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CHAPTER 1

Introduction
1.1. Overview
DNA is replicated and passed on to daughter cells in an error-free fashion to maintain genomic integrity. If damage to a cell is detected the cell responds by activating several DNA repair mechanisms. The DNA damage response (DDR) is the name given to the cellular response to DNA damage, which is conserved from yeast to human (Ciccia and Elledge, 2010). It allows the damaged DNA to be repaired efficiently. Failure to do so can lead to the development of cancer. DNA repair, similar to other processes involving DNA such as transcription or replication, occurs in a chromatin environment. The folding of DNA into chromatin hinders the ability of proteins to get access to the DNA to carry out these DNA transactions. But mechanisms such as covalent histone modifications (including phosphorylation, methylation or acetylation for example), ATP-dependent chromatin remodelling (which consist of nucleosome exchange, displacement and re-organisation) and incorporation of histone variant proteins have evolved to deal with this problem (Costelloe et al., 2006).

Studies have elucidated roles for post-translational modifications of histones in processes such as transcription and the DDR. In a screen carried out on histone mutants in this laboratory, the glutamate residue 50 on histone H3 which is located in the H3 αN helix, was found to be involved in resistance to DNA damaging agents that damage in S phase. Another residue histone H3 serine 28 when mutated to an alanine also appeared to be sensitive to CPT. Preliminary results obtained suggested that both H3E50 and H3S28 play a role in the DDR.

1.2. *Saccharomyces cerevisiae* cell cycle and budding morphology
The eukaryote mitotic cell cycle consists of four stages; G1 (Gap1), S, G2 (Gap2) and mitosis (M). The S and M phases are the most important phases during which DNA is fully and correctly replicated, and equally segregated between the 2 daughter cells respectively. *Saccharomyces cerevisiae* divides by budding (Figure 1.1). Cells in G1 are unbudded. Like in all eukaryotes, cells commit irreversibly into the next cell cycle once they pass the Start (or restriction point in higher cells) and enter S phase. At that point *S. cerevisiae* cells start bud growth. The bud will continue to grow during DNA replication in S phase and
the G2 phase, until it reaches 2/3 of the size of the mother cell early in M, at the metaphase/anaphase transition. Then, cells engage into mitosis and nuclear division and cytokinesis will occur to produce the mother and daughter cells. An asynchronous cell culture consists of one third unbudded cells in (G1), one third of small budded cells in S and one third of large budded cells in G2 or M (Figure 1.1A). Yeast cells can easily be synchronised in G1 and G2 by using α-factor (G1), a physiological peptide involved in mating and nocodazole which inhibits the polymerisation of microtubules (G2/M arrest) (Figure 1.1B).

![Diagram of the budding yeast cell cycle and the DNA damage checkpoints.](image)

**Figure 1.1: Saccharomyces cerevisiae cell cycle and the DNA damage checkpoints.** (A) The budding yeast cell cycle showing the points of action of the three principal checkpoint arrests. (B) Specific cell morphologies observed in the normal cell cycle, after synchronization in G1(α factor) and G2/M (nocodazole). Figure adapted from (O'Shaughnessy et al., 2006).
1.3. The DNA damage response

Cells are constantly under threat from damage from exogenous and endogenous sources such as UV, IR and reactive oxygen species (ROS), which if left untreated threaten the integrity of the genome. DSBs are the most cytotoxic form of damage a cell can incur. Therefore to maintain genomic integrity cells have evolved DNA repair mechanisms. If genomic integrity is not maintained this leads to the development of cancer. Following DNA damage cells have evolved signal transduction pathways, which transmit a signal to effectors once the damage has been detected. These signal transduction pathways are termed DNA damage cell cycle checkpoints which upon detecting damage initiate a DDR (Figure 1.2; (Harrison and Haber, 2006). Remarkably all the steps of checkpoint activation are conserved from yeast to humans (Lisby and Rothstein, 2009). After DNA double strand breaks (DSBs) two principal mechanisms of repair, Non homologous end joining (NHEJ) and Homologous Recombination (HR) have evolved to maintain the genomic integrity of the cell.

DSBs are sensed by both the Ku (yKu70/yKu80) and MRX (Mre11-Rad50-Xrs2) DSB repair complexes (Lisby et al., 2004; Martin et al., 1999; Wu et al., 2008), where both can bind directly to unprocessed DSB ends (Chen et al., 2001; Hopfner et al., 2001; Milne, 1996). The Ku complex inhibits 5’end resection which leads to repair of the DSB via non-homologous end joining (NHEJ) (Lee et al., 1998).

In contrast the MRX complex plays both a role in HR and NHEJ in S. cerevisiae (Rupnik et al., 2009). In S. cerevisiae, following formation of a DSB, the DNA ends are initially sensed by the MRX complex (Figure 1.2B Lisby et al., 2004). This binding of the MRX complex promotes the recruitment of the PIKK Tel1 to sites of damage via direct interaction between Tel1 and the C terminus of the Xrs subunit of the MRX complex (Figure 1.2C Falck et al., 2005; Nakada et al., 2003). Tel1 phosphorylates H2A on S129 and this kinase is also required for the DNA damage-induced phosphorylation of the Mre11 and Xrs subunits of the MRX complex and the Sae2 endonuclease (Baroni et al., 2004; Clerici et al., 2006; D'Amours and Jackson, 2001; Usui et al., 2001). Resection, which is initiated by the MRX complex and Sae2, occurs which gives rise to short 3’
ssDNA tails (Mimitou and Symington, 2008; Zhu et al., 2008). Further processing by Exo1 nuclease and/or Dna nuclease together with Sgs1 helicase results in the formation of long 3’ ssDNA tails (Gravel et al., 2008; Mimitou and Symington, 2008). Processive resection is followed by the disassociation of Sae2, the MRX complex, and Tel1 from the DSB and the concomitant binding of RPA to the 3’ ssDNA tails (Figure 1.2 D Lisby et al., 2004). Mec1 which associates with Ddc2 (Majka et al., 2006; Paciotti et al., 2000) recognises RPA coated ssDNA, which leads to localisation of Mec1-Ddc2 to sites of damage.

The 9-1-1 checkpoint clamp which comprises of Rad17-Mec3-Ddc1 proteins and the clamp loader, Rad24-rfc2-5 are independently recruited to RPA-coated ssDNA and are required to initiate the Mec1-dependent DNA damage checkpoint (Figure 1.2.E Kondo et al., 2001). The co-localisation of Mec1-Ddc2 and Ddc1 facilitates a stable association between Mec1-Ddc2 and Ddc1 which promotes the Mec1 dependent phosphorylation of Ddc2, the Ddc1 and Mec3 subunits of the 9-1-1 complex, Rad24 and RPA (Brush and Kelly, 2000; de la Torre-Ruiz et al., 1998; Majka et al., 2006; Paciotti et al., 2000; Paciotti et al., 1998). Subsequently Rad9 is phosphorylated and acts as a key regulator in the DNA damage checkpoint leading to activation of downstream targets (Emili, 1998; Schwartz et al., 2002; Vialard et al., 1998). Following checkpoint activation the downstream effector kinases Rad53 and Chk1 are activated (Blankley and Lydall, 2004; Gilbert et al., 2001; Sweeney et al., 2005).
Figure 1.2: DSB-induced checkpoint activation in *S. cerevisiae*. Following DSB formation (A), the DDR signaling cascade is initiated by binding of the MRX complex to the DSB ends (B). MRX recruits the Tel1 kinase, which phosphorylates histone H2A (C). After resection, the ssDNA is rapidly coated with RPA (D). This structure promotes the independent recruitment of both the Ddc1/Rad17/Mec3 complex that is loaded onto DNA by the Rad24/Rfc2-5 complex, and the Mec1/Ddc2 complex (E). Dpb11 is recruited via binding to phosphorylated Ddc1. Subsequently, Rad9 is recruited by either binding to γ-H2A and methylated H3K79 or through its association with Dpb11, and facilitates the PIKK-dependent phosphorylation of the Rad53 and Chk1 effector kinases (F). Activated Rad53 and Chk1 phosphorylate downstream effectors including Cdc5, Dun1, and Pds1, the end result being activation of the major biological responses to DNA damage, such as cell cycle arrest, DNA damage-induced transcription, DNA repair, and slowing of DNA replication. Figure adapted from (Finn et al., 2012).
1.3.1. Homologous recombination and non homologous end joining

In response to even one DSB cells initiate a cascade of events which leads to cell cycle arrest and repair (Harrison and Haber, 2006). In budding yeast HR is the preferred mechanism of repair, whereas in higher eukaryotes NHEJ performs a more important role. NHEJ is a potentially error prone form of repair which is generally restricted to the G1 phase of the cell cycle (reviewed in Weterings and Chen, 2008). While HR is an error free process in which genetic information is exchanged between DNA sequences that share homology, HR is active in S and G2 phases of the cell cycle after replication has occurred and a sister chromatid is available as a template for repair to occur.

Clb-CDK activity is essential for recombinational repair of DSBs and the choice of repair pathway is determined by CDKs (Aylon et al., 2004; Ira et al., 2004). Efficient HR requires genes which belong to the RAD52 epistasis group. These genes include *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *XRS2* and *MRE11* (Game and Mortimer, 1974).

