to the S phase of the cell cycle, which is the predominant synthesis period for histones (473). As a model system to validate our approach, TAP coupled to mass spectrometric analysis was applied to identify interacting partners of histone H4.1 (Hhf1). We successfully purified all the core histones. Besides, we have isolated a total of 14 proteins as genuine interactors of Hhf1, most of which have DNA-binding properties and chromatin-associated functions. Our results suggest this method is complementary to the classical biochemical or immunoprecipitation strategy. This experiment may serve as a foundation to better understand the compaction and rigidity of the chromatin structure. It may also serve as a starting point to determine the function of each identified protein in a particular biological process, especially through interacting with chromatin.

Figure 6.1. A schematic representation of tandem affinity purification.

The tandem affinity purification (TAP) consists of three components: a calmodulin-binding peptide (CBP), a tobacco etch virus (TEV) protease cleavage site and Protein A as an immunoglobulin G (IgG)-binding domain. Extracts are prepared from cells containing TAP-tagged protein(s) under mild conditions, and TAP is carried out. The first column consists of IgG beads. TEV protease cleaves the immobilized multi-protein complexes. Another round of binding is carried out on a second column that consists of calmodulin beads. The native complex is then eluted by chelating calcium using EGTA. This figure is adapted from Huber *et al.* (474).

6.2. Results

To utilize the TAP system, the construct for the C-terminal TAP tag addition to the target protein, herein, histone H4 (h4.1, or Hhf1), was generated. Since DNA synthesis is in synergic pace with histones, an additional copy of Hhf1 was made to ensure normal cell growth and function. This was achieved by preparing the PCR product including its promoter region of 400 bp, which

added up to ~700 bp (Figure 6.2A). To produce C-TAP-tagged Hhf1, the PCR product was inserted into the vector between the *Cla*I and *Bam*HI sites via electroporation. Colony PCR was used to select transformants containing *hhf1*⁺ (Figure 6.2B). Positive clones with correct orientation of plasmid DNA were confirmed by digestion with *Kpn*I. Since there are two *Kpn*I sites in pJK210-CTAP (Figure 2.3), successful transformation would lead to two restriction fragments of 5.4 kb and 870 bp after digestion, whereas two bands with 5.4 kb and 170 bp indicated the original vector without gene insertion (Figure 6.2C).

Figure 6.2. C-terminal TAP tagging of Hhf1.

(A) PCR-amplified product using primers as described in Materials and Methods, the size of PCR product is 712 bp. (B) Colony PCR to screen for bacterial colonies (lane 1-4) with successful ligation of plasmid C-TAP-Hhf1. Standard PCR reaction was used to determine the size of DNA cloned into vector pJK210-CTAP, purified PCR product (lane P) was recruited as a positive control. (C) RE digestion of the C-TAP-Hhf1 (lane 1-4) and the empty vector pJK210-CTAP (lane E) using *Kpn*I. Undigested C-TAP-Hhf1 (lane U) and purified PCR product (lane P) are included. 1 kb ladder (M) was used for all the electrophoresis, 3 kb, 1 kb and 500 bp are indicated.

After ensuring the protein coding sequence was free of mutation by DNA sequencing (B.4), the positive clones were then introduced into wild-type cells for protein expression. To integrate the tagged gene into the genome, C-

TAP-Hhf1 was linearized with the restriction enzyme *HpaI* for chromosomal integration.

To determine whether the constructs were functional, protein expression of C-TAP-Hhf1 was performed by immunoblotting the lysates using anti-Histone H4, anti- α -protein A and anti-CBP antibodies (Figure 6.3A). To this end, the tagged proteins displayed a mass shift of approximately 21 kDa, consistent with the addition of TAP tags. Both native and tagged Hhf1 were detected using anti-Histone H4. The tagged format was also detected using anti- α -protein A and Anti-CBP. These results suggested that C-TAP-tagged histone H4 was successfully expressed without interfering with its native expression.

TAP was then performed on the strain carrying the TAP tag and wild-type cells, and the protocol was adapted from Ohi *et al.* (256). The various steps in the affinity purification of TAP-tagged complexes were monitored by SDS-PAGE and Western blotting using the TAP tag as an epitope. To investigate whether the Hhf1 protein was really purified, the protein eluates were subjected to immunoblotting by anti-Histone H4 and anti-CBP antibodies (Figure 6.3B). As expected, the protein lysates before applying to IgG matrix displayed molecular mass of approximately 33 kDa (lane 1); and the IgG column eluate after TEV cleavage manifested a decreased mass shift of 17 kDa (lane 3), corresponding to the removal of protein A. The weak signal at ~16 kDa was presumably due to sample dilution, and the two bands above 37 kDa detected by anti-Histone H4 could be caused by non-specific binding introduced from either samples or reagents. Lane 7 and 8 display equal volume of calmodulin affinity column eluate before and after TCA precipitation. The striking enhancement of signal intensity in lane 8 suggested that the proteins of interest

were essentially concentrated. The wild-type cells were recruited as a negative control to ensure that the detected signals were not due to cross-reactivity of the antibodies.

Figure 6.3. Protein expression and purification of TAP-tagged Hhf1. (A) Protein expression of TAP-tagged histone H4. Proteins were extracted from wild-type (-) and hhf1-CTAP (+) cells and resolved on SDS-PAGE. The blot was incubated with monoclonal antibody anti-Histone H4 (upper panel) to detect Hhf1, anti- α -protein A (middle panel) and anti-CBP antibody (bottom panel) to detect TAP-tagged proteins. (B) Purification of TAP-tagged histone H4. Protein lysates were prepared from wild-type (*wt*) and hhf1-CTAP cells. Following purification over IgG sepharose, treatment with TEV protease and subsequent binding and release from calmodulin beads, a small portion from each purification step was resolved on SDS-PAGE and immunoblotted for the presence of hhf1 (upper panel) and CBP1 (bottom panel). Lane 1, cell lysate applied to IgG column from hhf1-CTAP; lane 2, cell lysate applied to IgG column from *wt*; lane 3, IgG column eluate after TEV protease cleavage from hhf1-CBP1; lane 4, IgG column eluate after TEV protease cleavage from *wt*; lane 5, calmodulin affinity column flow-through (FT); lane 6, calmodulin column wash FT; lane 7, calmodulin affinity column eluate before TCA precipitation; lane 8, TCA concentrated calmodulin eluate. Lane 5 to lane 8 all refer to hhf1-CBP1.

A small portion of each eluate was also examined by SDS-PAGE and silver staining as shown in Figure 6.4. From the TCA concentrated calmodulin eluate (lane 6), the pattern of all four core histones (H2A, H2B, H3 and H4) was observed, including the CBP-conjugated H4. Therefore, TAP tagging of histone H4 did not affect the protein expression of the other histones.

Figure 6.4. Silver-stained SDS-PAGE gel of C-TAP-Hhf1 purification. Lane 1, cell lysate applied to IgG column; lane 2, IgG column flow-through (FT); lane 3, IgG column eluate (TEV cleavage); lane 4, calmodulin affinity column FT; lane 5, calmodulin affinity column eluate; and lane 6, TCA concentrated calmodulin eluate. The TCA concentrated proteins were subjected to nanoLC-MS/MS. The core histones (H2A, H2B, H3 and H4) and their corresponding molecular weight are indicated. “H4 (hhf1-CBP)” represents histone H4 conjugated with calmodulin binding peptide (CBP).

In this study, the SDS-PAGE was employed to separate affinity purified protein mixtures prior to mass spectrometric analysis (Figure 6.5A). Gels were sequentially sliced as indicated unless there was no visible band at those positions. Except for the two protein bands around 250 kDa, the parallel mock purification performed from the parental cell line did not yield any significant materials. A total of 22 gel slides were digested with trypsin, and the resulting peptide mixtures were analyzed separately using nanoLC-MS/MS.

Figure 6.5. Proteins identified in the C-TAP-Hhf1. (A) Silver-stained 14% SDS-PAGE gel of proteins associated with TAP-tagged histone H4. TAP purification was performed as described in Materials and Methods, an untagged mock control (no TAP) was included. The gel was divided into 22 protein bands and subjected to proteome analysis individually. The corresponding identified proteins are indicated with red arrows. Although the same proteins could be found in multiple fractions, the proteins were only indicated once. The full lists of identified proteins from C-TAP-Hhf1 and mock control are presented in file 8 and 10 in DVD-R, respectively. (B) Pie chart indicating a high nuclear coverage of proteins identified from Hhf1-TAP. The localization of each associated proteins was assessed using fission yeast yellow fluorescent protein fusion localization database (251).

The peptides significantly matching sequences in the database were used to generate a list of candidate proteins co-purifying with C-TAP-Hhf1. As in TAP purification, the proteins identified with single unique peptide were eliminated to avoid false positive hits. To this end, 151 proteins were identified with validation (file 8 in DVD-R). Within the list, 23 of them were identified as protein groups, meaning the identified peptides are shared by two or more proteins. This kind of purification is always hampered by the highly abundant ribosomal proteins (259). In our case, more than one third of the detection (i.e.,

58 proteins) belonged to ribosomal proteins (red, file 9 in DVD-R), and they should be removed in prior to proceeding downstream analysis.

Within a particular purification, members of this protein complex should be isolated in approximately stoichiometric amounts and relatively enriched compared to the other detected proteins, except for abundant background proteins, such as ribosomal proteins. To distinguish genuine histone H4 interactors from background, the relative abundance (abundance index, or A-index) of each identified proteins was determined as described in (259) (see Materials and Methods). Therefore, we omitted another 31 proteins whose A-indices were below 1.75 (blue in file 9 in DVD-R). Furthermore, the proteins identified from both wild-type (i.e., negative control) and TAP-tagged sample were subtracted to avoid non-specific interactions (green in file 9 in DVD-R). Recently, a list of 263 proteins has been compiled as common background contamination of TAP purifications in *S. pombe* [Table S1 in (259)]. The most dominant contaminants are heat shock proteins, translation factors, and abundant housekeeping metabolic enzymes, majority of which are present in cytoplasm. Based on this list, Rpc40, Tdh1 and Srp72 were discarded although their A-indices were quite high (Purple in file 9 in DVD-R). Although Htb1 (histone H2B) was categorized as a common background protein, we did not ignore this protein. Given that we were isolating the histone protein complex, and its A-index was extremely high (i.e., 22.11), Htb1 is impossible to be a non-specific background protein. In other words, the detection of Htb1 was truly due to the Co-IP with histone H4.

Finally, a list of 14 proteins was generated to represent the proteins specifically interact with histone H4 (Table 6.1 and Figure 6.5A). The output is

reported as the number of independent peptides that correlated significantly to a protein in the database. As expected, all the core histone proteins (H2A, H2B, H3 and H4) were identified with high confidence, consistent with the fact that two of each of the core histones assemble to form one octameric nucleosome core (1). Overall, the four core histone proteins have been identified 932 times, which was consistent with the fact that histones were presented in many different modification forms and have multiple variants in the cell. On the other hand, none of these proteins were detected from mock control (file 10 in DVD-R), suggesting that the purification was specifically associated with histone H4. However, we failed to capture any known histone chaperones in the mixture of the histone H4-associated peptides. This might indicate that the histone assembly complex was disrupted and dissociated from the core histones. Both endogenous and exogenous histone H4 were detected, indicating that histone H4 no longer existed as a dimer with histone H3 in our preparation.

According to the list, more than 70% of these proteins are localized to the nucleus (Figure 6.5B). Based on the protein functions, majority of these nuclear-localized proteins are associated with chromatin (Table 6.2). Although these proteins may not represent the most classical histone-interacting partners, the association with histone H4 should imply their importance in chromatin-related functions.

Table 6.1. List of proteins identified by MS co-purifying with *S. pombe* C-TAP-Hhf1.

