<table>
<thead>
<tr>
<th>Title</th>
<th>Characterisation of novel Histone H3 RAD9 mutants defective in the DNA damage response in Saccharomyces cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Walsh, Mary</td>
</tr>
<tr>
<td>Publication Date</td>
<td>2013-02-28</td>
</tr>
<tr>
<td>Item record</td>
<td><a href="http://hdl.handle.net/10379/4254">http://hdl.handle.net/10379/4254</a></td>
</tr>
</tbody>
</table>

Some rights reserved. For more information, please see the item record link above.
Characterisation of novel Histone H3 and \textit{RAD9} mutants defective in the DNA damage response in \textit{Saccharomyces cerevisiae}

Mary Walsh

Genome Stability Laboratory, Centre for Chromosome Biology, Department of Biochemistry, School of Natural Sciences, National University of Ireland, Galway

A thesis submitted to the National University of Ireland, Galway for the degree of Doctor of Philosophy

Supervisor: Professor Noel F. Lowndes
Co-supervisor: Doctor Muriel Grenon

February 2013
# Table of Contents

**LIST OF FIGURES**............................................................................................................v

**LIST OF TABLES**.............................................................................................................vi

**ABBREVIATIONS**.............................................................................................................vii

**ACKNOWLEDGEMENTS**...............................................................................................viii

**DECLARATION**................................................................................................................ix

**ABSTRACT**.........................................................................................................................x

**CHAPTER 1: Introduction**..............................................................................................1

1.1. Overview.........................................................................................................................2

1.2. *Saccharomyces cerevisiae* cell cycle and budding morphology................................2

1.3. The DNA damage response ........................................................................................4

1.3.1. Homologous recombination and non homologous end joining..........................7

1.3.2. HO-endonuclease system in yeast..............................................................................10

1.3.3. The DNA damage checkpoints; The S phase DNA damage response......................12

1.4. RAD9, the prototypical DNA damage checkpoint gene............................................13

1.4.1. Domain structure of Rad9..........................................................................................13

1.5. DNA damaging agents which perturb S phase progression......................................15

1.5.1. CPT is a topoisomerase I poison..............................................................................15

1.5.2. Cisplatin forms inter and intrastrand crosslinks......................................................15

1.5.3. MMS is a DNA alkylating agent which blocks replication......................................13

1.5.4. HU blocks replication by inhibiting dNTPs.............................................................13

1.6. Nucleosome structure and function..........................................................................17

1.6.1. Histone H3 αN helix is highly conserved and important for viability.....................19

1.6.2. H3T45 and H3K56, two residues located at the H3 αN helix are important for replication.................................................................................................................21

1.6.3. MMS22 and MMS1 play an important role along with H3K56ac in genomic integrity.................................................................................................................................23

1.7. Histone post-translational modifications..................................................................24

1.7.1. Histone post-translational modifications in transcriptional regulation and the DNA damage response..........................................................24

1.7.2. H3S10 and H3S28 phosphorylation are cell cycle regulated.................................25

1.8 Working hypothesis/Aim of thesis..............................................................................26

1.9 References....................................................................................................................29

**CHAPTER 2: Is H3 serine 28 phosphorylated in *Saccharomyces cerevisiae*?**.............40

2.1. Summary.......................................................................................................................41

2.2. Highlights......................................................................................................................41

2.3. Introduction..................................................................................................................42

2.4. Results.........................................................................................................................46

2.4.1. *h3S28A* is highly sensitive to DNA damaging agents....................................46

2.4.2. H3S28ph yeast specific antibody and commercial H3S28ph human antibodies cannot detect H3S28 phosphorylation in *S. cerevisiae*........................................48

2.4.3. NI-H3S28ph recognises both H3S28 and H3S10 phosphorylation.........................53
2.4.4. Mass spectrometry analysis cannot detect H3S28 phosphorylation