Unlike HR, NHEJ does not require a homologous sequence as a template for repair. In NHEJ the broken DNA ends are ligated irrespective of sequence, which can result in the loss of DNA bases around the site of the break. In yeast the proteins required for NHEJ are yKu70 and yKu80 (in mammalian cells the Ku70/Ku80 heterodimer is also associated with DNA-PKcs); the MRX complex; and a DNA ligase complex comprised of Dnl4 (DNA ligase IV in humans), Lif1 (XRCC4 in humans), and Nej1 (XLF/Cernunnis in humans) (Figure 1.3A-D).

In budding yeast the preferred mechanism of repair is HR. The MRX complex is the earliest sensor of DNA damage breaks. Mre11 is the first protein detected at the site of a DSB (Lisby et al., 2004) which signals checkpoint activation through Tel1/Atm and regulates 5-3’ resection at the break. The MRX complex and Sae2 are required for the initiation of 5-3’ resection at the site of the break. Endonucleases such as Sgs1,Exo1 and Dna1 have all been implicated in carrying out further resection for the generation of ssDNA at the break (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008).
Resection leaves 3’ DNA ends which are rapidly covered by RPA protein (Figure 1.3F; Alani et al., 1992). Generation of RPA coated ssDNA provides a platform for the recruitment of further checkpoint machinery. Subsequent binding of Rad52 to RPA-coated ssDNA results in the formation of the Rad51 nucleoprotein filament by displacing RPA and facilitating the concomitant binding of Rad51 (Krejci et al., 2002; Miyazaki et al., 2004; Song and Sung, 2000). Rad52, the Rad55-Rad57 heterodimer and Rad59 mediate this Rad51 nucleofilament step (Davis and Symington, 2003). Rad51 is required for homology search and strand invasion. Once homology is found the Rad51 nucleofilament together with the Rad55-57 heterodimer and Rad54 invades the homologous (donor) strand (Figure 1.3H; (Alexeev et al., 2003; Petukhova et al., 2000; Sugawara et al., 2003; Sung, 1997; Van Komen et al., 2002), resulting in strand exchange and formation of structures called Holliday junctions. Srs2 helicase mediated disassembly of the the Rad51 nucleoprotein filament is then required to allow DNA synthesis to proceed (Figure 1.3I; Krejci et al., 2003; Veaute et al., 2003). Following DNA synthesis, resoultion of the Holliday junctions is preformed by Yen1 (human GEN1) endonuclease (Ip et al., 2008) and the Slx4-Slx1 (human SLX4-SLX1) flap endonuclease (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen and Harper, 2010). Following resolution of the intermediates, Rad52 and Rad55-Rad57 promote re-annealing of the newly synthesised DNA (Figure 1.3J; Miyazaki et al., 2004).
Figure 1.3: Model of DSB repair by NHEJ and HR pathways in *S. cerevisiae*. Following DSB creation, the DSB ends are tethered by the MRX complex and the Ku complex (A). In NHEJ, DSB ends are further stabilised by MRX and yKu70/yKu80 (B). The Dnl4-Lif1-Nej1 ligase complex is then recruited (C), the broken DNA ends are ligated, and the lesion repaired (D). In HR, resection is initiated by Sae2 and the MRX complex, followed by a more processive resection catalysed by Exo1 and/or Dna2 in collaboration with the Sgs1 helicase (E). RPA binds to ssDNA generated by resection (F). RPA-coated ssDNA is a substrate for Rad51 filament formation, involving Rad52, Rad55-Rad57, and Rad54 (G). Rad51 filament homology search and strand invasion lead to the formation of a D-loop (H). This is followed by disassembly of the Rad51 filament mediated by the Srs2 helicase, and subsequent DNA synthesis (I). Resolution of the Holliday junction is followed by re-annealing and ligation of the newly synthesised DNA (J). Figure adapted from (Finn et al., 2012).
1.3.2. HO-endonuclease system in yeast

Recombination between homologous sequences is a vital process which is involved in DNA recombination, repair and replication. In yeast we can study DSB repair by using a widely studied homologous recombination event which is mating type (MAT) switching (Haber, 2012). The mating of yeast occurs between haploid cells which are either a or α. The mating type is determined by two different alleles of the mating-type (MAT) locus. MATa and MATα are located on chromosome III.

Mate type switching is as result of a genetic recombination event intitated by specific DNA leavage by the HO endonuclease at the MAT locus. Purified HO protein cleaves a 24 base pair recognition site which leaves a 4bp, 3’ overhang ends (Montelone, 2002). Introducing mutations into the HO recognition site prevents cleavage and mating type switching. The ability of HO endonuclease to make specific, unique DSBs in chromosomal DNA has been exploited to study recombinaton both at the mating type locus and elsewhere in the yeast genome.

In the cell the homolgous recombination machinery repairs the cut by using one of the silent copies of the mating type information resident at the HM loci to replace the information at the active MAT locus. The 3’ overhangs generated following HO endonuclease cleavage are further processed by endonucleases to produce single stranded 3’ tails. These 3’ tails can interact with Rad51 to create a nucleoprotein complex that searches for homologous DNA sequences, which exist at the HM loci. The free ends then invade the homologous double-stranded region at HML or HMR and initiate a recombination event which results in the mating type information resident at the silent locus being copied to the MAT locus. The DSB repaired is repaired from one or two heterochromatic donors (HML and HMR) (Haber, 2012; Montelone, 2002).

Many studies have been performed using yeast mating-type gene switching (MAT switching) to induce DSBs. It has been proposed that Rad52 carries out three distinct functions during MAT switching (Miyazaki et al., 2004). Rad52
was shown to be involved in the presynaptic, synaptic and postsynaptic stages during *MAT* switching (Miyazaki et al., 2004).

HO-induced breaks, like γ-irradiation induced damage preferentially generates Rad52 foci in S phase. The number of Rad52 foci formed *in vivo* is independent of the number of DNA DSBs regardless of whether they were induced by HO-endonuclease or γ-irradiation (Lisby et al., 2001). This suggests that one focus represents the repair of multiple DNA lesions within a limited certain of centers in the nucleus (Lisby et al., 2001). In addition it has been shown that Rad52 foci whether spontaneous or induced by DNA damage are strongly enriched in the nuclear interior (Bystricky et al., 2009).
1.3.3. The DNA damage checkpoints; The S phase DNA damage response

Two checkpoints operate during S phase and have been termed the Intra S phase checkpoint and the replication checkpoint (Segurado and Tercero, 2009). Although genetically separable, both checkpoints partially overlap as they both utilize similar or common DNA structures (Zegerman and Diffley, 2009). The Intra S phase checkpoint slows down S phase progression following damage induced by DNA damaging agents.

In S.cerevisiae, checkpoint activation is dependent on Mec1 and Rad53 (Paulovich and Hartwell, 1995). The intra S checkpoint slows replication by inhibiting polymerase α-primase (pol α primase) (Longhese et al., 1996; Marini et al., 1997). DNA primase pol α primase required for initiating DNA synthesis is involved in both S phase checkpoints. Polα is activated by phosphorylation in a cell cycle dependent manner and activated Rad53 blocks this phosphorylation thereby inhibiting the action of Polα and preventing initiation of DNA synthesis (Longhese et al., 1996; Marini et al., 1997).

The DNA replication checkpoint inhibits replication when replication forks stall due to perturbations or a depletion of dNTPs as a result of ribonucleotide reductase inhibiton. Interestingly unlike the intra S checkpoint, Rad9 does not play a role in the DNA replication checkpoint but rather Mrcl (mediator of replication checkpoint) is the adaptor protein which is responsible for activation of Rad53 (Alcasabas et al., 2001). Upon activation of the replication checkpoint, Mrcl forms a stable complex with the S phase checkpoint protein complex Tof1-Csm3 (equivalent to Timelss (Tim) and Tipin in human cells), preventing uncoupling of the replisome from the site of DNA synthesis (Katou et al., 2003). Mec1-Ddc2 is subsequently recruited to stalled replication forks, allowing for phosphorylation of Mrcl which in turn leads to Rad53 phosphorylation (Naylor et al., 2009; Osborn and Elledge, 2003). In the absence of Mrcl, Rad9 can fulfil this role, indicating that the replication stress can be converted into a DNA damage signal (Alcasabas et al., 2001; Foss, 2001), leading to Rad9 mediated activation of Rad53 and Chk1 (Alcasabas et al., 2001).
Maintaining the genomic integrity of the cell during replication is paramount to the cell and it is not surprising the degrees of complexity which are involved in the S phase pathways (Finn et al., 2012).

1.4. RAD9, the prototypical DNA damage checkpoint gene

RAD9 was the first DNA damage checkpoint gene discovered (Weinert and Hartwell, 1988). Rad9 regulates multiple cellular processes that influence genomic integrity. Rad9 plays a fundamental role in the DNA damage and loss of Rad9 results in sensitivity to DNA damaging agents, defective transactivation of DNA repair and replication genes, defective activation of DNA damage checkpoints and increased genomic instability (Aboussekhra et al., 1996; Myung and Kolodner, 2002; Toh and Lowndes, 2003; Weinert and Hartwell, 1988; Weinert and Hartwell, 1990).

Rad9 is not just involved in one phase of the cell cycle but is required for the G1, S and G2/M arrests in response to DNA damage where it promotes Mec1/Tel1-dependent phosphorylation of both Rad53 and Chk1 effector kinases in response to DNA damage (Gilbert et al., 2001; Paulovich et al., 1997; Sanchez et al., 1999; Siede et al., 1993; Sweeney et al., 2005; Weinert and Hartwell, 1988).