Subunit	Systematic name	MW (kDa)	A-index ^a	Unique peptides	Total spectra	Sequence coverage [%]
Hhf1(Hhf2/Hhf3)	SPAC1834.03c (SPBC8D2.03c/SPBC1105.12)	11.4	55.33	12	664	53.4
Htb1	SPCC622.09	13.8	22.11	9	199	35.7
Sap1	SPCC1672.02c	29.1	4.13	8	33	31.1
Hta2(Hta1)	SPAC19G12.06c (SPCC622.08c)	13.7	9.00	4	36	29.8
Hht1(Hht2/Hht3)	SPAC1834.04 (SPBC8D2.04/SPBC1105.11c)	15.3	46.00	7	322	28.7
Rim1	SPAC2F3.04c	17.1	5.00	3	15	20
Tpi1	SPCC24B10.21	27.2	2.33	3	7	12.9
Sty1	SPAC24B11.06c	40.2	3.00	2	6	5.4
--- ^b	SPCC736.03c	50.1	19.00	2	38	4.2
Sec6	SPCC1235.10c	83.3	3.00	3	9	4
Psd3	SPAC31G5.15	109.7	2.33	3	7	3.5
Mis4	SPAC31A2.05c	180.2	2.50	2	5	2.4
Dna2	SPBC16D10.04c	157.7	9.00	2	18	2.4
--- ^b	SPCC1183.11	114.2	12.50	2	25	1.7

Note: Only proteins identified with at least two unique peptides were included. The columns correspond to their names, systematic names, abundance indices, theoretical molecular weights, unique peptides, total spectra and sequence coverage from LC-MS/MS analysis. The table is ordered from high to low sequence coverage. ^a A-index (Relative Abundance Index) was calculated as a ratio between the total number of MS/MS spectra and the number of unique peptides matched to the protein sequence; ^b No gene symbol is assigned to the corresponding gene, because the function of the gene is based on prediction or dubious. For the complete list, please see file 8 in DVD-R.

Table 6.2. Localizations and functions of the proteins identified by MS co-purifying with *S. pombe* C-TAP-Hhf1.

Subunit	ORF No.	Localization	Main function
Hhf1(Hhf2/ Hhf3)	SPAC1834.03c (SPBC8D2.03c/ SPBC1105.12)	nuc	nuclear nucleosome; chromatin assembly or disassembly
Htb1	SPCC622.09	nuc	nuclear nucleosome; chromatin assembly
Sap1	SPCC1672.02c	nuc	mating-type switching; chromosome organization; replication fork arrest
Hta2	SPAC19G12.06c (SPCC622.08c)	nuc	nuclear nucleosome; chromatin assembly
Hht1(Hhf2/ Hht3)	SPAC1834.04 (SPBC8D2.04/ SPBC1105.11c)	nuc	nuclear nucleosome; chromatin assembly or disassembly
Rim1	SPAC2F3.04c	cyt	mitochondrial DNA replication; mitochondrial genome maintenance
Tpi1	SPCC24B10.21	cyt-nuc	carbohydrate biosynthesis; gluconeogenesis
Sty1	SPAC24B11.06c	cyt-nuc	positive regulation of mitotic cell cycle; regulation of histone acetylation
---	SPCC736.03c	ND	mitochondrial translation; phenylalanyl-tRNA aminoacylation
Sec6	SPCC1235.10c	nuc	cytokinetic cell separation
Psd3	SPAC31G5.15	cyt	phosphatidylcholine biosynthetic process; regulation of cell cycle cytokinesis
Mis4	SPAC31A2.05c	cyt-nuc	cohesin loading factor; sister chromatid cohesion
Dna2	SPBC16D10.04c	cyt-nuc	DNA replication and repair; telomere maintenance
---	SPCC1183.11	cyt	ion transmembrane transport

Note: The columns correspond to gene symbols, systematic names, localizations, and main functions. The table is ordered the same as Table 6.1. * No gene symbol was assigned to the corresponding gene, because the function of the gene is based on prediction or dubious. For the complete list, please see file 8 in DVD-R.

Except for the core histone subunits, Sap1 is the most confident hit. It is an essential DNA-binding protein responsible for efficient mating-type switching and chromosome stability. Lack of this protein hampers chromosome cohesion and condensation process during cell cycle (475, 476). It also binds to replication fork barrier 1 (RFB1), and leads to replication fork blockage (477).

As mentioned, Sty1 is a MAP kinase that transmits osmotic and other stress signals, requiring for mitotic checkpoint control in response to actin cytoskeletal damage (426, 478, 479). Although it is not constitutively present in the nucleus, its role in chromatin remodelling and histone modification might explain the Co-IP of Sty1 with histone H4 (263).

Another two identified proteins are also found to be associated with chromatin. The cohesion loader Mis4 is a structural CAP responsible for establishing sister chromatid cohesion (480). In fission yeast, mutation of Mis4 is synthetically lethal with HDAC deletion mutations (481). Mis4 mutant cells confer hypersensitivity to the class I and II HDAC inhibitor trichostatin A (TSA) (482); and TSA treatment results in decreased chromatin-bound cohesins in the chromosome arm region of Mis4 mutant cells. It also binds many chromatin-modifying enzymes such as Clr3 and Hos2, which might explain its coprecipitation with Histone H4.

Dna2 is a DNA2/NAM7 helicase family member required for DNA replication. It has a direct role in coordinating the multienzyme process of the Okazaki fragment elongation and maturation (483). Dna2 mutant cells exhibit aberrant mitosis when combined with checkpoint mutations. Furthermore, ChIP assay has revealed that Dna2 binds to telomere DNA for generating the telomeric single-strand overhang (484).

A mitochondrial protein was identified with high confidence, supporting the fact that histones are dual-targeted proteins that can be found in both nucleus and mitochondria (485, 486). As a single-stranded DNA binding (SSB) protein, Rim1 is involved in mitochondrial DNA replication, and its ortholog in *S. cerevisiae* is essential for maintaining mitochondrial genomic stability (487, 488). Rim1 is also likely to play a role in DNA repair and recombination (489).

Interestingly, an exocyst complex subunit Sec6 appeared in our list. Previous studies suggested that Sec6 is present in SPB and crucial for cytokinetic cell separation in *S. pombe* (490). An unexpected finding was Tpi1, which is a triosephosphate isomerase involving in various biosynthetic events (491). Other findings were more unexpected, including Cc736.03c, Psd3 and Cc1183.11. Whether they are *bona fide* histone H4 interacting proteins need to be further confirmed by Western blotting.

6.3. Discussion

TAP is a powerful strategy to identify novel components from complex assemblies up to the whole proteome. As a pilot study, the TAP approach was applied to obtain the affinity-purified histone H4 complex, gel electrophoresis was used to fractionate the proteins followed by in-gel digestion and mass spectrometric identification. We have perfectly isolated all the core histone members. Some novel histone H4-interactors are also discovered, which are related to a series of chromatin-associated functions, such as DNA replication, cell cycle regulation and chromatin organization. Although the known structural and functional properties of the isolated proteins have established a propensity

to be associated with Hhf1, the large scale pull-down is always not compatible with immunoprecipitation. To ensure accuracy, sequential tagging and immunoblotting of candidate interactors should be performed to reciprocally validate these interactions.

In this study, we utilized a newly developed procedure (259) to filter the data. Ribosomal proteins should always be removed from the list at the beginning of the data processing. The common background proteins were sorted out and distinguished from proteins specifically enriched in the TAP isolation using A-index. A-index is a semi-quantitative estimates of protein abundance, indicating the average number of MS/MS events triggered by the precursor ion matched to a given protein sequence. To place our priority upon accuracy, we have employed this stringent concept to clear up the protein list. The utilization of A-index facilitates a fast and substantial identification of interacting partners without further confirmation using immunoblotting with specific antibodies. The drawback is some potential histone H4 interacting partners might have been removed according to the A-index (259). For instance, we removed Set6, a histone lysine methyltransferase which should be a genius histone chaperone. Therefore, the cut-off of A-index at 1.75 is empirical and arbitrary in our scenario. To solve this problem, we should introduce biological and technical replicates to ensure our identified proteins are truly associated with histone H4.

We realize that our TAP fails to identify the known interacting partners of Hhf1 in *S. pombe* (492), and this could be due to several reasons. First, some histone chaperones are low in abundance, so that their identifications have been impeded by those of highly abundant proteins; for example, the histone H3 methyltransferase Clr4 has extremely low expression which is undetectable

using protein reverse array (251). Second, the conditions we used to purify the protein complex are not optimal. To solubilize the DNA-protein complex, we conducted the immunoprecipitation of histone H4 complex using conventional protocols under denaturing condition. However, the large DNA-protein macrocomplexes are easily lost during clarification step as they pellet along with the DNA. As a result, most of the proteins found to be associated with DNA might not be available for subsequent immunoprecipitation step, and would have escaped from being identified (269, 271). Third, buffer conditions, especially the salt concentration in elution and washing buffers, are critically important to isolate and differentiate *bona fide* interactors of histone H4 from non-specific ones. On the one hand, low salt in elution buffer is not efficient to elute the proteins which are tightly associated with calmodulin beads. On the other hand, low salt in washing buffer is not strong enough to remove highly abundant contaminants, but can preserve weak interacting partners. It has been claimed that histone H4 incorporated into chromatin is not effectively extracted with salt below 400 mM (471). Given that we applied physical salt concentration (i.e., 150 mM NaCl) to wash the protein complex, it is plausible that the identified proteins are more prone to interact with non-chromatin bound histones; so that we were unable to identify histone chaperones which are mainly associated with deposition complex. Last but not least, the expression of histone and histone chaperones are controlled in a cell cycle-dependent manner, in which the protein levels are low during the G2/M phase and peak during S phase of the cell cycle (493). For example, Asf1 is highly expressed during S phase, because this protein is required for acetylation of H3K56, loss of which

causes DNA damage sensitivity (494). Therefore, the inability of detecting Asf1 could be due to its low abundance at the G2/M boundary.

Given that we used conventional immunoprecipitation protocol to purify histone H4, the identified protein complex may only represent soluble fraction of CAPs. Therefore, the protocol requires optimization to seek a balance between specific and non-specific interactions without losing weak interacting partners. To retain chromatin-bound protein complex in solution, mChIP could be an alternative good choice (211). This method involves a single affinity purification step, in which the conventional ChIP is modified by performing a mild DNA shearing step in prior to gentle centrifugation, so that the integrality of chromatin-bound complexes are maintained. Therefore, this method is superior to enhance the solubility of DNA-protein complex without drastically reducing the number of associated proteins purified with the bait of interest. To expand the knowledge of H4 interaction networks, the Histone Association Assay may serve as a straightforward platform to detect proteins that bind to chromatin *in vivo* (210). This technique involves ChIP by using histone H3- or H4-specific antibodies to precipitate bulk chromatin from crosslinked WCE. It takes the advantage of isolating associated protein without considering its innate solubility. Furthermore, this assay enriches not only H4 interactors, but also chromatin DNA interactors, which may help better analyze the architectures and functions of chromatin.

We noted that about 40% of the identified proteins are ribosomal subunits. During MS analysis, highly abundant ribosomal and background proteins are great confounding factors that severely interfere with the identification of chromatin-bound proteins with low abundance. The common

contaminants from human (e.g., keratin) also distract the real protein hits from being detected. To ameliorate this problem, we can introduce a “precursor ion exclusion list” to exclude the m/z values of the peptides belonging to the highly abundant background contaminants. To this end, the exclusion list is a “blacklist” to prevent those “ m/z ” from being acquired during subsequent runs, so that low abundant peptides will gain more opportunities to be identified. If experimental replicates are involved, the “precursor ion exclusion list” can be extended by flagging all identified peptides in preceding runs, which spares the MS capacity to identify additional peptides.

Overall, we have identified a series of novel CAPs which potentially interact with Hhf1. Further investigations are needed to confirm these physical interactions and characterize their functional associations with chromatin if the interactions are real. Considering the low abundance and high insolubility of DNA-binding proteins, we chose gel-based approach to resolve the purified protein complex. Howbeit identification of proteins from polyacrylamide gels offers a number of important advantages (495), this approach has disadvantages including poor separation, sample carryover, and sample loss during gel electrophoresis or extraction. Gel-based method is also unfavourable for proteins with extremely high or low molecular weight, and those with diffused migration or poor binding. To conquer the problem associated with gel fractionation, we should also apply an improved technique by combining TAP approach with MudPIT, which is also termed TAP/DALPC (Direct Analysis of Large Protein Complexes).