2.5. Discussion

2.6. Conclusion

2.7. Materials and Methods

2.7.1. Media and growth conditions

2.7.2. Selective media and genotoxic treatments

2.7.3. Yeast strains and Plasmids

2.7.4. Acid extracted histones

2.7.5. Western blotting and antibodies

2.7.6. Recombinant histone mutants

2.7.7. Mass spectrometry

2.8. Supplementary Data

2.8.1. Supplementary Figures

2.8.2. Supplementary Tables

2.9. References

CHAPTER 3: A role for H3E50 in the DNA Damage Response

3.1. Summary

3.2. Highlights

3.3. Introduction

3.4. Results

3.4.1. Generation of histone H3 glutamate 50 mutants

3.4.2. h3E50G and h3E50A cells do not recover efficiently from an intra-S phase checkpoint

3.4.3. H3E50 is not required for 5'-3' DNA end resection

3.4.4. h3E50G and h3E50A mutant cells form persistent Rad52 foci after exposure to genotoxic agents

3.5 Discussion

3.6 Conclusion and Future work

3.7. Materials and Methods

3.7.1. Media and growth conditions

3.7.2. Cellular growth and genotoxic treatments

3.7.3. Yeast strains and Plasmids

3.7.4. G1 arrest and release experiment

3.7.5. Immunoblotting

3.7.6. Chromatin Immunoprecipitation

3.7.7. Microscopy analysis to detect Rad52-YFP foci

3.7.8. Fluorescence Activated Cell Sorting (FACS)

3.8. Supplementary Data

3.8.1. Supplementary Tables

3.9. References

CHAPTER 4: A role for Rad9 CDK sites S494 and S618 in the DNA Damage Response

4.1. Summary

4.2. Highlights

4.3. Introduction

4.4. Results

4.4.1. Rad9 CDK sites S494 and S618 are required for Rad9 cell cycle phosphorylation

4.4.2. Rad9 CDK sites S494 and S618 are required for recovery from the G2/M checkpoint
4.4.3. \textit{rad9S494A+S618A} cells are proficient in repair and DNA resection ................................................................. 139
4.4.4. Rad9 CDK sites S494 and S618 contribute to an efficient NHEJ repair process ................................................................. 142
4.5. Discussion ........................................................................... 143
4.6. Conclusion and Future work ..................................................... 146
4.7. Materials and Methods ............................................................ 148
  4.7.1. Media and growth conditions ................................................. 148
  4.7.2. Genotoxic treatments .......................................................... 148
  4.7.3. Drop test methodology ....................................................... 149
  4.7.4. Strains used in this study ...................................................... 149
  4.7.5. Cell cycle and checkpoint experiments ................................. 150
  4.7.6. Western blotting and antibody conditions ............................... 151
  4.7.7. Non homologous End-joining ligation assay ............................. 151
  4.7.8. DNA Resection assay ......................................................... 151
  4.7.9. FACS ................................................................................. 152
4.8 Supplementary Data: ................................................................. 153
  4.8.1. Supplementary Tables ........................................................ 153
4.9 Reference ............................................................................. 154
CHAPTER 5: Conclusions and Future Directions ................................. 159
  5.1 Chromatin and its vital role in the DDR ................................. 160
  5.2 References ............................................................................ 163
APPENDIX A: Laboratory Protocols .................................................. 165
APPENDIX B: Authors Funding and Contributions ............................. 178
LIST OF FIGURES