1.4.1. Domain structure of Rad9

Rad9 is a phospho-protein which becomes phosphorylated in S and G2 phases (Vialard et al., 1998). The RAD9 gene encodes a protein of 1309 amino acids with a predicted mass of 148kDa (Schiestl et al., 1989; Weinert and Hartwell, 1990). Rad9 contains a number of domains that are important for its function in checkpoint control (Figure 1.4). The N terminus of Rad9 comprises the Chk1 Activation Domain (CAD), corresponding to the first 231 amino acids of the protein (Blankley and Lydall, 2004) The CAD region is required for Rad9-dependent activation of Chk1 (Blankley and Lydall, 2004) and specific CDK sites on Rad9 are necessary for Chk1 activation in response to DNA damage (Abreu et al., 2013).

Rad9 contains 14 potential PIKK phosphorylation sites (SQ/TQ), six which are clustered together in the centre of the protein and referred to as the SQ/TQ
cluster domain (SCD) (Schwartz et al., 2002). These sites are required for Mec1-dependent Rad9 hyperphosphorylation following DNA damage (Schwartz et al., 2002). Rad9 also contains a tandem Tudor domain that is required for recruiting Rad9 to chromatin via binding of its Tudor domain to methylated lysine 79 of histone H3 (Grenon et al., 2007; Huyen et al., 2004). The C terminal domain of Rad9 contains tandem BRCT (BRCA1 carboxyl terminal) repeats. The Rad9 BRCT domain mediates the oligomerisation of Rad9 following DNA damage (Soulier and Lowndes, 1999; Usui et al., 2009).

Figure 1.4: Schematic of domain structure of budding yeast Rad9. Structural motifs on the Rad9 protein include CAD: Chk1 activation domain; SCD: serine/threonine cluster domain; Tudor: tandem Tudor domain; BRCT: tandem BRAC1 carboxy terminus domains. Also depicted are putative PIKK phosphorylation sites and putative CDK sites.
1.5. DNA damaging agents which perturb S phase progression

1.5.1. CPT is a topoisomerase I poison
Cellular DNA is tightly compacted and extensively supercoiled. In order for transcription, replication, recombination and repair to be carried out the two strands of duplex DNA must be separated to form a template for these processes to proceed. Topoisomerase I (Top1) enzymes change the topology of the DNA to facilitate these functions by cleaving and religating the DNA. Camptothecin (CPT) is a Top1 poison which induces DSBs specifically in S phase by preventing the transient cleavage and religation of DNA Top1. In a normal S phase, Top1 acts to prevent the superhelical strain around the elongating replication fork by transiently cleaving and religating a single strand of duplex DNA via a covalent 3’phosphotyrosyl enzyme-DNA intermediate. Normally these cleavage complexes are short-lived intermediates, but CPT stabilizes this complex by slowing the rate of DNA religation (Pommier, 2006). DSBs are generated when this CPT-induced stabilized complex blocks a replication fork. Although CPT induces damage in S phase, this damage does not induce an intra S-phase checkpoint. Instead, where possible “on-the-fly and checkpoint blind repair” of CPT induced lesions is carried out (Redon et al., 2003). Cells may also pass through S phase and eventually pause in the G2/M checkpoint phase of the cell cycle to complete repair.

1.5.2. Cisplatin forms inter and intrastrand crosslinks
Cisplatin forms covalent bonds with the N7 position of purine bases to form 1,2- or 1,3 covalent-intrastrand crosslinks and a lower percentage of interstrand crosslinks. The formation of these cisplatin induced crosslinks interferes with DNA replication and transcription. The intrastrand and interstrand crosslinks interfere with the structure of the DNA and these alterations are recognised by the cellular proteins which repair cisplatin induced DNA damage. The primary pathway involved in repairing cisplatin induced damage is nucleotide excision repair (NER). Mismatch repair (MMR) pathway and the homology-directed DNA repair (HR) pathway have also been implicated in the repair of cisplatin induced damage (Basu and Krishnamurthy, 2010).
1.5.3. MMS is a DNA alkylating agent which blocks replication
Methyl methanesulfonate (MMS) is a DNA alkylating agent which modifies both guanine (to 7-methylguanine) and adenine (to 3-methyladenine) leading to base mispairing and replication blocks, respectively (Sedgwick, 2004). MMS damage is primarily repaired by Base excision repair (BER) and DNA alkyltransferases. RAD52 epistasis group mutants are sensitive to MMS (Prakash and Prakash, 1977) therefore raising the idea that MMS leads to DSBs which are repaired by HR. Evidence suggests that MMS does not induce DSBs directly but rather MMS-induced DSBs are the by product of collapsed replication forks (Lundin et al., 2005; Nikolova et al., 2010).

MMS induces DSBs primarily in S phase of the cell cycle. HR and not NHEJ protect against MMS induced damage. HR is the major pathway of repair in response to damage by MMS (Nikolova et al., 2010). Mms22, Mms1, Rtt101, Rtt109 and Asf1 are required for MMS induced HR. Mms22 and Mms1 are not required for HR induced by DSBs therefore these proteins are specifically required for HR involving blocked replisomes. Mms1 and Mms22 are important for maintaining the integrity of the replisome when replication progression is slowed by HU (Duro et al., 2008; Vaisica et al., 2011).

1.5.4. HU blocks replication by inhibiting dNTPs
HU blocks DNA synthesis by inhibiting ribonucleotide reductase (RNR), thus preventing the reduction of ribonucleotides to deoxyribonucleotides. High doses of HU (200 mM) prevents the accumulation of dNTPs that occurs as cells enter S phase, impeding S phase progression and activating a checkpoint response (Chabes et al., 2003; Koc et al., 2004). Replication stress induced by HU affects both elongation and origin activation. In response to HU-induced replication stress, the S phase checkpoint acts to slow down S phase progression so that activations from all origins are delayed and late origin activation is repressed in the presence of HU (Alvino et al., 2007; Santocanale and Diffley, 1998). The S phase checkpoint leads to activated Rad53 which triggers several downstream events to regulate DNA synthesis.
1.6. Nucleosome structure and function

The fundamental subunit of chromatin is the nucleosome (Luger et al., 1997a), which consists of 146bp DNA wrapped around a histone octamer. This histone octamer consist of two copies of each histone H2A, H2B, H3 and H4. A H2A-H2B dimer attaches on each side of the (H3-H4)$_2$ tetramer within the core nucleosome (Figure 1.5A; Luger et al., 1997a; White et al., 2001). The repeating nucleosome core assembles into higher order chromatin structures which are stabilised by the linker histone H1 and these compact linear DNA overall by a factor of 30-40. This higher order structure which is the principal packaging element of DNA within the nucleus primarily determines DNA accessibility. It is within this chromatin environment in which processes such as recombination, transcription and replication occur. The 146bp of DNA are wrapped around the histone octamer in 1.65 turns of a flat, left-handed superhelix (Luger et al., 1997a).

Histones are made up of two structural regions, a histone fold domain which participates in histone-histone interactions and DNA-histone interactions, and flexible tails which protrude out from the nucleosome core and are which often modified by post translational modifications. Although tails do not contribute to the overall stability of the nucleosome (Luger et al., 1997b) they are involved in a number of processes which can affect the higher order structrue of chromatin (Luger and Richmond, 1998).
Figure 1.5: Nucleosome core particle and Histone H3 sequence. (A) Nucleosome core particle: Ribbons representing the structure of the nucleosome, showing DNA in brown and green and eight histone protein chains in yellow: H3; blue: H4; cyan: H2A; grey: H2B. The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle. The H3E50 residue located on the H3 αN helix, represented on the nucleosome by CPK colouring, is located at the DNA entry and exist site of the nucleosome. (Nucleosome core particle schematics courtesy of Andrew Flaus) (B) Histone H3 sequence of *S. cerevisiae*. The α-helices are underlined and L1 and L2 loops are depicted by a broken line and named L1 and L2 underneath. H3S28 and H3E50, the two residues on histone H3 studied in this thesis have both been highlighted by a blue box.
Histones consist of similar secondary structures. They each contain three α-helices (α1, α2 and α3) and 2 loops (L1 and L2) (Figure 1.5B; (Luger et al., 1997a). In addition to these similar structures H3 and H2B each have an extra αN and αC helix respectively, while H2A has short αN and αC helices. Each histone has an N and C terminus tail. The N terminal tail lies outside the core globular domain. These N terminal tails are often heavily modifiable and are involved in a number of process. Histone residues in the tail and globular core have been shown to modified and play important roles such as transcription and DNA damage response (Berger, 2007; Costelloe et al., 2006; Downs et al., 2007; Masumoto et al., 2005; Toh et al., 2006; Wurtele and Verreault, 2006). Histone mutant screens involving site directed mutagenesis on histone residues have been completed in S. cerevisiae (Dai et al., 2008; Hyland et al., 2005; Matsubara et al., 2007) and this is discussed in more detail in chapter 3.

1.6.1. Histone H3 αN helix is highly conserved and important for viability
One region in histone H3, the H3 αN helix, has been reported to be involved in many cellular responses. The positioning of the H3 αN helix, which is located at the entry and exist site of the DNA, is one reason why this area is important for cellular responses (Figure 1.5A). Screens carried out where all residues in the αN helix were mutated to alanines showed all but one residue being sensitive to DNA damaging agents (see chapter 3 for further discussion) (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). These screens also identified H3E50 when mutated to an alanine to be sensitive to CPT and MMS. H3E50 is located in the middle of the H3 αN helix and this residue is conserved between species (Figure 1.6).
In vitro studies with *Xenopus laevis* nucleosomes have shown deletions of the H3 tail result in changes to the behaviour of nucleosomes (Ferreira et al., 2007). H3 αN helix, which is adjacent to the H3 tail, makes important contacts with the DNA at the edge of the nucleosome so alterations to the H3 tail may affect the conformation of the H3 αN helix from which the tail protrudes (Ferreira et al., 2007). To investigate this Ferreira et al carried out a study to investigate the effects of alanine mutagenesis within the H3 αN helix on nucleosome dynamics (Ferreira et al., 2007). Interestingly, they found nucleosome sliding was increased in many of the mutants generated but to varying degrees. The sensitivity of nucleosomes to alterations in the H3 αN region is consistent with studies carried out in yeast (see chapter 3 for further discussion). *h3L48A, h3I51A* and *h3Q55A* were unable to form octamers, a result comparable to yeast.