CHAPTER 7. GENERAL CONCLUSION

7.1. Conclusion of Chapter 3

CAPs are important for functions such as chromatin remodelling, transcription, DNA replication and repair (137, 148, 257, 263). Malfunctioning of CAPs leads to a series of devastating diseases, including cancer. Most of non-histone CAPs were identified based on the availability of specific antibodies or nucleotide probes. We aim to know whether non-histone CAPs can also be identified without prior knowledge. However, traditional chromatin fractionation assay cannot effectively deplete the highly abundant non-CAPs. To resolve this issue, we have coupled the chromatin fractionation assay to the SILAC-mediated proteomic approach, which is used to compare the relative protein levels before and after chromatin fractionation. By using the ratio between chromatin extract and WCE, we have shown that the ribosomal proteins are effectively depleted from the top SILAC-ranked proteins. This result suggests SILAC-mediated proteomic analysis of chromatin extract allow identification of non-histone CAPs without prior knowledge. Consistent with this, we have shown that the nucleus-localized proteins (i.e., potential CAP candidates) are greatly enriched in the top SILAC-ranked proteins, and only the SILAC-enriched nucleus-localized proteins can be real non-histone CAPs. Significantly, the SILAC-enriched nucleus-localized proteins exhibit the high likelihood of requirement for growth fitness in DNA damage stress. Therefore, our results suggest that this strategy is capable of identifying non-histone CAPs

without prior knowledge. To our knowledge, this work is the first attempt to perform the high-throughput analysis of non-histone CAPs without assistance of protein antibodies or DNA probes using quantitative proteomic approach.

For chromatin fractionation assays, the advantages of nuclease digestion and high-salt extraction complement each other. We should combine these two or more means to expand the power of our SILAC-mediated analysis by rescuing those relatively insoluble non-histone CAPs, which have escaped from our previous analysis. Furthermore, chromatin fractionation assay can be extended to enrich non-histone CAPs specifically associated with euchromatin or heterochromatin (224). Given that euchromatin is lightly packed with more nucleotides exposed outside, it is more accessible to nuclease digestion than heterochromatin (496). By using DNase I digestion, only euchromatin but not heterochromatin should be enriched, provided that the nuclease activity has been quenched and additional centrifugation steps have been involved (497). This is based on the fact that euchromatin is predominantly digested to mononucleosomes, while heterochromatin is mainly digested into larger DNA oligomers (224). Chromatin states partially rely on differential histone PTMs, which could be a hallmark to indicate that the two types of chromatin have been separated from each other. For example, acetylation of histone H3 and H4 is generally correlated with active euchromatin; while trimethylation of histone H3 on lysine 9 or 27 is correlated with silent heterochromatin (498-500).

Based on the growth assays, the chance of obtaining proteins involved in DNA replication and repair is significantly higher from the SILAC-enriched proteins than that from genome-wide screening. Mutations in these proteins can interfere with genomic stability, and the corresponding human orthologs might

be relevant to malignant tumour formations. In budding yeast, SILAC has been used to analyze compositional changes of chromatin in response to DNA damage (501). As such, this strategy may provide information about normal and pathological changes to fission yeast chromatin, provided that the effects on chromatin composition of mutations in genomic stability have been tested.

7.2. Conclusion of Chapter 4

A novel HIRA protein Hip4, the histone chaperone requiring for chromatin assembly, has been significantly enriched in our SILAC analysis, and its chromatin association has been confirmed by traditional chromatin fractionation assay. As mentioned, chromatin fractionation assay can be improved by recruiting additional steps to separate euchromatic from heterochromatic components. Therefore, it is interesting to check whether Hip4 is present in euchromatic or heterochromatic fraction. The presence of Hip4 on heterochromatin and absence from euchromatin will provide direct evidence to suggest this protein is functionally related to heterochromatin. Further experiment such as ChIP-chip or ChIP-seq should be applied to locate its binding region(s) to heterochromatin.

We have shown that Hip4 has a global role in maintaining genomic stability, confirming a connection between the DDR and chromatin modifications. We have noted that deletion of *hip4*⁺ does not abolish the signalling transduction of DSB-induced G2/M DNA damage checkpoint in the wild-type genetic background, suggesting Hip4 is dispensable for sensing DNA damage at early stage. Therefore, it will be interesting to know whether this

protein is required for restoration of chromatin structure after DNA damage and during DNA repair. Recently, most studies investigate DDR and chromatin assembly separately. To gain better knowledge about the relationship between DDR and chromatin structure associated with Hip4, a systematic approach should be employed by combining genetic screening, transcriptional profiling, and proteomic analysis. These techniques may serve for better understanding of the chromatin structural modifications in response to genomic insults and the spatiotemporal organization of the DDR.

7.3. Conclusion of Chapter 5

Recently, the Class III Zn(II)₂Cys₆ zinc finger proteins have been extensively studied in *S. cerevisiae* (369), while the understanding of their roles in *S. pombe* is still poor. From the SILAC top-ranked protein, we selected a novel zinc cluster protein Bc530.05, for subsequent investigation. We have found that *BC530.05Δ* cells display unique hypersensitivity to CPT, which may indicate its defect to maintain the structure of replication fork. However, the precise role of Bc530.05 in protecting the replicative intermediates remains to be determined. To this end, the comet assay would be straightforward to measure the heterogeneity of *BC530.05Δ* in response to different DNA damaging agents, and reveal the associated DNA damage-induced replication fork regression (502). It is also important to investigate whether *BC530.05⁺* is involved in preventing replication fork collapse or repairing broken replication forks. This piece of information may help to dissect the chromatin structure alteration before, during, and after DNA repair.

We have confirmed that Bc530.05 is required for cellular tolerance to caffeine and toxic cations, but we could not observe significant protein activation/accumulation or obvious protein modifications for combating these stresses. In *S. pombe*, the stress response proteins are activated via translocation from cytoplasm to nucleus. For example, the transcription factor Pap1 is translocated into the nucleus upon induction by oxidative stresses, which concomitantly activate an adaptive anti-oxidant response (430, 503). Therefore, it will be interesting to investigate whether Bc530.05 is translocated from cytoplasm to nucleus in response to the toxicities.

Based on our analysis, we hypothesize Bc530.05 may function as a transcriptional regulator of calcineurin, which is responsible for maintaining cell integrity. Recent reports also revealed the involvement of calcineurin in antifungal tolerance, cell morphogenesis and virulence. Therefore, additional tools are required to decipher the precise roles for this protein in cellular homeostasis; for example, Microarray or RNA-seq. As crucial regulators of fungal physiology, more and more zinc cluster proteins have been identified and characterized. Discovery of these proteins is of great importance for eukaryotic transcription. Furthermore, their functions may also provide mechanistic insights into the disease prevalence in human fungal pathogens.

7.4. Conclusion of Chapter 6

In this study, we have employed TAP to successfully identify all the core histone proteins and some interesting non-histone CAPs. Proteins associated with chromatin DNA have the dual nature of participating in conventional

soluble protein-protein interactions as well as participating in DNA-protein macrocomplexes. To obtain a holistic view of the physical interaction with histone H4 and gain better knowledge about epigenetic information encoded by histones, we may also adopt the newly developed mChIP using histone antibodies, allowing the purification of histone interactors which are less abundant and less soluble. The identified CAPs may facilitate expanding our knowledge about chromatin network. Understanding their interactions with histone H4 may provide new mechanistic insights into histone metabolisms and chromatin organization.

To our knowledge, although the TAP strategy has been well-established in the distantly related fungi *S. cerevisiae*, no systematic biochemical isolation of protein complex has yet been performed in *S. pombe*. Nowadays, TAP coupled to MS has been a very useful approach to identify the proteomic organizations and link proteins to certain pathways. Worth of mentioning, TAP coupled to MS can expand its power to a systematic analysis, which will facilitate proteome-wide characterization of protein interaction networks.

7.5. Future perspectives

Not only DNA-binding proteins, but also RNA-binding proteins are frequently identified from our SILAC analysis, including ATP-dependent RNA helicases and U3 small nucleolar ribonucleoprotein (snoRNP). Since RNA-binding proteins are highly associated with chromatin for their functions (264), investigation of RNA-binding CAPs might provide the mechanistic insight into the connection between RNA and chromatin worlds. In both fission yeast and

higher eukaryotes, the RNAi machinery is recognized to nucleate the formation of constitutive heterochromatin. The outbreak of ncRNA studies and the description of new mutations in the epigenetic machinery have highlighted the increasing importance of RNA and RNA-mediated regulation in the epigenetic field (504-508). Therefore, it would be of great value to also investigate the RNA-interacting proteins in the context of chromatin. Recently, a so called “quantitative RNA pull-downs” have been applied to examine the protein interactions that are mediated by ncRNA in the nuclear environment of mammalian cells (509).

Chromatin biology is one of the most popular field in which intensive research efforts are being spent. Given that the chromatin structure and dynamics are highly conserved from fission yeast to human, our discovery provides a platform to unravel the mechanisms of chromatin-associated factors, which may also hold great promise for building novel approaches to combat human diseases, such as cancer. Recent MS-based proteomics has gained tremendous interest in investigating diseases processes (510). By involving this highly sensitive technology, the knowledge gaps between disease pathogenesis and therapeutic strategies can be filled. Meanwhile, more convenient diagnostic methods and effective remedies will be developed for the treatment of devastating human diseases.

APPENDIX A. SUPPLEMENTAL MATERIALS AND METHODS

A.1 General rules of fission yeast nomenclature

In this thesis, standard conventions were used to denote gene and protein names in the fission yeast *S. pombe*. For wild-type cells, gene names were expressed as a three italicized letter, followed by a number, and a plus superscripted in normal text (e.g., *rad3*⁺). For deletion mutant, the plus was replaced by symbol delta (e.g., *rad3*Δ) as short hand. The rules of expressing mutant in the strain tables are: 1) for deletion alleles constructed with an insertion of uracil (*ura4*⁺) or kanamycin (*kan*⁺) was written as *rad3*Δ::*ura4*⁺ or *rad3*Δ::*kan*⁺; 2) for deletion alleles constructed without an insertion, we used an allele designation beginning with D (e.g., *ura4-D18* represents a deletion of *ura4*⁺), or with some numbers (e.g., *leu1-32* is a allele-specific mutation). Proteins were indicated by roman text with the first letter capitalized (e.g., Rad3). For those proteins without protein symbols, their systematic names were used instead, and “SP” was omitted in the text to save the space (e.g., the wild type, deletion and protein were written as *BC530.05*⁺, *BC530.05*Δ and Bc530.05, respectively); specifically, the deletion mutants in Figure 3.9 were expressed by keeping only one capital letter in front of the number (e.g., *BC530.05*Δ was written as *C530.05*Δ).

A.2 Construction of HA-tagged strain

A PCR-mediated gene-disruption protocol was adapted to construct HA tagged strains (511). Briefly, the uracil (*ura4⁺*)-containing PCR-fragments, flanked by the 3'-end sequence minus the stop codon of target genes fused to a 3HA-6his sequence in the vector pBS(SK)-3HA6HIS-(lp)*ura4⁺*(lp), were transformed into LJY188 to generate C-terminal tagged -3HA-6His strains. All constructed alleles were verified by primer-specific PCR assays, appropriate tagging of strains was confirmed by immunoblotting (Figure B.2). The oligonucleotides used for HA tagging and validation are listed in Table A.2.