Figure 1.1: Saccharomyces cerevisiae cell cycle and the DNA damage checkpoints.............3
Figure 1.2: DSB-induced checkpoint activation in S. cerevisiae........................................6
Figure 1.3: Model of DSB repair by NHEJ and HR pathways in S. cerevisiae.................9
Figure 1.4: Schematic of domain structure of budding yeast Rad9...............................14
Figure 1.5: Nucleosome core particle and Histone H3 sequence.................................18
Figure 1.6: The H3 αN helix is highly conserved from fission yeast to humans.............20
Figure 1.7: Histone H3, H3S10 and S28 are preceded by an ARK sequence.................26
Figure 2.1: Histone H3, H3S10 and S28 are preceded by an ARK sequence.................43
Figure 2.2: Targeted mutagenesis of modifiable but previously uncharacterised residues of
H3 and H4 and their preliminary characterisation.........................................................47
Figure 2.3: Histone H3 alignment..................................................................................49
Figure 2.4: Analysis of sensitivity and specificity of Nl-H3S28ph and commercially
available antibodies raised to H3S28 phosphorylation................................................52
Figure 2.5: Histone H3 phosphorylation by Aurora B kinase..........................................53
Figure 2.6: Nl-H3S28ph antibody recognises both H3S10 and H3S28
phosphorylation..............................................................................................................55
Figure 2.7: Histone preparations sent for mass spectrometry analysis...........................57
Figure S2.1: h3S28A mutant contains two extra mutations.............................................66
Figure 3.1: Nucleosome core particle and Amino acid substitutions..............................81
Figure 3.2: H3E50 mutants show slow growth phenotypes along with varying degrees of
sensitivity to DNA damaging agents that cause damage in S phase.........................86
Figure 3.3: DNA damage checkpoint and recovery analysis of h3E50G and h3E50A
mutants...............................................................................................................................88
Figure 3.4: h3E50G and h3E50A cells have S phase recovery defects in response to MMS
and CPT damage............................................................................................................91
Figure 3.5: H3E50 is not required for 5′-3′ DNA end resection....................................93
Figure 3.6: Rad52 foci occur spontaneously in h3E50G and h3E50A cells.....................95
Figure 3.7: Rad52 foci persist in h3E50G and h3E50A cells after treatment with genotoxic
agents...............................................................................................................................97
Figure 3.8: Rad52 foci persist in h3E50G and h3E50A cells after treatment with HU......99
Figure 3.9: H3/H4 tetramer illustrating positive charges surrounding H3E50.............105
Figure 4.1: Rad9 is phosphorylated in an unperturbed cell cycle..................................126
Figure 4.2: CDK sites S494 and S618 are required for Rad9 cell cycle phosphorylation in
G2....................................................................................................................................130
Figure 4.3: Mutation of Rad9 CDK sites S494 and S618 mildly affects cell cycle........134
Figure 4.4: rad9S494+S618 cells have a G2/M checkpoint recovery defect......................138
Figure 4.5: CDK sites S494 and S618 are not required for survival after DNA damage or
DNA resection................................................................................................................141
Figure 4.6: Partial decreased efficiency of non homologous end joining in rad9S494+S618
cells................................................................................................................................143
LIST OF TABLES

Table S2.1: Antibodies used in this study.................................................................66
Table S2.2: Yeast strains used in this study.............................................................67
Table S2.3: Plasmids used in this study.................................................................69
Table S2.4: Primers used in this study.................................................................70
Table 3.1: H3E50 mutants show slow growth phenotypes in the presence and absence of damage.................................................................101
Table 3.2: Amino acid residue structure and propensity scale.................................103
Table 3.3: h3E50G, h3E50A and h3K56R cells are defective in S phase checkpoint recovery after MMS and CPT damage as measured by persistent Rad52 foci.....107
Table 3.4: Formation of persistent Rad52 homologous structures after MMS, CPT and HU treatment.................................................................108
Table S3.1: Strains used in this study.................................................................116
Table S3.2: Plasmids used in this study.................................................................117
Table S3.3: Primers used in this study.................................................................118
Table 4.1: Rad9 phosphorylation is perturbed in rad9S494, rad9S618 and rad9S494+S618 cells....................................................................................134
Table S4.1: Strains used in this study.................................................................153
Table S4.2: Plasmids used in this study.................................................................153
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast and ovarian cancer type-1 susceptibility gene</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1 carboxyl terminal</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum album</td>
</tr>
<tr>
<td>CAD</td>
<td>Chk1 activation domain</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CPT</td>
<td>Camptothecin</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>G1</td>
<td>Gap phase 1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap phase 2</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>γ-H2A</td>
<td>Phosphorylation of histone H2A on serine 129</td>
</tr>
<tr>
<td>HHT2</td>
<td>Histone H3 Copy-II</td>
</tr>
<tr>
<td>HHF2</td>
<td>Histone H4 Copy-II</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>M</td>
<td>Mitosis</td>
</tr>
<tr>
<td>MDC1</td>
<td>Mediator of DNA damage checkpoint 1</td>
</tr>
<tr>
<td>MMS</td>
<td>Methylmethane sulphonate</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MRX</td>
<td>Mre11-Rad50-Xrs2</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphoinositide 3-kinase related kinase</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>RCAF</td>
<td>Replication-Coupling assembly factor</td>
</tr>
<tr>
<td>RFC</td>
<td>Replication factor C</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>SCD</td>
<td>SQ/TQ cluster domain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TopI</td>
<td>Topoisomerase I</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my supervisors, Noel Lowndes and Muriel Grenon for all their guidance, support and encouragement over the last few years. I am thankful for all the help and support you provided me throughout the course of my PhD. Thanks to all the members of the Lowndes laboratory past and present for your help and friendship throughout the past few years. I am grateful to have worked and become friends with a great group of people. I would sincerely like to thank Anne for all your help throughout my PhD. I would like to thank Aoife for all her encouragement and friendship throughout my studies. I would also like to thank Danielle for providing help with experiments I carried out. I am grateful to all the help I received from the Flaus lab from advice to use of equipment. Thanks to Andrew Flaus for all your advice on histones. Additionally I would like to thank Jessica Downs and her laboratory for allowing me visit to learn techniques. Also I am grateful to Haico Van Attikum and his laboratory for accommodating me in thier laboratory and a special thanks to Thomas for all his help and experimental advice on my visit to Leiden. I am grateful to the Anatomy department for allowing me to use their microscopy equipment and to Kerry Thompson and Peter Owens for all their support and advice on microscopy. And finally I would like to thank my close friends and family for their great support and understanding throughout my PhD. Thanks to Brendan for always supporting and encouraging me and to John for all your encouragement. Lastly thanks to my parents whom without you none of this would be possible. Thanks to you all.
DECLARATION