![Figure 1.6: The H3 αN helix is highly conserved from fission yeast to humans.](image)

Alignment of the H3 αN helix, residues 45 to 56 on histone H3 from *S. pombe, S. cerevisiae, C. elegans, D. melonogaster, M. musculus, R. norvegicus, H. sapiens*. The H3 αN helix is highly conserved from fission yeast. H3E50, which is conserved through evolution, is located in the middle of the αhelix and is coloured in pink. H3T45 and H3K56, two residues located in the αhelix, which play roles in replication are highlighted in blue.

*In vitro* studies with *Xenopus laevis* nucleosomes have shown deletions of the H3 tail result in changes to the behaviour of nucleosomes (Ferreira et al., 2007). H3 αN helix, which is adjacent to the H3 tail, makes important contacts with the DNA at the edge of the nucleosome so alterations to the H3 tail may affect the conformation of the H3 αN helix from which the tail protrudes (Ferreira et al., 2007). To investigate this Ferreira et al carried out a study to investigate the effects of alanine mutagenesis within the H3 αN helix on nucleosome dynamics (Ferreira et al., 2007). Interestingly, they found nucleosome sliding was increased in many of the mutants generated but to varying degrees. The sensitivity of nucleosomes to alterations in the H3 αN region is consistent with studies carried out in yeast (see chapter 3 for further discussion). *h3L48A, h3I51A* and *h3Q55A* were unable to form octamers, a result comparable to yeast.
studies carried out which report these three mutants to be lethal in yeast (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008).

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

The H3 αN helix has been shown to be important for nucleosome stability and viability (Dai et al., 2008; Ferreira et al., 2007; Matsubara et al., 2007; Seol et al., 2008). Two residues, H3T45 and H3K56, which are present on either side of the αN helix are reported to be involved in the DDR (Baker et al., 2010). H3T45 is phosphorylated \textit{in vivo}, and H3T45 when mutated to alanine, \textit{h3T45A}, is highly sensitive to CPT and MMS (Baker et al., 2010; Matsubara et al., 2007). H3T45ph functions in apoptosis in higher cells but no such function exists in yeast (Hurd et al., 2009). This modification which is present in S phase is induced by replication stresses such as CPT and HU (Baker et al., 2010).

H3K56, the last residue of the H3 αN helix is well characterised and has been shown to be acetylated in lower and higher eukaryotes. H3K56 acetylation is cell cycle regulated, maintained upon replicative damage in a checkpoint dependent manner and is essential for surviving DNA damage (Celic et al., 2006; Hyland et al., 2005; Maas et al., 2006; Masumoto et al., 2005; Millar and Russell, 1992; Recht et al., 2006; Wurtele et al., 2012). Although both H3T45ph and H3K56ac are present in S phase, they function independently of each other. In \textit{S. cerevisiae}, 20-30\% of H3K56 is acetylated in asynchronous cells (Masumoto et al., 2005), but occurs at much lower levels in human cells (Das et al., 2009; Miller et al.; Tjeertes et al., 2009; Vempati et al.; Xie et al., 2009; Yuan et al., 2009). In \textit{S. cerevisiae}, H3K56ac has been reported to be present in the vast majority of new H3 molecules deposited behind replicating forks (Celic et al., 2006; Masumoto et al., 2005).

H3K56 methylation has also been identified but is unique to higher eukaryotes. H3K56me1, which contributes to replication control through its interaction with PCNA, exists in a small but significant fraction of H3 (Yu et al., 2012).
H3K56ac both increases upon genotoxic stress and localises to DNA damage foci in higher eukaryotes (Das et al., 2009; Yuan et al., 2009). Interestingly Yu et al., found in human cells unlike H3K56ac, H3K56me1 remains unchanged and does not localise to DNA damage after IR, thus indicating that H3K56ac and H3K56me1 must have distinct cellular functions.

H3K56 acetylation is dependent on the histone acetyltransferase (HAT) Rtt109 as well as the histone chaperone Asf1 (Driscoll et al., 2007; Han et al., 2007; Recht et al., 2006). HDACS Hst3/Hst4 are responsible for deacetylation of H3K56 as cells pass through S phase. Mutations that result in abrogated H3K56 acetylation such as \textit{asf1Δ}, \textit{rtt109Δ}, \textit{h3K56R}, \textit{hst3Δ/hst4Δ} lead to many cellular phenotypes such as increased doubling times, failure of a subpopulation of cells to undergo mitosis, defective S phase progression, sensitivity to genotoxic agents (Cellic et al., 2006; Cellic et al., 2008; Chen et al., 2008; Driscoll et al., 2007; Hyland et al., 2005; Kim and Haber, 2009; Masumoto et al., 2005; Recht et al., 2006; Thaminy et al., 2007; Wurtele et al., 2012).

Asf1 is a highly conserved histone chaperone that co-purifies \textit{in vitro} with histone H3 and H4 in a complex known as the replication-coupling assembly factor (RCAF). Specific acetylation patterns found on H3 and H4 in the RCAF complex are identical to those found in newly synthesised histones (Tyler et al., 1999). Both RCAF and Caf-1 act as histone chaperone for H3 and H4 and function synergistically to assemble chromatin on to newly synthesised DNA (Tyler et al., 1999). In \textit{S. cerevisiae} CAF-1 consists of three subunits, Cac1, Cac2 and Cac3 of which Cac1 interacts with histones H3 and H4. Histone deposition onto DNA is assisted by Asf1 and Rtt106 (Huang et al., 2005; Tyler et al., 2001).

Asf1 is required for chromatin assembly following DSB repair and the absence of Asf1 leads to cell death (Chen et al., 2008). Lack of H3K56ac results in a defective checkpoint recovery phenotype in which cells are unable to recover from the DNA damage checkpoint. Although \textit{asf1Δ} cells are proficient for repair they display a persistent checkpoint after repair is complete which ultimately leads to cell death (Chen et al., 2008). Recent evidence argues that defects in
DNA damage checkpoint deactivation are unlikely to account for the loss of viability of H3K56ac-deficient cells (Wurtele et al., 2012).

H3K56ac-deficient cells have problems in completing homology-dependent recombination (Wurtele et al., 2012). In the absence of genotoxic agents asf1Δ cells accumulated DSBs as measured by Rad52 foci during S and G2/M phase of the cell cycle, with 53% Rad52 foci occurring spontaneously compared to 24% Rad52 for WT cells in S phases and G2/M cells (Prado et al., 2004). In response to genotoxic damage, h3K56R cells formed persistent Rad52 foci for over 6 hours following a transient exposure to MMS, and CPT, whereas WT cells had significantly decreased levels of Rad52 foci at 3-4 hours after damage (Wurtele et al., 2012). The persistence of these foci indicates problems in H3K56ac–deficient cells in completing homology dependent recombination.

1.6.3. MMS22 and MMS1 play an important role along with H3K56ac in genomic integrity
A genetic analysis in S. cerevisiae identified a pathway involving the MMS22, MMS1, RTT101, ESC4/RTT107, ASF1 and RTT109 genes as being significant players to cell survival after replisome stalling (Collins et al., 2007; Pan et al., 2006). Similar to H3K56ac deficient cells, Mms22 and Mms1 mutant cells are sensitive to agents that perturb replisome progression (Araki et al., 2003; Chang et al., 2002; Hanway et al., 2002; Hryciw et al., 2002; Prakash and Prakash, 1977; Vaisica et al., 2011) and have an S phase recovery defect as measured by persistent Rad53 phosphorylation after damage induced by MMS (Duro et al., 2008). Cells lacking Mms22 in both S. cerevisiae and S. pombe showed elevated levels of Rad52/Rad22 foci in damaged and undamaged cells compared to WT (Dovey and Russell, 2007; Duro et al., 2008).

Cullins are a family of proteins that provide scaffolds for the assembly for ubiquitin E3 ligases. S. cerevisiae encodes three cullin proteins: Cdc5, Cul3 and Rtt101. Mms1 and Mms22 were identified as functional subunits of an Rtt101-based uniquitin ligase that promotes the completion of DNA replication after MMS exposure (Duro et al., 2008; Zaidi et al., 2008). Sequence analysis suggests that the Rtt101Mms1/Mms22 complex is similar to the mammalian Cul4Ddb1 complex
Cul4\textsuperscript{Ddb1} is a ubiquitin ligase with a number of roles in genome maintenance (reviewed in Jackson and Xiong, 2009). Rtt107 (regulator of Ty1\textsuperscript{transposition 107}; Esc4) is also necessary for the completion of DNA replication after MMS exposure. Deletion of RTT109 affects the Rtt101-dependent recruitment of Rtt107 to chromatin in response to MMS damage but deletion of ASF1 or a h3K56R mutation does not (Roberts et al., 2008; Rouse, 2004). Therefore, it has been speculated that Rtt109, in addition to acetylating H3K56, may acetylate other targets that regulate the Rtt101-Mms1-Mms22 complex. But deletion of RTT101, MMS1, or MMS22 does not exacerbate the MMS sensitivity of rtt109\textsuperscript{Δ} mutant cells, implying that Rtt109 and H3K56ac function in the same pathway as the Rtt101-Mms1-Mms22 Ub ligase to confer resistance to MMS (Wurtele et al., 2012).