A.3 Construction of deletion mutant strain

A PCR-mediated deletion approach was used to construct targeted open reading frame (ORF) deletion mutants (236). In brief, each synthesized primer (Integrated DNA Technologies, Coralville, IA; Table A.2, Disruption primers) contains 80 nucleotides complementary to a particular ORF's flanking regions, followed by a barcode (20 nucleotides in length) and finally 18 nucleotides homologous to a *ura⁺* or kanamycin (*kan⁺*) selection marker cassette. About 16 µg of the synthesized PCR fragment specific to a particular ORF was transformed into either *h⁻* or *h⁺* haploid cells by the lithium acetate method (243). *ura⁺* or *kan⁺* colonies were then selected on EMM agar plates lacking uracil or containing 120 µg/ml of G418 (Geneticin[®], Invitrogen). Colonies were examined using allele-specific primer pairs (Table A.2, Validation primers for deletion) to ensure deletion and replacement of the target gene by the *ura4⁺* or *kan⁺* gene.

A.4 P-value calculation using binomial test

Based on the SILAC ratio, the occurrence of ribosomal proteins and nucleus-localized proteins in the top ~10% (or second top 10%), and background level were calculated. The null hypothesis was used to test whether the two categories are equally likely to occur, and the p-values were calculated in the software R2.14.0 using the one-tailed test.

A.5 Statistical analysis of protein enrichment

Based on the ranking (i.e., protein ratio or abundance), proteins were divided into twenty percentile intervals (5th percentile, 10th percentile, 15th percentile, etc). To determine the fold enrichment of nucleus-localized proteins in each interval, the odds ratio of observed protein number belonging to this group over the background level was calculated; the background level is the appearing frequency of nuclear proteins in the corresponding protein list. The same algorithm was applied to cytoplasm-localized, dual-localized and ribosomal proteins. For GO enrichment analysis, the GO term Biological Process (BP) was considered. Any term with less than five associated proteins were filtered out. The fold enrichment of each GO term was determined in the same way.

A.6 ¹⁵N labelling

To prepare ¹⁵N-containing heavy medium, unlabelled NH₄HCl was replaced with ¹⁵N-labelled NH₄HCl (NLM-467-5, Cambridge Isotope

Laboratories) in EMM2 without adding amino acid supplements (^{15}N -labelled medium for short). To test whether the autotrophic strain (LJY95) can grow normally in ^{15}N -labelled medium, cells were cultured overnight at 30°C, refreshed in both unlabelled and ^{15}N -labelled medium to $\text{OD}_{600}\sim 0.1$, and the cell growth was monitored every 2 h over a 10-h period.

For testing the incorporation efficiency of ^{15}N into proteins in *S. pombe*, the overnight liquid cultures from unlabelled medium were inoculated into ^{15}N -labelled medium to refresh the cells to $\text{OD}_{600}\sim 0.4$. Once the OD_{600} reached 0.8, half of the cells were harvested and designated as DT1 (i.e., one doubling time), the other half were diluted with equal volume of ^{15}N -labelled medium and were cultured for another DT (DT2). The passage was continued in ^{15}N -labelled medium to obtain cultures up to DT6. The cells of $\text{OD}_{600}\sim 0.8$ collected from unlabelled medium (DT0) were used as normal sample without ^{15}N labelling. Cell lysates from each sample were prepared by beads beating (2.5.1). For testing the accuracy of quantitative analysis, normal (DT0) and ^{15}N -labelled cell lysates (DT6) were adjusted to equal concentration, and then mixed with various volumes (4:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:4) to generate the pre-mixed protein samples with expected ratio between the light and heavy peptides or proteins. Protein samples from both experiments were subjected to SDS-PAGE, in-gel digestion and nanoLC-MS/MS as previously described in 2.5.

The IP2 (version 1.01, Integrated Proteomics Inc, San Diego, California, USA) was used to perform data analysis from database searching to peptide/protein quantification (Figure A.1). The identified and quantified peptides/proteins are listed in file 12 and 13 in DVD-R.

Figure A.1. A schematic overview of the IP2 workflow.

All RAW spectra files were converted to the ms1 and ms2 format. ms2 files were used for peptide and protein identification, and ms1 files were used for quantification. Uninterpreted MS/MS spectra were searched against the *S. pombe* protein databases by ProLuCID in IP2, the search parameters were as follows: the requirement of tryptic cleavage on both ends of a peptide (allowing 1 missed cleavage site), minimum peptide length of 7 amino acids, maximal precursor ions mass deviation of 10 ppm, peptide mass tolerance of ± 0.5 Da, static modification on Cys of +57.0215 Da, differential modification on Met of 15.9945 Da. To validate identified peptides and proteins, DTASelect 2.0 were used for validation and quantification of identified peptides and proteins, the parameters were adjusted to ensure the peptide FDR < 1% and the protein FDR < 5%. Census was employed to quantify ^{15}N labelling by selecting “high resolution mode”.

Table A.1. Bioneer deletion strains used in this study.

Strain	Relevant genotype ^a	BG ID ^b	Plate ^c	Position ^d
1	<i>urg3Δ::kan⁺</i>	BG_0035H	S22	D06
2	<i>SPAC1071.02Δ::kan⁺</i>	BG_0061H	S20	H04
3	<i>get3Δ::kan⁺</i>	BG_0100H	S15	E08
4	<i>swr1Δ::kan⁺</i>	BG_0121H	S3	C07
5	<i>rpl32Δ::kan⁺</i>	BG_0150H	S20	F05
6*	<i>SPAC13A11.06Δ::kan⁺</i>	BG_1625H	S6	E04
7	<i>rps11Δ::kan⁺</i>	BG_0241H	S23	B06
8	<i>gcv2Δ::kan⁺</i>	BG_0245H	S24	D02
9	<i>rps61Δ::kan⁺</i>	BG_0246H	S30	E06
10	<i>ssa1Δ::kan⁺</i>	BG_0256H	S28	D08
11	<i>ade2Δ::kan⁺</i>	BG_0279H	S24	D08
12	<i>SPAC1486.01Δ::kan⁺</i>	BG_0295H	S5	H09
13	<i>atp1Δ::kan⁺</i>	BG_0318H	S28	B07
14	<i>sdh1Δ::kan⁺</i>	BG_0324H	S24	E09
15	<i>cdd1Δ::kan⁺</i>	BG_0326H	S24	E10
16	<i>pmm1Δ::kan⁺</i>	BG_0329H	S30	B10
17	<i>SPAC15A10.07Δ::kan⁺</i>	BG_0345H	S24	F03
18	<i>SPAC1687.21Δ::kan⁺</i>	BG_0408H	S6	A02
19	<i>SPAC17G6.15cΔ::kan⁺</i>	BG_0536H	S1	C04
20	<i>SPAC17H9.14cΔ::kan⁺</i>	BG_0569H	S4	H08
21	<i>cki3Δ::kan⁺</i>	BG_0579H	S3	C12
22	<i>shm2Δ::kan⁺</i>	BG_0633H	S25	A01
23	<i>rps7Δ::kan⁺</i>	BG_0642H	S28	G03
24	<i>SPAC19G12.09Δ::kan⁺</i>	BG_0706H	S27	D09
25	<i>plb1Δ::kan⁺</i>	BG_0717H	S21	H05
26	<i>kap95Δ::kan⁺</i>	BG_0726H	S28	G11
27*	<i>wis2Δ::kan⁺</i>	BG_0736H	S27	B06
28	<i>SPAC1F12.02cΔ::kan⁺</i>	BG_0772H	S5	C08
29*	<i>mug161Δ::kan⁺</i>	BG_0789H	S30	H05
30	<i>fio1Δ::kan⁺</i>	BG_0811H	S25	C09
31	<i>SPAC20G8.09cΔ::kan⁺</i>	BG_0840H	S30	H08
32	<i>lys3Δ::kan⁺</i>	BG_0894H	S5	E04
33	<i>dak1Δ::kan⁺</i>	BG_0905H	S27	E12
34*	<i>SPAC22A12.16Δ::kan⁺</i>	BG_0910H	S25	D08
35	<i>rps12Δ::kan⁺</i>	BG_0984H	S30	E07
36	<i>SPAC23A1.17Δ::kan⁺</i>	BG_1001H	S30	F09
37*	<i>cdb4Δ::kan⁺</i>	BG_1098H	S26	B07
38	<i>SPAC24H6.11cΔ::kan⁺</i>	BG_1139H	S1	C03
39	<i>his1Δ::kan⁺</i>	BG_1176H	S26	C12
40	<i>SPAC26H5.02cΔ::kan⁺</i>	BG_1221H	S17	E05
41	<i>SPAC27D7.06Δ::kan⁺</i>	BG_1239H	S4	A06

Table A.1. Bioneer deletion strains used in this study (continued).

Strain	Relevant genotype^a	BG ID^b	Plate^c	Position^d
42*	<i>SPAC27F1.06cΔ::kan⁺</i>	BG_1264H	S17	G04
43	<i>SPAC29A4.02cΔ::kan⁺</i>	BG_1287H	S23	D07
44	<i>pom1Δ::kan⁺</i>	BG_1353H	S3	A02
45	<i>SPAC2F7.09cΔ::kan⁺</i>	BG_1359H	S2	C12
46	<i>SPAC30C2.04Δ::kan⁺</i>	BG_1403H	S27	G05
47	<i>gef2Δ::kan⁺</i>	BG_1438H	S16	C10
48	<i>rps111Δ::kan⁺</i>	BG_1444H	S27	G11
49	<i>SPAC31G5.04Δ::kan⁺</i>	BG_1445H	S29	G04
50	<i>gcv1Δ::kan⁺</i>	BG_1456H	S6	B10
51	<i>tps1Δ::kan⁺</i>	BG_1474H	S18	C08
52	<i>SPAC32A11.02cΔ::kan⁺</i>	BG_1484H	S18	C09
53	<i>SPAC3A11.07Δ::kan⁺</i>	BG_1512H	S6	C05
54	<i>rpl21Δ::kan⁺</i>	BG_1528H	S25	H11
55	<i>SPAC3C7.07cΔ::kan⁺</i>	BG_1543H	S23	A11
56	<i>obr1Δ::kan⁺</i>	BG_1550H	S17	C07
57	<i>abc2Δ::kan⁺</i>	BG_1562H	S15	G03
58	<i>rpl72Δ::kan⁺</i>	BG_1618H	S27	A07
59	<i>spo2Δ::kan⁺</i>	BG_1634H	S26	D07
60	<i>isp6Δ::kan⁺</i>	BG_1646H	S2	B11
61	<i>itr1Δ::kan⁺</i>	BG_1699H	S1	D01
62	<i>rpl91Δ::kan⁺</i>	BG_1743H	S6	G01
63	<i>SPAC513.07Δ::kan⁺</i>	BG_1769H	S6	G08
64*	<i>pabpΔ::kan⁺</i>	BG_1822H	S30	E05
65	<i>adh4Δ::kan⁺</i>	BG_1842H	S6	H11
66	<i>SPAC607.06cΔ::kan⁺</i>	BG_1856H	S2	A01
67	<i>acp2Δ::kan⁺</i>	BG_1875H	S21	D05
68	<i>cit1Δ::kan⁺</i>	BG_1970H	S18	E03
69	<i>SPAC6C3.08Δ::kan⁺</i>	BG_1974H	S1	F03
70	<i>idp1Δ::kan⁺</i>	BG_2017H	S12	H06
71	<i>ndk1Δ::kan⁺</i>	BG_2071H	S5	B08
72	<i>SPAC821.05Δ::kan⁺</i>	BG_2078H	S28	E12
73	<i>dmc1Δ::kan⁺</i>	BG_2156H	S1	H06
74	<i>SPAC8E11.10Δ::kan⁺</i>	BG_2151H	S18	F01
75*	<i>msh3Δ::kan⁺</i>	BG_2161H	S3	A04
76	<i>fas1Δ::kan⁺</i>	BG_2198H	S21	H04
77	<i>rps43Δ::kan⁺</i>	BG_2207H	S28	G01
78*	<i>SPAC977.14cΔ::kan⁺</i>	BG_2223H	S7	F02
79*	<i>SPAC9G1.05Δ::kan⁺</i>	BG_2247H	S20	G05
80	<i>SPAC9G1.10cΔ::kan⁺</i>	BG_2252H	S21	A09
81	<i>SPACUNK4.10Δ::kan⁺</i>	BG_5177H	S12	B04
82	<i>SPACUNK4.16cΔ::kan⁺</i>	BG_5171H	S13	H10

Table A.1. Bioneer deletion strains used in this study (continued).