I, Mary Walsh declare that the work presented in the thesis has been acquired and analysed by myself with the exception of:

Chapter 2:
- Figure 2.2A and 2.2B. Histone mutant screen and drop test analysis was performed by Thomas Costelloe.

Chapter 3:
- Figure 3.5 was performed by Thomas Costelloe.

Chapter 4:
- Figure 4.1 was performed by Noel Lowndes
- Figure 4.2 was performed by Jean Soulier
- Figure 4.3 was performed by Aisling O'Shaughnessy
- Figure 4.4 was performed in part by Aisling O'Shaughnessy
- Figure 4.5A was performed by Aisling O'Shaughnessy and Figure 4.5B by Achille Pellicioli
ABSTRACT

The DNA damage response (DDR) is a signal transduction cascade, which regulates cell cycle progression, gene transcription and DNA repair. Histones also play a vital role in the activation of the DDR and have been shown to be necessary for checkpoint protein interactions with chromatin, including Rad9, the first checkpoint protein discovered. Here, we study the role of these two types of proteins in the DDR.

In a screen to identify DNA damage sensitive histone mutants, specific residues of histone H3 were found to be involved in resistance to Camptothecin (CPT). We investigated further the role of two of these residues. We found that serine 28, which is highly conserved and phosphorylated in higher cells, is neither phosphorylated nor necessary to the DDR. However, when glutamate residue 50 was mutated to glycine, we observed defective growth phenotype, a slower S phase progression and sensitivity to a range of S phase damaging agents. H3E50 is located in the H3 αN helix, a region known to be important for cell viability and nucleosome stability. We observed that h3E50G and h3E50A cells have a strong checkpoint recovery defect along with the prolonged presence of Rad52 foci in agreement with chromatin structure being key to DSB repair in S phase.

We also studied mutations in Rad9 that induce a strong defect in checkpoint recovery from the G2/M checkpoint. These mutations affect two of the 20 possible CDK phosphorylation sites on Rad9 and are important for cell cycle phosphorylation. The Rad9 protein contains 20 putative Cdk phosphorylation sites. We have identified two of these sites important for recovery of cells from the G2/M checkpoint. Our results suggest that rad9S494A+S618A might be defective for specific Rad9 function including non homologous end joining, the other pathway of choice for DNA double-strand break repair.

Taken together, this work provides new leads to study the connection between chromatin structure and the cellular response to DNA damage.