1.7. Histone post-translational modifications

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

Histones are subjected to post translational modifications such as methylation, acetylation, phosphorylation, sumoylation and glycosylation. These PTMs play roles in transcription, replication, chromosome segregation, recombination and repair. Histones comprise of a high content of lysines and arginines and a significant number of serines and threonines. These residues, by their polar nature, are typically located on the exposed surfaces of the histone octamer. The amine and hydroxyl functional groups of these side chains are reactive under biochemical conditions and can readily undergo post translational modifications. Differences in the electrostatic properties of unmodified and modified residues can significantly alter the interactions between the histones and DNA.

Acetylation of lysines in the N-terminal tails of H3 and H4 removes the positive charge of the side chain which results in destabilized higher order chromatin structure. (Narlikar et al., 2002). Acetylation dependent relaxation of higher order chromatin structure occurs in transcriptionally active regions. For H3, acetylation and methylation of lysines is correlated with active and silent chromatin. Methylation of H3 residue 4, 36 or 79 is associated with active chromatin whereas methylation residue 9 or 27 is correlated with silent
chromatin. H3K56ac, as mentioned above plays a role in replication but this mark is has also been implicated in transcription (Rufiange et al., 2007; Xu et al., 2005).

H3S10 and H3S28 phosphorylation in higher cells correlates with active chromatin. H3S10ph is not associated with any specific chromatin fractions and is found equally in transcriptionally active and repressed chromatin regions, whereas H3S28ph is found in fractions containing transcribed chromatin (Sun et al., 2007).

DNA repair occurs in a chromatin environment. In recent years roles for post-translational modifications in the DDR have been elucidated. One characterised mark on chromatin is the phosphorylation of serine 139 on the C-terminal tail of histone H2AX, or the equivalent residue of yeast, serine 129. H2A(X), is phosphorylated by phosphatidyl inositol kinase-like kinase (PIKK), is induced after DNA damage and has become a standard mark of DSBs (Downs et al., 2007; Toh et al., 2006). H2AS129ph is involved in the recruitment of chromatin remodeling enzymes to DSBs, including INO80, Swr1 and NuA4 chromatin remodeling complexes (Downs et al., 2000; Morrison et al., 2004; van Attikum et al., 2004).

H3K79me is a mark which is constitutively present on chromatin, but following DNA damage plays a role in checkpoint activation. (Giannattasio et al., 2005; Grenon et al., 2007; Huyen et al., 2004; Wysocki et al., 2005), H3K79me is required for efficient checkpoint signaling and Rad9 recruitment onto chromatin. Following DNA damage, Rad9 is recruited via binding of its Tudor and BRCT domains to methylated H3K79 and γ-H2A, respectively (Grenon et al., 2007; Hammet et al., 2007; Huyen et al., 2004).

1.7.2. H3S10 and H3S28 phosphorylation are cell cycle regulated
Biological functions of H3S10 and H3S28 phosphorylation marks have yet to be elucidated fully but these modifications appear to be related to transcriptional activities (Banerjee and Chakravarti, 2011; Cerutti and Casas-Mollano, 2009).
H3S10 and H3S28 phosphorylation are both used as cell cycle markers in mitosis. Neither of these marks have been implicated to have a role in the DDR.

H3S10 and H3S28 are both phosphorylated in higher cells in vivo by the Aurora B kinase and the mitogen- and stress- activated kinase 1/2 (MSK1/2) (Dyson et al., 2005; Goto et al., 2002; Hsu et al., 2000; Soloaga et al., 2003). The yeast Aurora B homologue Ipl1 can phosphorylate both H3S10 and H3S28 in vitro (Sugiyama et al., 2002). No evidence exists for H3S28 phosphorylation in yeast but H3 is highly conserved between yeast and humans (Figure 1.7). Therefore it is probable that H3S28 is phosphorylated in yeast. Ipl1, the budding yeast equivalent of Aurora B, phosphorylates H3S10 in *S. cerevisiae* (Sugiyama et al., 2002). Human Aurora B, which is expressed in the G2/M phase of the cell cycle (Bischoff et al., 1998) is an arginine directed kinase which requires an arginine at the -2 position. Interestingly, both H3S10 and H3S28 sequences are preceeded by an ARK sequence, with arginine being present in the -2 position (Figure 1.7). Although in similar sequence context Sugiyama et al., and colleagues reported that the phosphorylation efficiency of Aurora B for H3S28 was approximatley 15% that of H3S10. One residue immediately following H3S28 (+1 position) is alanine, where as threonine is at the +1 position of H3S10. Interestingly when the threonine at the +1 position to H3S10 was mutated to alanine a similar in vitro reduction in phosphoryaltion was seen to that of H3S28 (Sugiyama et al., 2002).

**Figure 1.7:** Histone H3, H3S10 and S28 are preceeded by an ARK sequence. Schematic representing residues 1-37 of human and *S. cerevisiae* histone H3. H3S10 and S28 are preceeded by an ARK sequence. with arginine being present in the -2 position. Aurora B/Ipl1 is an arginine directed kinase which requires an arginine to be present at the -2 position.
If H3S28 is phosphorylated in yeast candidate kinases could be Ipl1 or Sch9, which is believed to be an orthologue of Msk1 in yeast. H3S28 phosphorylation status in *S. cerevisiae* was investigated in this thesis but remains to be determined.

### 1.8 Working hypothesis/Aim of thesis

Histones are highly conserved from lower to higher eukaryotes. Many posttranslational modifications are also highly conserved through species. In addition to the core histone fold domain (α1-L1-α2-L2-α3) H3 has an additional αN helix which is important for nucleosome stability. The H3 αN helix, which is located at the DNA entry and exit site of the nucleosome, regulates nucleosome stability. In a screen performed in our laboratory we found that when H3E50 was mutated to glycine the resulting strain was viable but dramatically sensitive to CPT. In general mutations in the H3 αN helix are highly sensitive to DNA damaging agents, highlighting the importance of this region for genome stability.

To investigate the role of H3E50 in cellular viability both in perturbed and unperturbed conditions, we generated five H3E50 mutants, *h3E50G*, *h3E50A*, *h3E50K*, *h3E50Q* and *h3E50D*. We predicted that mutating residue 50 from a glutamate to an aspartate residue would not have severe implications for the cells as glutamate and aspartate are similar in structure and charge. On the other hand, the other four mutations, due to differences in structure and charge would distort the H3 αN helix structure which could affect nucleosome dynamics and impact upon genomic stability.

*h3E50G* cells are highly sensitive to DNA damaging agents that damage during replication. Therefore, we predict that an intact H3E50 residue is important for the DNA damage response during S phase. H3K56 is another residue located on the H3 αN helix. H3K56 acetylation is a known mark which plays a role in the DNA damage response. Cells lacking H3K56 acetylation have previously been reported to be sensitive to CPT and MMS but not to acute doses of IR (Driscoll et al., 2007; Masumoto et al., 2005; Pommier, 2006; Wurtele et al., 2012). *h3E50G* cells, similar to H3K56ac deficient cells are sensitive to MMS and CPT.
but not to doses of HU which activate a checkpoint response. $h3E50G$ cells and H3K56ac deficient cells have defects in completing homologous recombination-mediated DSB repair after MMS and CPT lesions (Chapter 3 and Wurtele et al., 2012). This suggests that $h3E50G$ and $h3K56R$ cell death after exposure to genotoxic agents is not due to problems in checkpoint deactivation but rather due to problems in HR mediated repair after MMS and CPT damage. Rtt109 and H3K56ac function in the same pathway as the Rtt101-Mms1-Mms22 Ub E3 ligase (equivalent to human CUL4$^{Ddb1}$) to confer resistance to MMS (Wurtele et al., 2012). Due to $h3E50G$ cells behaving similarly to $h3K56R$ cells, we believe an intact H3E50 residue could be important for this pathway.

Earlier studies performed in the laboratory suggested that H3S28, when mutated to an alanine is highly sensitive to CPT. H3S28 has been shown to phosphorylated in higher cells but no such evidence for H3S28 phosphorylation exists in lower eukaryotes. We predicted due to the sensitivity of cells to genotoxic agents in the absence of H3S28 and the high conservation of this residue, that H3S28 would also be phosphorylated $S.cerevisiae$. Therefore we carried out detailed experiments to investigate the phosphorylation status of H3S28 in yeast.

With respect to H3, this thesis aimed to understand the role that H3E50 plays in the DDR, and secondly to determine whether H3S28 phosphorylation exists in $S. cerevisiae$.

Additionally, we investigated the role played by CDK sites on Rad9 in the DDR. The Rad9 protein contains 20 putative Cdk phosphorylation sites and we identified two of these sites to play an important role in the recovery of cells from the G2/M checkpoint. These two sites, S494 and S618, are important for Rad9 cell cycle phosphorylation and have been shown to induce a strong checkpoint recovery defect when mutated, with the exit from the damage-dependent. We aim to elucidate a role for Rad9 CDK sites in the DDR.
1.9 References


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CHAPTER 2

Is H3 serine 28 phosphorylated in Saccharomyces cerevisiae?
2.1. Summary
In the cell, chromatin is the physiological template for all DNA transactions including transcription, replication, and DNA repair. The fundamental subunit of chromatin is the nucleosome consisting of 147bp of DNA wrapped around a histone octamer comprised of two molecules each of the histones H2A, H2B, H3, and H4. Recent studies have elucidated a role for post-translational modifications of histones in this DNA damage response (DDR).