Strain	Relevant genotype^a	BG ID^b	Plate^c	Position^d
83	<i>tpp2Δ::kan⁺</i>	BG_2298H	S21	G10
84	<i>SPAPB24D3.08cΔ::kan⁺</i>	BG_5265H	S14	C01
85	<i>lys4Δ::kan⁺</i>	BG_2354H	S7	H10
86	<i>SPBC1198.08Δ::kan⁺</i>	BG_2398H	S8	A09
87	<i>mrt4Δ::kan⁺</i>	BG_2419H	S26	H08
88	<i>spf38Δ::kan⁺</i>	BG_2452H	S11	A10
89	<i>SPBC12C2.07cΔ::kan⁺</i>	BG_2465H	S18	G01
90	<i>dnm1Δ::kan⁺</i>	BG_2464H	S22	A01
91	<i>glo1Δ::kan⁺</i>	BG_3130H	S19	A10
92	<i>fkbp39Δ::kan⁺</i>	BG_2484H	S4	G12
93	<i>SPBC13G1.02Δ::kan⁺</i>	BG_2513H	S23	F05
94	<i>myo1Δ::kan⁺</i>	BG_2537H	S21	H11
95	<i>kap123Δ::kan⁺</i>	BG_2559H	S29	A10
96	<i>pho2Δ::kan⁺</i>	BG_2601H	S21	B01
97*	<i>rps122Δ::kan⁺</i>	BG_2643H	S8	D01
98	<i>SPBC16A3.02cΔ::kan⁺</i>	BG_2675H	S14	F02
99	<i>vps132Δ::kan⁺</i>	BG_2678H	S29	B04
100	<i>qcr6Δ::kan⁺</i>	BG_2684H	S29	B06
101*	<i>SPBC16D10.08cΔ::kan⁺</i>	BG_2697H	S5	C05
102	<i>SPBC16G5.05cΔ::kan⁺</i>	BG_2722H	S29	G12
103	<i>bag11Δ::kan⁺</i>	BG_2728H	S8	D10
104	<i>rps3Δ::kan⁺</i>	BG_2731H	S30	C08
105	<i>sks2Δ::kan⁺</i>	BG_2773H	S26	A08
106	<i>SPBC1711.08Δ::kan⁺</i>	BG_2795H	S23	A03
107	<i>his2Δ::kan⁺</i>	BG_2800H	S28	A06
108	<i>SPBC17D1.07cΔ::kan⁺</i>	BG_2874H	S21	D01
109	<i>rga5Δ::kan⁺</i>	BG_2887H	S2	D05
110	<i>tif213Δ::kan⁺</i>	BG_2896H	S23	D06
111	<i>rpl112Δ::kan⁺</i>	BG_2897H	S15	F04
112	<i>ape2Δ::kan⁺</i>	BG_2953H	S8	G06
113	<i>ubr1Δ::kan⁺</i>	BG_2972H	S26	D03
114*	<i>uve1Δ::kan⁺</i>	BG_2979H	S30	E12
115	<i>leu1Δ::kan⁺</i>	BG_3035H	S30	C11
116	<i>SPBC1E8.03cΔ::kan⁺</i>	BG_3031H	S29	C01
117	<i>arg4Δ::kan⁺</i>	BG_3071H	S18	A10
118	<i>SPBC215.11cΔ::kan⁺</i>	BG_3074H	S8	H08
119*	<i>rad3Δ::kan⁺</i>	BG_3084H	S31	B09
120	<i>car2Δ::kan⁺</i>	BG_3107H	S15	H07
121	<i>SPBC21C3.19Δ::kan⁺</i>	BG_3118H	S9	A03
122	<i>his5Δ::kan⁺</i>	BG_3139H	S5	E02
123	<i>egd2Δ::kan⁺</i>	BG_3215H	S28	F05

Table A.1. Bioneer deletion strains used in this study (continued).

Strain	Relevant genotype^a	BG ID^b	Plate^c	Position^d
124	<i>sua1Δ::kan⁺</i>	BG_3261H	S9	C03
125	<i>fib1Δ::kan⁺</i>	BG_3336H	S31	D03
126	<i>clr1Δ::kan⁺</i>	BG_3343H	S3	E01
127	<i>hmt2Δ::kan⁺</i>	BG_3386H	S19	B10
128	<i>erilΔ::kan⁺</i>	BG_3397H	S21	G03
129	<i>SPBC30D10.05cΔ::kan⁺</i>	BG_3415H	S9	E06
130	<i>pdb1Δ::kan⁺</i>	BG_3407H	S19	B11
131	<i>rpl12Δ::kan⁺</i>	BG_3402H	S27	B05
132	<i>gpx1Δ::kan⁺</i>	BG_3451H	S9	F02
133	<i>tif512Δ::kan⁺</i>	BG_3472H	S23	D05
134	<i>cho1Δ::kan⁺</i>	BG_3493H	S5	F07
135	<i>SPBC365.01Δ::kan⁺</i>	BG_3538H	S2	G03
136	<i>SPBC3B8.03Δ::kan⁺</i>	BG_3576H	S13	A07
137*	<i>hsp16Δ::kan⁺</i>	BG_3616H	S20	H09
138	<i>leu3Δ::kan⁺</i>	BG_3739H	S9	H11
139	<i>SPBC3F6.01cΔ::kan⁺</i>	BG_3630H	S21	A05
140	<i>ade7Δ::kan⁺</i>	BG_3676H	S13	A11
141	<i>eca39Δ::kan⁺</i>	BG_3693H	S20	E01
142	<i>arg12Δ::kan⁺</i>	BG_3696H	S9	H04
143	<i>meu6Δ::kan⁺</i>	BG_3698H	S13	B04
144	<i>SPBC428.11Δ::kan⁺</i>	BG_3702H	S13	B05
145*	<i>SPBC530.05Δ::kan⁺</i>	BG_3760H	S3	D08
146	<i>utp2Δ::kan⁺</i>	BG_3796H	S31	C04
147	<i>pob3Δ::kan⁺</i>	BG_3833H	S21	H10
148*	<i>sds23Δ::kan⁺</i>	BG_3847H	S30	E04
149	<i>SPBC713.03Δ::kan⁺</i>	BG_5319H	S2	G07
150	<i>ura5Δ::kan⁺</i>	BG_3926H	S5	E11
151	<i>ste13Δ::kan⁺</i>	BG_3938H	S30	A03
152	<i>atb2Δ::kan⁺</i>	BG_3953H	S22	A03
153	<i>rpl432Δ::kan⁺</i>	BG_3963H	S14	H04
154*	<i>hhf2Δ::kan⁺</i>	BG_4023H	S10	C08
155	<i>SPBC8D2.18cΔ::kan⁺</i>	BG_4037H	S29	D06
156*	<i>SPBC947.08cΔ::kan⁺</i>	BG_4063H	S4	C10
157	<i>ilv1Δ::kan⁺</i>	BG_4118H	S10	E02
158	<i>SPBP4H10.12Δ::kan⁺</i>	BG_4141H	S10	E07
159	<i>rps232Δ::kan⁺</i>	BG_4142H	S26	H11
160	<i>rpp21Δ::kan⁺</i>	BG_4156H	S23	C06
161	<i>SPBP8B7.11Δ::kan⁺</i>	BG_4161H	S30	B02
162	<i>cyp4Δ::kan⁺</i>	BG_4175H	S17	G01
163	<i>gpt1Δ::kan⁺</i>	BG_5386H	S17	H08
164	<i>SPCC1183.02Δ::kan⁺</i>	BG_4202H	S23	D09

Table A.1. Bioneer deletion strains used in this study (continued).

Strain	Relevant genotype ^a	BG ID ^b	Plate ^c	Position ^d
165*	<i>nmt1Δ::kan⁺</i>	BG_4222H	S5	A08
166	<i>rps182Δ::kan⁺</i>	BG_4250H	S28	G04
167*	<i>SPCC1281.07cΔ::kan⁺</i>	BG_4286H	S11	C10
168	<i>SPCC132.01cΔ::kan⁺</i>	BG_4289H	S11	C12
169	<i>rpl233Δ::kan⁺</i>	BG_4304H	S5	A06
170*	<i>SPCC1450.12Δ::kan⁺</i>	BG_4356H	S20	B12
171	<i>arz1Δ::kan⁺</i>	BG_4367H	S11	E06
172	<i>SPCC16C4.10Δ::kan⁺</i>	BG_4466H	S13	C06
173	<i>SPCC1827.03cΔ::kan⁺</i>	BG_4532H	S19	F09
174	<i>bgs4Δ::kan⁺</i>	BG_4538H	S13	C09
175	<i>SPCC1840.09Δ::kan⁺</i>	BG_4545H	S19	F11
176	<i>SPCC18B5.05cΔ::kan⁺</i>	BG_4567H	S5	A11
177	<i>SPCC191.02cΔ::kan⁺</i>	BG_4760H	S29	E08
178	<i>SPCC24B10.18Δ::kan⁺</i>	BG_4635H	S29	D11
179	<i>rpl122Δ::kan⁺</i>	BG_4686H	S26	A02
180	<i>SPCC330.06cΔ::kan⁺</i>	BG_4710H	S11	H04
181	<i>SPCC338.14Δ::kan⁺</i>	BG_4728H	S25	H07
182*	<i>nap1Δ::kan⁺</i>	BG_4741H	S15	H11
183	<i>SPCC584.15cΔ::kan⁺</i>	BG_4874H	S15	C03
184	<i>snd1Δ::kan⁺</i>	BG_4961H	S4	E06
185	<i>lat1Δ::kan⁺</i>	BG_5073H	S13	G08
186	<i>efl1a-aΔ::kan⁺</i>	BG_5075H	S13	G09
187	<i>rps121Δ::kan⁺</i>	BG_5111H	S27	A03
188	<i>ade1Δ::kan⁺</i>	BG_5393H	S29	F09
189*	<i>mug161Δ::kan⁺</i>	BG_1215H	S22	H07
190*	<i>but2Δ::kan⁺</i>	BG_3601H	S14	G02
191*	<i>eng2Δ::kan⁺</i>	BG_1051H	S22	F09
192*	<i>SPCC736.13Δ::kan⁺</i>	BG_5008H	S13	F11
193*	<i>aru1Δ::kan⁺</i>	BG_1605H	S6	E01
194*	<i>asp1Δ::kan⁺</i>	BG_4413H	S11	F02
195*	<i>ddb1Δ::kan⁺</i>	BG_0565H	S20	H01

Note: ^a the mating type of Bioneer mutant strains are *h⁺*; ^b background ID; ^c 96-well plate number; ^d position in the corresponding 96-well plate. * strains used for plating assay. Strain 189-195 were not applicable for calculating GFS_{MMS} , either because they do not have defined localizations, or the growth curve does not merely reply on ΔT_{50} .

Table A.2. List of primers for construction and validation of HA-tagged strains and deletion mutant strains.

Primer	Sequence
<i>Tagging primers</i>	
SPBC3E7.02c_Foward	5'-CCAACTTCAGTAATGGCCTATTGACTGTCACCT GCCCAAGGTCGAGAAGTCCCAAATAAGAAACAA ATTGCTATTAAGTGGATCCCCGGGTAAATTAA-3'
SPBC3E7.02c_Reverse	5'-AATATATGCTCACAATCCAACAAGATTGGTCTCA TAACCATTTCGAAACTATCATATTAATTCGGAAG AGACGATTTTACTCACTATAGGGCGAATTGG-3'
SPCC18.05c_Foward	5'-TCTTTGCGGTGGATTGGTCTCCGGATGGACAGCG TGTCGCTTCTGGAGGCGCTGATAAAGCAGTCCGTA TATGGTCACACTGGATCCCCGGGTAAATTAA-3'
SPCC18.05c_Reverse	5'-AACCGTAAAAGAAAACCAAATTCCTGAAATTAG TTCTAAATTTTAAACAACAATATGCTACCGAAAG CGTAAATATCACTCACTATAGGGCGAATTGG-3'
SPAC8F11.03_Foward	5'-CAAACTATTAATGATTCTTAATGCCGAAGAGAT TGATTTTAAAGGCTTTTCTATGATCTTACCGCTTT CGAAGAAATCTGGATCCCCGGGTAAATTAA-3'
SPAC8F11.03_Reverse	5'-TGCTTAAATTTATTTATACAGTAAAATCTTATGT GTTGAGTATCCTAATAAAGTAGTTAATTCAGTAAT GCTCTCTCTCACTCACTATAGGGCGAATTGG-3'
SPBC19C7.09c_Foward	5'-ATTATCCGCCCCGAGCTGAAAAGCGTTTAACTGC AAGAAAAAGGCGTAGTAGAAAAGAAGAAGTAGAA GAGGATGAAAAATGGATCCCCGGGTAAATTAA-3'
SPBC19C7.09c_Reverse	5'-GCCAAAAAGACAAAAATGATTGCATTGAAAATG CAGATGGCTAAATTATGCCATAAATCAAAAAGTAT GACGGATTTTACTCACTATAGGGCGAATTGG-3'
SPAC1B1.03c_Foward	5'-GAAAAACGAAAATATCTTCGCAGCAAATAAGG ATTTGGCTCGTTGGGCTACGGAGCAGGTTAAGCGT CAAGCTAGAGCTTGGATCCCCGGGTAAATTAA-3'
SPAC1B1.03c_Reverse	5'-TTTGATCGAATTAGCATAAATATCTAATTTTTCC CCTTCTATTAACCTACACTTCACGAGTATATTTT AACACAAATTACTCACTATAGGGCGAATTGG-3'
SPAC16.02c_Foward	5'-CTTCGGAAGAACAACAGCCTTTGCAAAACCATTC TGATGTAGGTAACGGTAGCGCTGAAGGACAGGTCG CTGCTGAATGGTGGATCCCCGGGTAAATTAA-3'
SPAC16.02c_Reverse	5'-AAGACCACAGACATATGACAGGAAAATCTAGTA AGCAGACACTAAAAAATAGTTTACCAAAGGAAAA ATTTCTATGTTTACTCACTATAGGGCGAATTGG-3'
SPBC530.05_Foward	5'-ATTTTGCTGCTGCTAATGTTTACAATCCTAATGTT TTTGATGATATGGGTTTGGATTGTAGTTTTATGGG AACGGTCTATGGATCCCCGGGTAAATTAA-3'
SPBC530.05_Reverse	5'-AAAACAAAACAAAAGGATTATCATATCATGCGC ATCTAGACAACGACGTCTATTTAATAAAACATAAT TTAGGTAAATTACTCACTATAGGGCGAATTGG-3'
SPBC947.08c_Foward	5'-TTCTTTCTCAGAATGCATCCTTACCCGTTGATGA ACATCAAGCTGAACCAGAGAAATCAATTCCAATA GTAGCATCCCATGGATCCCCGGGTAAATTAA-3'
SPBC947.08c_Reverse	5'-ATTAACGAAAAAATATGATATAAAAAATAATAT ACAGAAATCGATTGACAGATCTTCAGGTCACATA TATGTATAATTACTCACTATAGGGCGAATTGG-3'

Table A.2. List of primers for construction and validation of HA-tagged strains and deletion mutant strains (continued).

Primer	Sequence
Validation primers for tagging^a	
Ura_C	5'-CATCTTAATTATACCTCACAGA-3'
HA_D	5'-CGTATGGGTAGCCAGCGTAATC-3'
SPBC3E7.02c_C	5'-TACTATTCCAGCCAAGATTGAT-3'
SPBC3E7.02c_D	5'-ATCTACACATATTACGATAGAG-3'
SPCC18.05c_C	5'-TTGGTCAGTTCTTCTCAAGATA-3'
SPCC18.05c_D	5'-TACGAAGTAGATAAAAGTAATCC-3'
SPAC8F11.03_C	5'-TTTAAGTCGTGCAACCGAAATT-3'
SPAC8F11.03_D	5'-GTTTATGCTCTTCTTCGTTAAA-3'
SPBC19C7.09c_C	5'-GATCCAACGGCGATTTCTGGGAT-3'
SPBC19C7.09c_D	5'-CGACAATACCAATATGACCGGAATTGC-3'
SPAC1B1.03c_C	5'-AAGGCGAACTTAAATCATATTT-3'
SPAC1B1.03c_D	5'-CTAATGGTGAAATATGTTCAAA-3'
SPAC16.02c_C	5'-TGAAGGTTCCAAATCCTATTCA-3'
SPAC16.02c_D	5'-CTCTCATCAAACTATGTGA-3'
SPBC530.05_C	5'-CAAATGACCAGTATAAGTTTGA-3'
SPBC530.05_D	5'-GTCAATCAAGAAAGACAATCTC-3'
SPBC947.08c_C	5'-CAAGTGCTTTAACTAATCCTTC-3'
SPBC947.08c_D	5'-TTAACAAGGAGGTTTGGGTTTA-3'
Disruption primers	
SPBC947.08c_Forward	5'-CATTTTCGCTTGTTAGGATTACTTTTCCTTGCAAA TTTTTCTTCTACGTTTTCACATACTTCGCTTTTGT TCGTCATGTAACCCTCACTAAAGGGAAC-3'
SPBC947.08c_Reverse	5'-ATTAACGAAAAAATATGATATAAAAAATAATAT ACAGAAATCGATTGACAGATCTTCAGGTCACATA TATGTATAATTACTCACTATAGGGCGAATTGG-3'
SPBC530.05_Forward	5'-ATTGAAAAAATTGTTACCGGATGGAAAAATTGA AATCAGCATCGCCGGAGGAAGACTCACCTCGCCAC GGGGATAACATGTAACCCTCACTAAAGGGAAC-3'
SPBC530.05_Reverse	5'-AAAACAAAACAAAAGGATTATCATATCATGCGC ATCTAGACAACGACGTCTATTTAATAAAACATAAT TTAGGTTAATTACTCACTATAGGGCGAATTGG-3'
Validation primers for deletion^b	
Kana_F	5'-TCTGGGCCTCCATGTCGCTGG-3'
Kana_R	5'-GTCAATCGTATGTGAATGCTGG-3'
Ura_B	5'-AAACATGCTCCTACAACATTAC-3'
SPBC947.08c_A	5'-TACCATCCTCCTAATCAAATTA-3'
SPBC947.08c_B	5'-TCAAATTAGCGACCAACGAATT-3'
SPBC530.05_A	5'-TCGGATTGATATAGTAATCTAC-3'
SPBC530.05_B	5'-TTTAGGTCTAGCTGTAAATACG-3'

Note: This list includes four parts: i) Tagging primers for tagging the genes with haemagglutinin (HA), ii) Validation primers for verifying appropriate tagging, iii) Disruption primers for constructing the deletion DNA cassette, and iv) Validation primers for verifying appropriate deletion alleles. All sequences are listed from 5' to 3'.

^a primer pairs [HA_D, C; Ura_C, D; C,D] were used for validating HA tagging; ^b primer pairs [Ura_B, A; A, B; C, D] or [kana_F, kana_R, A, B; C, D] were used for validating deletion.

Figure A.2. Plasmid maps of pREP1 and pREP41. pREP1 (upper) and pREP41 (bottom) contain the *S. pombe* origin of replication *ars1* (512) and are maintained extrachromosomally at high copy number. The promoter strength is *nmt1* > *nmt41*. Both are constructed with the *S. cerevisiae* *LEU2* gene (513) as selectable marker, which encodes β -isopropylmalate dehydrogenase and complements the *S. pombe* *leul-32* allele. Restriction sites are indicated. This figure is adapted from <http://www.riken.jp/SPD/pREP.html> with modification.

APPENDIX B. SUPPLEMENTAL DATA

B.1 Results of SILAC analysis

Figure B.1. Analysis of chromatin extract via non-SILAC proteomic analysis. (A) Ribosomal proteins are overrepresented in the top 10% most abundant proteins in chromatin extract. X-axis indicates the percentile ranks based on protein abundance, Y-axis indicates enrichment or depletion of various types of proteins based on the observed number over the background level. (B) Gene ontology analysis of the top 10% abundant proteins in chromatin extract. Asterisk indicates the occurrence (i.e., < 5) of the relative GO term associated genes is too low to perform statistical analysis.

Figure B.2. Expression of HA-tagged fusion proteins used for chromatin fractionation assay. To ensure the HA-tagged strains can normally express the tagged proteins, wild-type strain without HA tagging (nc, negative control) and HA-tagged strains were grown to mid-log phase, cell lysates were extracted, and equal amounts of protein were resolved by SDS-PAGE. Protein expression was determined using anti-HA antibody. HA-tagged Bc530.05 (pc, positive control) was used to ensure the antibody was working. For each tested protein, corresponding protein ratio from SILAC analysis and molecular weight are indicated.

Figure B.3. Mini-culture growth curve assays for sensitivity to 0.008% (v/v) MMS. (A) Mini-culture growth curves of the two-fold serial diluted cultures. The upper panel shows Mini-culture growth curves of wild-type cells with OD₆₀₀ from 0.4 to 0.0125 in a two-fold serial dilution, the bottom panel shows that the half-maximal-concentration time T_{50} of each culture is proportional to the concentration of its starting culture. (B) Mini-culture growth curve of wild-type cells in medium without or supplemented with 0.002, 0.004 and 0.008% (v/v) MMS.

B.2 Optimization of spheroplasting in chromatin fractionation assay

In fission yeast, the most critical step in chromatin fractionation assay is spheroplasting. To optimize the condition for spheroplasting, cell walls were digested with zymolyase, and the reaction was stopped at three different points; the results were monitored by DAPI staining, immunoblotting and mass spectrometry (Figure B.4). DAPI staining suggested that 45 min of zymolyase treatment generated better spheroplasts, in which most of the nuclei are morphologically intact and out of the cell remnant (Figure B.4A). Consistent with this, the results of Western blotting analysis showed that histone H4 was significantly enriched and β -tubulin was extensively depleted when spheroplasts were collected at 45 min (Figure B.4B, middle panel). On the contrary, 30 min of zymolyase treatment was not sufficient to isolate majority of β -tubulin from the soluble fraction (Figure B.4B, left panel); while proteins from spheroplasts with 60-min treatment started to degrade and β -tubulin became undetectable (Figure B.4B, right panel). Furthermore, 45 min of zymolyase treatment led to identification of 50% more proteins than that with 30 min based on mass spectrometric analysis (Figure B.4C). The number was also twice as much as that with 60 min, presumably due to extensive protein degradation with prolonged spheroplasting. Collectively, 45 min of zymolyase treatment is considered as a proper condition for spheroplasting. Nevertheless, it is arbitrary to conclude that spheroplasts should be isolated at 45 min, as cell wall conditions are variable between different samples. Therefore, spheroplasting should be optimized for each new cell sample or condition.

Figure B.4. A time course study to test conditions for spheroplasting. 45 min of zymolyase treatment leads to proper digestion of cell walls and better extraction of chromatin. (A) Microscopy images of fission yeast spheroplasts. Cells were treated with Zymolyase 20T, and spheroplasts were isolated at 30, 45, and 60 min. The spheroplasts were stained with DAPI as described in Materials and Methods. Cells without zymolyase treatment (0 min) were included as a negative control. (B) Immunological characterization of proteins in soluble fraction (S1) and chromatin (C) from different zymolyase treatment as in (A). To analyze the proteins by Western blotting, histone H4 and β -tubulin (TAT1) were used as controls for chromatin-bound and non-chromatin bound proteins, and the amount in S1 and C from this assay was determined by immunoblotting with anti-Histone H4 polyclonal antibody or anti-tubulin monoclonal antibody, respectively. (C) Venn diagram showing that more proteins are identified from chromatin with 45 min of zymolyase treatment. The chromatin extract was subjected to tryptic digestion and proteome analysis. The total number of identified proteins from each condition is indicated in the parentheses.