In this study we aim to identify novel histone marks involved in the DDR. We focus on previously uncharacterised, modifiable residues on both the amino- and carboxyl-terminus of histones H3 and H4. Using a plasmid shuffle system we have generated a collection of histone mutants to screen for defects in the DDR. The results of this screen identified a specific residue of histone H3 involved in resistance to camptothecin (CPT). H3 serine 28 (when mutated to alanine) is highly sensitive to a range of DNA damaging agents. H3 serine 28 has been studied in higher cells but the specific roles of this residue have yet to be elucidated. H3S28 has been shown to be phosphorylated in vitro and in vivo in higher cells. Here we present results of our histone mutant screen and also we investigate whether H3S28 is phosphorylated in *Saccharomyces cerevisiae*.

From our studies we conclude that H3S28 is not phosphorylated in *S. cerevisiae*. However we cannot rule out that H3S28 may be phosphorylated at low levels with which our range of detection methods are not sufficient for identification of this phosphorylation mark.

2.2. Highlights
- Histone mutant screen identifies novel histone mark involved in the DNA damage response.
- Range of techniques established to investigate if H3S28 is phosphorylated in *S. cerevisiae*.
2.3. Introduction

In the cell, chromatin is the physiological template for all DNA transactions including transcription, replication, and DNA repair. The fundamental subunit of chromatin is the nucleosome consisting of 147bp of DNA wrapped around a histone octamer comprised of two molecules each of histone H2A, H2B, H3, and H4. In response to DNA damage, detection and repair of lesions must occur in this chromatin environment. DNA is tightly packaged into chromatin which alters accessibility of the DNA to proteins which are involved in DNA transactions. Therefore specialised mechanisms have evolved to deal with the chromatin-packaged state of DNA. These mechanisms include covalent histone modifications, ATP dependent chromatin remodelling and histone variant incorporation (Berger, 2002; Costelloe et al., 2006; Lydall and Whitehall, 2005; Saha et al., 2006).

Histones are subjected to post translational modifications such as acetylation, phosphorylation, methylation, ubiquitylation, sumoylation and ADP ribosylation (Kouzarides, 2007). In recent years, histone covalent modifications involved in the DDR have been identified (Figure 2.1). Many roles have been elucidated for histone marks in the DDR. One commonly studied mark of the DDR is the phosphorylation of serine 139 on the C terminal tail of histone H2AX, also known as γ-H2A in S. cerevisiae (Downs et al., 2000). γ-H2A(X) promotes efficient double-strand break (DSB) repair through the recruitment of cohesin, histone modifiers and chromatin remodelling complexes, in addition this mark also promotes the accumulation and retention of checkpoint and repair proteins at the sites of DNA damage (Huertas and Jackson, 2009). S. cerevisiae cells devoid of this mark are sensitive to DSB-inducing agents and exhibit impaired DSB repair (Downs et al., 2000; Redon et al., 2003; Toh et al., 2006).

Another histone modification identified to play a role in the DDR is H3K56 acetylation (H3K56ac). h3K56R cells are highly sensitive to DNA damaging agents and H3K56ac has been shown to be required for an effective DDR response (Chen et al., 2008; Hyland et al., 2005; Li et al., 2008; Masumoto et al., 2005; Recht et al., 2006; Wurtele et al., 2012).
The phosphorylation of histones, and in particular the phosphorylation of histone H3 has been an area of interest in post translational modifications. In higher cells several sites on histone H3 have been shown to be phosphorylated (Cerutti and Casas-Mollano, 2009). Two of these sites include H3 serines 10 and 28 which are both phosphorylated in higher cells in vivo by the Aurora B kinase and the mitogen- and stress- activated kinase 1/2 (MSK1/2) (Dyson et al., 2005a; Goto et al., 2002; Hsu et al., 2000; Soloaga et al., 2003). The yeast Ipl1 homologue Aurora B can phosphorylate both H3S10 and H3S28 in vitro (Sugiyama et al., 2002). Although evidence exists in higher eukaryotes for H3S28 phosphorylation (H3S28ph) (Dyson et al., 2005; Goto et al., 2002; Hsu et al., 2000; Soloaga et al., 2003) no evidence exists in lower eukaryotes such as in the model system S. cerevisiae. In contrast to this, Ipl1 has been shown to phosphorylate H3S10 (H3S10ph) in budding yeast in vivo (Hsu et al., 2000). Additionally in vitro H3S10 and H3S28 have also been shown to interact with the 14-3-3 protein, with H3S28 having a stronger binding affinity than H3S10 for this protein (Winter et al., 2008). In vivo H3S10ph is believed to mediate the recruitment of 14-3-3 to chromatin during transcription activation (Karam et al., 2010; Macdonald et al., 2005).

H3S10 and H3S28 have been shown to be phosphorylated in higher cells in a cell-cycle dependent manner (Hsu et al., 2000). Despite both marks being
phosphorylated by the Aurora B kinase in mitosis, differences in the phosphorylation of these residues have been reported in higher cells. In mammalian cells H3S28ph occurs during early mitosis and coincides with mitotic chromosome condensation (Goto et al., 1999). The phosphorylation begins in prophase but starts to decrease at the metaphase/anaphase transition disappearing in late anaphase. In contrast, H3S10 persists until late anaphase (Goto et al., 1999). The levels of H3S10ph and H3S28ph are not only regulated by Aurora B in mitosis but also by phosphoprotein phosphatase 1 (PP1) (Goto et al., 2002; Hsu et al., 2000; Murnion et al., 2001). Due to the actions of PP1, the level of H3S28ph is undetectable prior to entry to mitosis (Goto et al., 2002). Okadaic acid is a potent inhibitor of PP1, PP2, and PP5 (Cohen-Fix and Koshland, 1997) and interestingly Goto et al. found that, unlike H3S10ph, H3S28ph could not be seen in the absence of okadaic acid. Therefore, H3S28ph appears to be much more sensitive to phosphatases than H3S10ph (Goto et al., 1999) since even in the presence of okadaic acid, dephosphorylation of H3S28ph occurred.

Biological functions of these phosphorylation marks have yet to be elucidated fully but they appear to be related in part to transcriptional activities (Banerjee and Chakravarti, 2011; Cerutti and Casas-Mollano, 2009). Phosphorylation of H3S10 has also been shown to be required for proper chromosome condensation in Tetrahymena (Wei et al., 1999). However, the requirement for both H3S10ph and H3S28ph in chromosome condensation in yeast and higher cells remains disputed. Hendzel and Wei both reported that H3S10ph and H3S28ph are required for proper chromosome condensation in higher cells (Hendzel et al., 1997; Wei et al., 1999). However, mutations of one or both these residues does not have an effect on chromosome condensation in S. cerevisiae (Hsu et al., 2000).

Histone H3 variants exist in higher cells. H3 in mammalian cells has three canonical histones, H3.1, H3.2 and H3.3. H3.3 is the only variant expressed outside of S phase and incorporated into chromatin in a replication-independent pathway (Ahmad and Henikoff, 2002). Drosophila H3.3 is relatively enriched for modifications associated with transcriptional activity (McKittrick et al.,
Budding yeast has only one canonical H3 histone, equivalent to H3.3. In mouse fibroblasts, all canonical and the H3.3 variant are phosphorylated at H3S10 and H3S28 (following stimulation of the Ras-MAPK pathway by 12-0-tetradecanoylphorbal 13-acetate (TPA)). Although the temporal induction pattern of H3S28ph and H3S10ph was similar, H3S28 phosphorylation occurred independently of H3S10 phosphorylation (Dunn and Davie, 2005). Although H3S28 and H3S10 phosphorylation is induced by the same stimuli and these marks disappeared within the same time frame, one phosphorylation event is not dependent upon the other (Dunn and Davie, 2005).

H3S10ph and H3S28ph are often studied together as both marks are preceded by an ARK sequence (discussed in chapter 1) and their phosphorylation is induced by the same stimuli. Although these two marks are similar in many ways, evidence shows that these two phosphorylation events are biologically different. Both phosphorylation events occur in a rapid and transient manner which take place at distinct chromatin regions (Dunn and Davie, 2005). Dunn et al reports that in response to TPA stimulated phosphorylation all three H3 canonical histones are phosphorylated at H3S10 and H3S28. They also report that H3S10ph and H3S28ph in response to TPA stimulation do not occur in the same chromatin regions (Dunn and Davie, 2005). In chicken erythrocytes, H3S28ph is associated with destabilized nucleosomes while phosphorylation at H3S10 is associated with more stable nucleosomes. It was also found that H3S28ph but not H3S10ph qualified as an active mark and that H3.3 is the principal H3 variant histone phosphorylated at H3S28, whereas H3S10 was phosphorylated to the same extent in all three H3 variants (Sun et al., 2007). This data provides evidence that although these two marks are both present in mitosis and quite similar in surrounding sequence, they play different roles in the cell.