B.3 Incorporation testing and validation of ^{15}N Labelling

^{15}N labelling is an alternative metabolic labelling approach of SILAC. Similar to SILAC, Incorporation of the “heavy” nitrogen source occurs through cell growth, protein synthesis, and turnover. By introducing “heavy” salt to the cells (e.g., NH_4Cl), ^{15}N labelling allows “light” and “heavy” proteomes to be distinguished by MS without disrupting the chemical and physical properties of proteins. It is routinely used to label a variety of organisms ranging from bacteria to flies and worms (201, 202, 514). As a cost-effective approach, ^{15}N labelling has been extensively applied to the labelling of mammals with ^{15}N -enriched diet (203). Comparing with ^{15}N labelling, SILAC is uniquely analytical and practical, because the stable isotopes are confined with specific amino-acid residues. By using different isotopes, multiple samples can be mixed and analyzed simultaneously.

In our SILAC analysis, only lysine was labelled. In order to make every peptide quantifiable, it would be better to label cells with both lysine and arginine. However, a problematic conversion of isotope-coded arginine to proline (arg-pro interconversion) compromises the quantitative analysis of arginine-ended peptides (515, 516). This problem is exacerbated in fission yeast, because the labelled arginine is also converted extensively to other amino acids expect for proline, such as glutamate, glutamine, and lysine (275). Recently, this issue in fission yeast has been resolved by genetic manipulation through deleting genes responsible for arginine metabolism and modifying the growth medium to facilitate arginine uptake in mutant (275). Nevertheless, our results indicate that the arginase mutant *aru1* Δ is sensitive to DNA damaging agents; it

is unknown whether the above genetic modification would affect the downstream analysis using SILAC-mediated approach.

A new software tool called Census has been developed to handle the intricate data generated using ^{15}N labelling (322). Because this strategy has never been utilized in fission yeast, we are curious to know whether it is applicable to this organism. Since the growth rate of autotrophic strain in ^{15}N -labelled medium was compatible to that in unlabelled medium (Figure B.5A), we continued to check the incorporation efficiency of ^{15}N labelling. Liquid culture sample was collected after each DT, the remaining cells were further refreshed into ^{15}N -labelled medium, and the cycle was repeated until sample from DT6 was obtained. A gel slice (~50 kDa) from each sample was subjected to the proteomic analysis after in-gel trypsinization. According to the results, the quantification efficiency of peptide was continuously decreased from DT1 to DT6 (Figure B.5B), and became less than 10% in DT5 and DT6, indicating that more than 90% of peptides were completely labelled with ^{15}N at these two stages. The ratios between light and heavy peptide levels in all peptide pairs from DT1 to DT4 were calculated. The peptide ratios were continuously decreasing from DT1 to DT4, and ~99% of them were found to be below -3 in log2 scale in DT4 (Figure B.5C), indicating that the ^{15}N was uniformly incorporated into all the proteins. To ascertain proteins were fully incorporated with ^{15}N , cell lysates derived from DT6 were applied as the heavy isotope labelled sample in this analysis.

To assess the accuracy of relative levels between light versus heavy peptides/proteins in ^{15}N labelling, the ^{15}N -labelled protein lysates (i.e., DT6, heavy) were mixed in various ratios with its natural counterpart (light). All the

samples obtained above 75% quantification efficiency (Table B.1), suggesting the numbers of quantifiable peptides were dramatically improved in ^{15}N labelling compared to that in SILAC labelling with single amino acid (i.e., below 25%). By estimating the median of observed ratios for all quantifiable peptides/proteins in a given pre-mixed sample, we found a strong linear correlation between the observed and the expected ratios in all the pre-mixed samples (Figure B.6). Therefore, it took only six DTs for the whole proteomes to be incorporated with ^{15}N , which is easy and fast to manipulate.

As a result, ^{15}N labelling is highly accurate and reliable in *S. pombe*, and it should be applicable for the quantitative analysis of chromatin extract. Moreover, the extensive amino-acid conversion problem is avoidable. As the cells grow normally in ^{15}N -labelled media without morphological changes, this method can be extensively used in any quantitative proteomic analysis in this organism.

Figure B.5. Analysis of ¹⁵N labelling incorporation.

(A) The autotrophic strain (LJY95) has similar growth rate in unlabelled medium and ¹⁵N labelled medium. Overnight cells were inoculated into unlabelled (solid circle) and ¹⁵N labelled (open circle) medium to OD₆₀₀~0.1, and the cell growth was recorded every 2 h over a 10-h period. (B) The bar chart showing the quantification efficiency of peptide was continuously decreased from DT1 to DT6, and became less than 10% in DT5 and DT6. (D) The boxplot showing the ratio of light and heavy (¹⁴N:¹⁵N) peptide levels in DT1 to DT4. The peptide ratios were continuously decreasing from DT1 to DT4, and ~99% of them were found to be below -3 in log₂ scale in DT4. The red open circles represent the 95th (upper) and 5th (bottom) percentile outliers.

Table B.1. Quantification efficiencies obtained using Census within the IP2 for 7^{15}N pre-mixed samples analyzed using LTQ-Orbitrap.

Sample (L:H*)	Log2 (L:H)	# Peptides Identified	# Peptides Quantified	Quantification Efficiency (%)
4:1	2	2853	2223	77.92
3:1	1.58	2541	2121	83.47
2:1	1	2458	2101	85.48
1:1	0	2440	2042	83.69
1:2	-1	2446	2077	84.91
1:3	-1.58	2552	2138	83.78
1:4	2	2711	2202	81.22

Note: * light (^{14}N) peptide versus heavy (^{15}N) peptide. The full peptide lists are in file 12 in DVD-R.

Figure B.6. The observed and expected ratio of the light and heavy peptide/protein levels in the pre-mixed samples are highly correlated in ^{15}N labelling. (A) Correlation between the median of the observed ratio and the expected ratio of light (^{14}N) and heavy (^{15}N) peptides. X-axis and Y-axis indicate the observed ratio and the expected ratio of light and heavy peptide levels, respectively. (B) Correlation between the median of the observed ratio and the expected ratio of light and heavy proteins. The display is identical to (A). The correlation coefficient R is shown. The ratios are in log2 scale. The full peptide and protein lists are in file 12 and 13 in DVD-R.

B.4 Sequencing results

Sequencing results of pREP1-*hip4*⁺ using primer pREP_F

CCTTCATGTGATATTATTTCAATCTCATTCTCACTTTCTGACTTATAG
TCGCTTTGTAAATCATATGTCGACATGTCGCTTTTGTCTGGCGACCA
ACGAATTATTTGCTTGGAAATTCCTTTGCAAGGCAAAGAAAACGTAC
ATATTAATTTTGCAAAAAGAAGTGGAAAACTTTATGGACCCTCTGTT
CAGGAGAATTCAAAAAAAAAATGATCTTTCAGACTCAGCGGATTCTG
AGGGCGAAAGTACACATGTTGATGAGCAACAAGCAGCTAATGGGGC
GGCCGATGGTACACAGACCATAAAGAAAAAGAAAAGGAAAAGGAG
GTACGCTGACGAATACTACGATAGAACAGATCCCTTTATTGATGATG
CTGAGCTCTACATTGAAGAGAAGGCTGCCGCTACCAAAGATGGATT
TTTTGTATTTAGCGGTCCACTCGTGGCCGAAGGAGATACTGTGAAAA
TCGAAAGATCAAAGAAGACTAAGAAAAAGAAAACATCTTTATC
CAATGCTACACACCCTGCGCCAGCTGTCTCTGCGGTGGTGGCTTCAG
CGGATGCTAGCTTTGACGATAGTAGGGATATTGAATCTGAGGACGA
GCAGCCATTAAGGACTTTGTCCATTGAAATGGCTGCTAAGAATGCTT
TAAAAGAGGCAAAACGGGAAAAATGCAAAGGTTCCCAAGGATGTCTC
TAAGAAGGAAGCAAAAACAACGAAAAGCAAAGAGAAAGCCACAAA
AAAGACATCCTCTTCAGTGCCCAAACAAGTACGGGAGAAAATACC
AAAAAGGCTGTAAACTTGAGACACCTCTTACATCTACACCCCTAT
ACCACCTCTCTCTGAACCAAAGCATTCTCCCGCTACTGTTAATGAGC
ACATTTCTCCTCCAAGTGCTTTAACTAATCCTTCTACGGAGGAACTC
AAACCTTCGACCTCCCTACCTATTGATCAGGGCAATGCTTCTGTCTG
TTCGAAACCTACTCAACAAGTAAGCATCGTTCTTTCTCAGAATGCAT
CCTTACCCGTTGATGAACATCAAGCTGAACCAGAGAAATCAATTCCA
ACTAGTAGCATCCCATGGATCC

Sequencing results of pREP1-*hip4*⁺ using pREP_R primer

CCGTGCTTTGAAGAAAACCTAGCAGTACTGGCAAGGGAGACATTCC
TTTTACCCGGGGACCATGGGATGCTACTAGTTGGAATTGATTTCTCT
GGTTCAGCTTGATGTTTCATCAACGGGTAAGGATGCATTCTGAGAAAG
AACGATGCTTACTTGTTGAGTAGGTTTCGAAACGACAGAAGCATTGC
CCTGATCAATAGGTAGGGAGGTGCAAGGTTTGAGTTCCTCCGTAGA
AGGATTAGTTAAAGCACTTGGAGGAGAAATGTGCTCATTAACAGTA
GCGGGAGAATGCTTTGGTTCAGAGAGAGGTGGTATAGGGGGTGTAG
ATGTAAGAGGTGTCTCAAGTTTAAACAGCCTTTTTTGGTATTTTCTCCCG
TACTTTGTTTGGGCACTGAAGAGGATGTCTTTTTTGTGGCTTTCTCTT
TGCTTTTCGTTGTTTTTGGCTTCCTTCTTAGAGACATCCTTGGAACCT
TTGCATTTTCCCGTTTTGCCTCTTTTAAAGCATTCTTAGCAGCCATTT
CAATGGACAAAGTCCTTAATGGCTGCTCGTCCTCAGATTCAATATCC
CTACTATCGTCAAAGCTAGCATCCGCTGAAGCCACCACCGCAGAGA
CAGCTGGCGCAGGGTGTGTAGCATTGGATAAAGATGTTTTCTTCTTT
TTCTTAGTCTTCTTTGATCTTTTCGATTTTTCACAGTATCTCCTTCGGCCA
CGAGTGGACCGCTAAATACAAAAAATCCATCTTTGGTAGCGGCAGC
CTTCTCTTCAATGTAGAGCTCAGCATCATCAATAAAGGGATCTGTTC

TATCGTAGTATTCGTCAGCGTACCTCCTTTTCCTTTTCTTTTCTTTAT
GGTCTGTGTACCATCGGCCGCCCATTAGCTGCTTGTTGCTCATCAA
CATGTGTACTTTCGCCCTCAGAATCCGCTGAGTCTGAAAGATCATTT
TTTTTTGAATTCTCCTGAACAGAGGGTCCATAAAGTTTTTCCACTTCT
TTTGCAAAATTAATATGTACGTTTTCTTTGCCTTGCAAAGGAATTTCC
AAGCAAATAATTCGTTGGTCGCCAGACAAAAGCGACTTTTCGACTT