H3S10 and H3S28 phosphorylation are both used as cell cycle markers in mitosis in higher cells. Neither of these marks have been implicated to have a role in the DDR and reports suggest that both H3S10ph and H3S28ph is reduced following DNA damage (Monaco et al., 2005; Ozawa, 2008; Tang et al., 2008). H3S10 is phosphorylated in S. cerevisiae cells but H3S28 phosphorylation in S. cerevisiae has yet to be identified. Histone H3 is highly conserved between yeast and
humans so we predict H3S28 is phosphorylated in yeast. Ipl1 phosphorylates H3S10 in *S. cerevisiae* (Sugiyama et al., 2002). Msk1, which phosphorylates H3S28 in higher cells (Soloaga et al., 2003) is poorly conserved, although Sch9 is believed to be its yeast orthologue. If H3S28 is phosphorylated in yeast the kinases responsible could be Ipl1 or Sch9, although we cannot rule out that other kinases may be involved. H3S28 phosphorylation status in *S. cerevisiae* remains to be determined. We determined whether H3S28 phosphorylation exists in *S. cerevisiae* or if this mark is specific to higher eukaryotes.

2.4. Results

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

A histone mutant screen was performed in the lab to identify novel histone marks which play a role in the DDR (Figure 2.2A). Two histone mutants generated showed sensitivity to CPT (Figure 2.2B). Mutation of serine 28 of histone H3 to an alanine (*h3S28A*) is highly sensitive to CPT and a triple mutant generated, *h3K56R,S57A,T58A*, showed sensitivity to CPT. H3K56 is acetylated in yeast cells (Masumoto et al., 2005) and H3K56ac is known to play a role in the DNA damage response (Chen et al., 2008; Li et al., 2008; Maas et al., 2006; Masumoto et al., 2005; Recht et al., 2006; Wurtele et al., 2012). Comparisons between the triple *h3K56R,S57A,T58A* mutant and the single *h3K56R* mutant revealed no difference in sensitivity to DNA damaging agents indicating that the observed sensitivity was likely due to the mutation of the K56 residue (data not shown). *h3S28A* is sensitive to CPT, but not to acute doses of ionising radiation (IR) or ultra violet radiation (UV), similar to *h3K56R,S57A,T58A*. To further investigate if *h3S28A* is specifically involved in resistance to CPT, we performed drop tests to other DNA damaging agents. *h3S28A* is also highly sensitive to cisplatin, bleocin, zeocin and MMS all damaging agents which cause damage in S phase of the cell cycle (Figure 2.2C).
Figure 2.2: Targeted mutagenesis of modifiable but previously uncharacterised residues of H3 and H4 and their preliminary characterisation. (A) Sequence of Histone H3 and H4 showing all modifiable residues in the tails of these histones. Open boxes highlight histone residues characterised in the DDR at the time of this study. Colored boxes indicate modifiable residues not characterised in the DDR at the time of this study. Serines and Threonines were mutated to alanine, lysines were mutated to arginine. (B) and (C) Preliminary analysis of the histone mutant collection generated by drop test. \textit{h3S28A} and \textit{h3K56R,S57A,T58A} mutants displayed significant sensitivity to CPT-induced damage. \textit{h3S28A} is highly sensitive to CPT, Cisplatin, Bleocin and Zeocin.
2.4.2. H3S28ph yeast specific antibody and commercial H3S28ph human antibodies cannot detect H3S28 phosphorylation in *S. cerevisiae*.

H3S28 has been shown to be phosphorylated in higher eukaryotes but not in lower eukaryotes (Dyson et al., 2005a; Goto et al., 2002; Hsu et al., 2000; Soloaga et al., 2003). We have seen that mutating H3S28 to an alanine had severe effects on the cell's ability to deal with DNA damage (Figure 2.2B and C). Therefore we hypothesised that these phenotypes may be due to lack of phosphorylation in the h3S28A strain. No evidence exists for phosphorylation of H3S28 in *S. cerevisiae* but due to the high conservation of the H3 sequence between yeast and higher eukaryotes and the fact that H3S28 is phosphorylated in higher eukaryotes, we hypothesised that H3S28 in yeast is phosphorylated.

To investigate if this residue is phosphorylated in *S. cerevisiae*, we decided to take a range of approaches. In our first approach to investigate the existence of this phosphorylation mark in *S. cerevisiae*, we raised a yeast specific H3S28ph polyclonal rabbit antibody (NL-H3S28ph) (Figure 2.3A). Since no commercial yeast H3S28ph antibodies are available, we obtained three commercial H3S28ph antibodies raised to human H3 sequences (see supplementary table S2.1 for list of commercial antibodies). All commercial antibodies obtained (Abcam-H3S28ph, Sigma-H3S28ph and SantaC-H3S28ph) have been raised to peptides within the region of residue 23 to 37 on histone H3. Only residue 31 in this region differs between *Homo sapiens* and *S. cerevisiae*: H3 residue 31 being alanine in human and serine in yeast (Figure 2.3B). This sequence difference is close to our residue of interest, H3S28. Although this may be a problem when using these commercial antibodies we believed the sequence similarity may be strong enough for successfully using these antibodies on yeast extracts.
To determine the specificity of the H3S28 phosphorylated antibodies we performed a peptide competition assay (Figure 2.4A). Serial dilutions of unphosphorylated (unmodified) and phosphorylated H3S28 peptide from 10 µg to 1 ng were dotted onto nitrocellulose membrane. Antibodies were pre-incubated with unphosphorylated and phosphorylated forms of the peptide immunogen to determine the specificity of each antibody. We tested if the antibodies could recognise unphosphorylated peptide (Figure 2.4B; First lane on all membranes). Under all conditions tested (explained below) no H3S28ph...
antibody recognised unphosphorylated H3S28 peptide. We also tested the antibodies ability to recognise H3S28 phosphorylated peptide (Figure 2.4B; Second lane on very membrane). All antibodies tested recognised H3S28 phosphorylated peptide to varying degrees (Figure 2.4B; First membrane in every panel marked – Competition). We next tested if the H3S28ph antibodies were recognising just H3S28ph or if they also detected any unspecific or unphosphorylated sequence in the region if H3S28. To do this we pre-incubated the membranes with H3S28ph antibody and unphosphorylated H3S28 peptide (Figure 2.4B; Second membrane in every panel marked + Unphospho peptide). The antibody will bind to the unphosphorylated peptide if it recognises an additional sequence to H3S28 phosphorylation. This will leave specific H3S28ph molecules of the antibody to bind to the phosphorylated peptide on the membrane. All antibodies recognise H3S28 phosphorylation alone as incubation with unphosphorylated peptide was comparable to incubation of the membranes with H3S28 antibody alone (compare first and second membrane of every panel). We also incubated the membranes and H3S28ph antibodies with phosphorylated H3S28 peptide (Figure 2.4B; Third membrane in every panel). As expected, an immunizing peptide blocking with phosphorylated H3S28 peptide (+ Phospho peptide) resulted in the antibody being neutralised with an excess of peptide resulting in no signal on the nitrocellulose membrane.

Each antibody did recognise the H3S28 phosphorylated peptide but to varying degrees. Abcam-H3S28 appeared the most sensitive antibody detecting phosphorylated H3S28 peptide down to 5 ng concentration (Figure 2.4B). Nl-H3S28p, Sigma-H3S28p and SantaC-H3S28p all recognised up to 100 ng of phosphorylated H3S28 peptide. Peptide dot blotting indicated that our Nl-H3S28ph antibody along with commercial antibodies were recognising phosphorylated H3S28 peptide but none of the antibodies were highly sensitive in detecting this mark. The antibodies tested recognise phosphorylated H3S28, but they may not be highly sensitive for the detection of this mark in vivo.

Knowing the commercial antibodies and Nl-H3S28ph antibody detect phosphorylated H3S28 peptide we proceeded to investigate if we could detect this mark in vivo. We optimised western blotting conditions to attempt to detect
H3S28 phosphorylation in yeast protein extracts and histone preparations. Due to the low specificity of the H3S28ph antibodies, antibody testing was performed using histone preparations in western blotting to increase the amount of histone H3 present, therefore increasing the chances of detecting H3S28 phosphorylation in *S. cerevisiae* cells. A range of blocking and probing conditions were tested with H3S28ph antibodies to determine the best conditions to detect this mark in our extracts. Various concentrations of phosphate buffered saline (PBS) and Tris-buffered saline (TBS) along with condensed milk and bovine serum albumin (BSA) were tested with each antibody. Finally the conditions for western blotting were set up as follows; 5% Block BSA in TBS-Tween for 1 hour at room temperature followed by primary H3S28ph antibody in 5% BSA in TBS-Tween overnight at 4°C.