Sequencing results of pREP41-*hip4*⁺ using pREP_F primer

GTAAGTGATATTATTTCAATCTCATTCTCACTTTCTGACTTATAGTCG
CTTTGTAAATCATATGTCGACATGTCGCTTTTGTCTGGCGACCAAC
GAATTATTTGCTTGGAATTCCTTTGCAAGGCAAAGAAAACGTACAT
ATTAATTTTGCAAAAGAAGTGGA AAAACTTTATGGACCCTCTGTTCA
GGAGAATTCAAAAAAAAATGATCTTTCAGACTCAGCGGATTCTGAG
GGCGAAAGTACACATGTTGATGAGCAACAAGCAGCTAATGGGGCGG
CCGATGGTACACAGACCATAAAGAAAAAGAAAAGGAAAAGGAGGT
ACGCTGACGAATACTACGATAGAACAGATCCCTTTATTGATGATGCT
GAGCTCTACATTGAAGAGAAGGCTGCCGCTACCAAAGATGGATTTTT
TGTATTTAGCGGTCCACTCGTGGCCGAAGGAGATACTGTGAAAATCG
AAAGATCAAAGAAGACTAAGAAAAAGAAAGAAAACATCTTTATCCAA
TGCTACACACCCTGCGCCAGCTGTCTCTGCGGTGGTGGCTTCAGCGG
ATGCTAGCTTTGACGATAGTAGGGATATTGAATCTGAGGACGAGCA
GCCATTAAGGACTTTGTCCATTGAAATGGCTGCTAAGAATGCTTTAA
AAGAGGCAAAACGGGAAAATGCAAAGGTTCCCAAGGATGTCTCTAA
GAAGGAAGCAAAAACAACGAAAAGCAAAGAGAAAGCCACAAAAAA
GACATCCTCTTCAGTGCCCAAAACAAAGTACGGGAGAAAATACCAAA
AGGGCTGTAAACTTGAGACACCTCTTACATCTACACCCCCTATACC
ACCTCTCTCTGAACCAAAGCATTTCTCCCGCTACTGTTAATGAGCACA
TTTCTCCTCCAAGTGCTTTAACTAATCCTTCTACGGAGGAAC TCAA
CCTTCGACCTCCCTACCTATTGATCAGGGCAATGCTTCTGTCTGTTTCG
AAACCTACTCAACAAGTAAGCATCGTTCTTTCTCAGAATGCATCCTT
ACCCGTTGATGAACATCAAGCTGAACCAGAGAAATCAATTCCAAC T
AGTAGCATCCCATGGATCCCCGGGTAAAAGGAATGTCTCCCT

Sequencing results of pREP41-*hip4*⁺ using pREP_R primer

CGATTTAGAAAACCCTAGCAGTACTGGCAAGGGGAGACATTCCTTTT
ACCCGGGGGATCCATGGGATGCTACTAGTTGGAATTGATTTCTCTGGT
TCAGCTTGATGTTTCATCAACGGGTAAGGATGCATTCTGAGAAAGAA
CGATGCTTACTTGTTGAGTAGGTTTCGAAACGACAGAAGCATTGCCC
TGATCAATAGGTAGGGAGGTCGAAGGTTTGAGTTCCTCCGTAGAAG
GATTAGTTAAAGCACTTGAGGAGAGAAATGTGCTCATTAACAGTAGC
GGGAGAATGCTTTGGTTCAGAGAGAGGTGGTATAGGGGGTGTAGAT
GTAAGAGGTGTCTCAAGTTTAAACAGCCCTTTTGGTATTTTCTCCCGTA
CTTTGTTTGGGCACTGAAGAGGATGTCTTTTTTGTGGCTTTCTCTTTG
CTTTTCGTTGTTTTTGCTTCCTTCTTAGAGACATCCTTGGAACCTTT

GCATTTTCCCGTTTTGCCTCTTTTAAAGCATTCTTAGCAGCCATTTCA
ATGGACAAAGTCCTTAATGGCTGCTCGTCCTCAGATTCAATATCCCT
ACTATCGTCAAAGCTAGCATCCGCTGAAGCCACCACCGCAGAGACA
GCTGGCGCAGGGTGTGTAGCATTGGATAAAGATGTTTTCTTCTTTTT
CTTAGTCTTCTTTGATCTTTCGATTTTCACAGTATCTCCTTCGGCCAC
GAGTGGACCGCTAAATACAAAAAATCCATCTTTGGTAGCGGCAGCC
TTCTCTTCAATGTAGAGCTCAGCATCATCAATAAAGGGATCTGTTCT
ATCGTAGTATTCGTCAGCGTACCTCCTTTTCCTTTTCTTTTCTTTATG
GTCTGTGTACCATCGGCCGCCCCATTAGCTGCTTGTTGCTCATCAAC
ATGTGTACTTTTCGCCCTCAGAATCCGCTGAGTCTGAAAGATCATTTT
TTTTTGAATTCTCCTGAACAGAGGGTCCATAAAGTTTTTCCACTTCTT
TTGCAAAATTAATATGTACGTTTTCTTTGCCTTGCAAAGGAAATTCC
AAGCAAATAAATCGTTGGTCGCCAAACAAAAGCGACATGTCGACAT
ATGATTTAAAAAAGCGACTATAAGTCAGAAAGGGGAAATGGGAATT
GAAATAATTAATTC

Sequencing results of pJK210-CTAP-*hhf1* using T7 promoter primer

GTAAGGGCCCTCGAGGTCGACGGTATCGATAGCTGTTGTGCTACT
TTTGCCAATTGTTGATCTTTAGAATTAATGGAAACGCAAGCGTACTG
CAGATATTTGCTATATATATGTTTTGAATCCACCCGATCTCATCAA
AGCAGGGTTAGGGTTTTGATGCAGATTCAAAACAAATAGACAGCGT
CGCCCCAACTTCCCTCTCTTCAGACAATCATTTCTTTTAATTGTTTT
GAATACGGATAAAATCCTTTGTCAATTTGCATTGACTTTTATCCTACC
GTAAACATCTGTTGACATCTATAAATTCATTGTTTCCTTTGTTAGTCAA
CGGAAAACCCCTTTATCTTTATTATACTTAAAGAATTACGCGTTTTTT
TGCGTAGATTTTAATTTTTTCTACTTCTTAACATGTCTGGTCGTGGTA
AAGGTGGTAAAGGTTTGGGAAAAGGTGGTGCTAAGCGTCACCGTAA
AATTCTTCGTGACAACATTCAAGGTATTACTAAGCCTGCTATCCGTC
GTCTTGCTCGTCGTGGTGGTGTGAAGCGTATTTCCGCTTTGGTTTATG
AAGAGACTCGTGCTGTTCTCAAGCTCTTCTTAGAAAAATGTTATTCGC
GATGCCGTCACCTATACTGAGCATGCCAAGCGTAAGACTGTCACTTC
TTTGGACGTTGTCTATTCTTTGAAGCGTCAAGGCCGTACCATTATG
GTTTCGGTGGTAGATCCACTAGTTCTAGAAAGCGACGATGGAAAAA
GAATTCATAGCCGTCTCAGCAGCCAACCGCTTTAAGAAAATCTCAT
CCTCCGGGGCACTTGATTATGGTACCCCACTACTGCTTCTGAAAAT
CTATATTTTCAAGGTGAACTAAAACTGCTGCTTTGGCTCAACATGC
GATTAAAGCTGATGCGCAACAAAATAACTTCAACAAAGATCAACAA
AGCGCCTTCTATGAAATCTTGAACATGCCTAACTTAAACGAAGCGCA
ACGTAACGGCTTCATTCAAAGTCTTAAAGACAACCCAGGCCAAAGC
ACTAACGTTTTAGGTGAAGCTAAAAAATTAACCGAATCTCAAGCAC

Sequencing results of pJK210-CTAP-*hhf1* using T3 promoter primer

NNNNCNCTGCTCGAGCTCGGACCACTAGTAACGGCCGCCAGTGTGC
TGGAATTCGCCCTTGGGCCCAGATCTACCACCGAAACCATAAATGGT
ACGGCCTTGACGCTTCAAAGAATAGACAACGTCCAAAGAAGTGACA
GTCTTACGCTTGGCATGCTCAGTATAGGTGACGGCATCGCGAATAAC
ATTTTCTAAGAAGAGCTTGAGAGCAGCACGAGTCTCTTCATAAACCA

AAGCGGAAATACGCTTCACACCACCACGACGAGCAAGACGACGGAT
AGCAGGCTTAGTAATACCTTGAATGTCGTCACGAAGAATTTTACGGT
GACGCTTAGCACCACCTTTTCCCAAACCTTTACCACCTTTACCACGA
CCAGACATGTTAAGAAGTAGAAAAAATTAATCTACGCAAAAAA
CGCGTAATTCTTTAAGTATAATAAAGATAAAGGGGTTTTCCGTTGAC
TAACAAAGGAACAATGAATTTATAGATGTCAACAGATGTTAACGGT
AGGATAAAAGTCAATGCAAATTGACAAAGGATTTTATCCGTATTCA
AAAACAATTAAAAGAAATGATTGTCTGAAGAGAGGGAAGTTGGGGC
GACGCTGTCTATTTGTTTTGAATCTGCATCAAACCCTAACCCCTGCTT
TTGATGAGATCGGGTGGATTCAAACATATATATAGCAAATATCTGC
AGTACGCTTGCGTTTCCATTAATTCTAAAGATCAACAATTGGCAAAA
GTAGCACAACAGCTATCGATGCGCGCAAGGGCGAATTCTGCAGATA
TCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATT
CGCCCTATAGTGAGTCGTATTACAATTCAGTGGCCGTCGTTTTACAA
CGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC
AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGC
ACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGN
CGCGCCCTGTAGCGGCGCATTAAGCGCGGCCGGGGTTGTTGGTGGTT
TACCGCCGCCAGGCGGT

APPENDIX C. CONTENTS OF THE DVD-R

C.1 Instructions

There are 14 files in this DVD-R. To read these files, you will need Microsoft Excel for “.xlsx” files, Microsoft Word for “.docx” files, and Adobe Acrobat Reader for “.pdf” files.

- “.xlsx” files were created with Microsoft Excel 2007
- “.docx” files were created with Microsoft Word 2007
- “.pdf” files were created with Adobe Acrobat Standard 6.0

C.2 Contents

- 1) The file “content.docx” contains a thorough explanation of the DVD-R contents
- 2) The file “SILAC_interact.pep.xlsx” contains a complete list of $^{12}\text{C}_6$ -lysine and $^{13}\text{C}_6$ -lysine containing peptides of SILAC analysis based on the area of peaks by XPRESS within TPP
- 3) The file “SILAC_ratio.xlsx” contains a list of the enrichment level (ratio) of the chromatin extract proteins when compared to WCE
- 4) The file “Non-SILAC_interact.pep.xlsx” contains a complete list of peptides identified from non-SILAC analysis within TPP
- 5) The file “Non-SILAC_area.xlsx” contains a list of peak areas of the identified peptides from non-SILAC analysis quantified by PepQuan within Bioworks 3.3.1
- 6) The file “Non-SILAC_abundance.xlsx” contains a list of the absolute abundance of the identified proteins in chromatin extract
- 7) The file “GFS_MMS.xlsx” contains a list of average growth fitness scores of the tested Bioneer deletion strains
- 8) The file “hhf1_protein.xlsx” contains a list of proteins identified from tandem affinity purification (TAP) of C-TAP tagged hhf1
- 9) The file “hhf1_processing.xlsx” contains a list of proteins identified from tandem affinity purification (TAP) of C-TAP tagged hhf1 with data processing
- 10) The file “mock_control_protein.xlsx” contains a list of proteins identified from tandem affinity purification (TAP) of mock control
- 11) The file “p3_ratio.xlsx” contains a list of the ratio between light and heavy peptide levels in p3 (third passage, i.e., ~9 generations in the heavy lysine-containing medium)
- 12) The file “ ^{15}N _labelling_Peptide.xlsx” contains a total of 14 worksheets, each worksheet contains a list of quantified peptides of corresponding samples from ^{15}N labelling

- 13) The file “¹⁵N_labelling_Protein.xlsx” contains a total of 14 worksheets; each worksheet contains a list of quantified proteins of corresponding samples from ¹⁵N labelling
- 14) The file “Thesis.pdf” contains full text of my dissertation in PDF format

PUBLICATION RELATED TO THIS STUDY

H. Wang[#], Pornpimol, T.[#], L. Zhu, S. Y. Poon, K. Tang, and J. Liu. (2012). Chromatin-associated proteins revealed by SILAC-proteomic analysis exhibit a high likelihood of requirement for growth fitness under DNA damage stress. doi:10.1155/2012/630409 International Journal of Proteomics (# equal contribution)

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