*h3S28A* and *h3S10A* histone preparations were run on SDS page gels and probed with Ni-H3S28 and total H3 antibody (Figure 2.4C). No band was detected in wild type (WT) recombinant protein indicating that Ni-H3S28 antibody does not detect any unphosphorylated residues in H3. A band was detected at 16kDa using Ni-H3S28ph antibody. This band is present in both *h3S28A* and *h3S10A* cells (Figure 2.4C) suggesting the antibody can recognise phosphorylation events other than the phosphorylation of H3S28. Commercially available H3S28ph antibodies were also tested on yeast histone preparations to investigate the presence of this mark. (Supplementary table 1). Similarly to our Ni-H3S28ph antibody, we could not detect a band that was specific to H3S28 phosphorylation in yeast extracts using commercial H3S28ph antibodies (Data not shown).
Figure 2.4: Analysis of sensitivity and specificity of Nl-H3S28ph and commercially available antibodies raised to H3S28 phosphorylation. (A) Peptide sequences of histone H3, which were used in the peptide dot blotting and also in peptide competition assays. (B) Peptide dot blots which range from 1000 ng to 1 ng of H3S28 unphosphorylated and H3S28 phosphorylated antibody. Four antibodies, Nl-H3S28ph, Abcam-H3S28ph, Sigma-H3S28ph and Santa Cruz-H3S28ph were tested for their sensitivity and specificity to phosphorylated serine 28 peptide. Unphosphorylated peptide was used as a control. Antibodies tested in this assay showed them to be specific to H3S28 phosphorylation but not very sensitive detecting no less than 10 ng of H3S28 phosphorylated peptide. (C) Nl-H3S28ph antibody was tested on yeast histone preparations to investigate if H3S28 phosphorylation alone could be detected. No band corresponding to H3S28 phosphorylation could be detected.
2.4.3. Ni-H3S28ph recognises both H3S28 and H3S10 phosphorylation

Western blotting experiments showed that Ni-H3S28ph antibody recognises a band in both \textit{h3S10A} and \textit{h3S28A} mutant cells, suggesting that this antibody could detect phosphorylation events other than H3S28ph. Aurora B phosphorylates H3S10 and H3S28 \textit{in vitro} and \textit{in vivo} (Goto et al., 1999; Hsu et al., 2000; Sugiyama et al., 2002). To establish if the H3S28ph antibodies can really detect H3S28 phosphorylation, a kinase assay using commercial Aurora B was performed \textit{in vitro} on 100 ng of recombinant yeast and human H3 (generated by Martin Browne in Andrew Flaus Lab) in the presence or absence of 1 mM ATP (+/-) (Figure 2.5). Two H3S28ph antibodies, Ni-H3S28ph and Ab-H3S28ph (Supplementary table 1), were used to detect phosphorylated H3. Phosphorylated H3 in this section refers to phosphorylated H3 by Aurora B kinase. Ni-H3S28ph recognised phosphorylated yeast and human H3, although this antibody looks to be slightly more specific to phosphorylated yeast H3. Abcam H3S28ph antibody recognises phosphorylated human H3 and phosphorylated yeast H3 to a lesser extent. H3S10ph antibody recognised both phosphorylated yeast and human H3. This assay demonstrates that recombinant yeast and human H3 is phosphorylated by Aurora B kinase \textit{in vitro}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.5.png}
\caption{Histone H3 is phosphorylated by Aurora B kinase. (A) A kinase using commercial Aurora B was performed \textit{in vitro} on 100ng of recombinant yeast and human histone H3 in the presence or absence of ATP (+/-). Antibodies directed against histone H3 serine 28 phosphorylation and histone H3S10 phosphorylation were used to detect the presence of phosphorylated histone H3. * Note both no treatment (-) and -ATP were loaded on this lane.}
\end{figure}
To investigate whether our raised Nl-H3S28ph and commercial antibodies are specific to H3S10ph or H3S28ph we performed another in vitro kinase assay using commercial Aurora B kinase with recombinant yeast histone H3 mutants. Recombinant single mutants h3S10A and h3S28A, along with a double h3S10A,S28A mutant (generated by PhD student Martin Browne in the Flaus laboratory). An in vitro kinase assay using commercial Aurora B was performed with the H3 recombinant mutants. Nl-H3S28ph detected phosphorylated H3S28 in all recombinant proteins except the double mutant h3S10A,S28A. This indicates that this antibody detects phosphorylated H3S10 and phosphorylated H3S28 but detects neither mark alone, suggesting this antibody is not specific to H3S28 phosphorylation alone. The H3S10 antibody detects phosphorylation in the h3S28A single mutant but not in the single h3S10A mutant or double h3S10A,S28A mutant, suggesting this antibody is specific to phosphorylated H3S10 alone (Figure 2.6A).

Along with Nl-H3S28 antibody we tested two commercially available antibodies, Abcam-H3S28ph and Sigma-H3S28ph antibodies, on our recombinant histone mutants (Figure 2.6B). Similar to Nl-H3S28ph, Abcam-H3S28ph detects phosphorylated H3 on all mutants except the double h3S10A,S28A mutant showing this antibody is also not specific to H3S28 phosphorylation alone. Interestingly, Sigma-H3S28ph antibody detects phosphorylation in the h3S10A mutant but not in the h3S28A mutant or double h3S10A,S28A mutant. This suggests that this antibody is the only antibody specific to yeast H3S28 phosphorylation out of the 3 antibodies tested here.
Figure 2.6: NI-H3S28ph antibody recognises both H3S10 and H3S28 phosphorylation. A kinase assay using recombinant yeast histone H3 mutants in the presence or absence of ATP was performed. (A) NI-H3S28ph antibody recognised both phosphorylated H3S10 and H3S28 but neither alone. H3S10 was specific to H3S10 phosphorylation. (B) Abcam-H3S28ph, similar to NI-H3S28ph antibody recognised both H3S10 and H3S28 phosphorylation but neither alone. Sigma-H3S28ph antibody recognised phosphorylation in the h3S10A mutant but not in the h3S28A mutant or double h3S10A,S28A mutant suggesting this antibody is specific to H3S28 phosphorylation alone.
2.4.4. Mass spectrometry analysis cannot detect H3S28 phosphorylation

We can detect H3S28 phosphorylation \textit{in vitro} in yeast recombinant proteins but we failed to detect this mark \textit{in vivo}. Therefore we tried a more sensitive mass spectrometry approach. Histone preparations were prepared and sent to our collaborator Alain Verreault Universite de Montreal, for analysis. Histone preparations were performed under various conditions to try and maximise conditions for detecting H3S28 phosphorylation.

\textit{h3S28A} is highly sensitive to CPT. So we hypothesised that CPT would mount a DNA damage response which involves the phosphorylation of H3S28. We therefore prepared histones in the presence of CPT with a view of increasing the levels of H3S28 phosphorylation. We also prepared histones in the presence of nocodazole (Figure 2.7A). Since in in higher eukaryotes H3S28 has been shown to be phosphorylated in mitosis (Goto et al., 1999), so a M phase arrest could increase our chances of detecting H3S28 phosphorylation by arresting cells in a phase of the cell cycle that this mark is associated with. Unfortunately, in these conditions no phosphorylation at H3 serine 28 could be detected. On a second attempt we prepared acid extracted histones from WT and \textit{pdr5\textDelta} mutant cells in the presence of CPT (Figure 2.7B). Pdr5 is a plasma membrane ATP-binding cassette (ABC) transporter which is involved in multiple drug resistance (Leppert et al., 1990). We reasoned that a \textit{pdr5\textDelta} mutant would allow excess amounts of CPT into the cell, in turn mounting a considerable response which would result in detectable amounts of H3S28 phosphorylation. Again, in these conditions no phosphorylation at H3 serine 28 could be detected. Although this approach was quantitative to detect known post translational modifications no H3S28 phosphorylation could be detected. This approach identified H2A and H2B phosphopeptides in the same samples that were analysed to detect H3 serine phosphorylation but H3S28 phosphorylation could not be detected in these samples. These results indicate that if H3S28 phosphorylation occurs in \textit{S. cerevisiae}, it occurs at very low levels which are beyond the detection limits used.
In a search for novel histone marks which play a role in the DDR (Figure 2.2A), we identified H3S28, as well as previously defined H3K56 (Hyland et al., 2005; Masumoto et al., 2005) as being required for resistance to DNA damaging agents. Similar to h3K56R, h3S28A is sensitive to CPT, bleocin and zeocin but not to acute doses of UV or IR (Figure 2.2B and C). H3K56ac has been reported to be required for an efficient DDR (Chen et al., 2008; Hyland et al., 2005; Li et al., 2008; Masumoto et al., 2005; Recht et al., 2006; Wurtele et al., 2012). Due to the important roles for histone modifications in the cell cycle and in the DDR, we believed the phenotypes seen in the h3S28A mutant were due to a lack of phosphorylation at this residue. H3S28, along with H3S10 has been shown to be phosphorylated in higher cells (Dyson et al., 2005a; Goto et al., 2002; Hsu et al., 2000; Soloaga et al., 2003) but H3S28 has never been shown to be phosphorylated in lower eukaryotes in vivo. It has been reported that yeast H3S28 can be phosphorylated by Aurora B kinases in vitro (Figure 2.5 and Sugiyama et al., 2002). H3S28 and H3S10 phosphorylation occurs in a cell cycle dependent manner, occurring in late G2/early M and these two phosphorylation events are used as markers of mitosis. Both phosphorylation events have been

**Figure 2.7: Histone preparations sent for mass spectrometry analysis.** (A and B) Histone preparations performed in various conditions to increase detection of H3S28 phosphorylation. Under these conditions H3S28 phosphorylation could not be detected by mass spectrometry analysis.
implicated in transcription and condensation but their biological roles have yet to be fully elucidated (Banerjee and Chakravarti, 2011).

Therefore we hypothesised that H3S28 is phosphorylated in S. cerevisiae and this phosphorylation event is important for an efficient DDR after DNA damage. We set out to elucidate the phosphorylation status of this residue in S. cerevisiae. Firstly we raised a yeast specific polyclonal H3S28ph antibody (Figure 2.3A). We tested this antibody in peptide dot blotting analysis along with commercially available H3S28 antibodies (Figure 2.4A and B). Peptide dot blots indicated that our Ni-H3S28ph antibody along with commercial antibodies recognised phosphorylated H3S28 peptide but all antibodies tested were not highly sensitive in detecting phosphorylated H3S28.

Antibodies were then tested on histone preparations to try and identify H3S28 phosphorylation in vivo. We are aware that H3S28 and H3S10 are similar in sequence content (both are preceded by an ARKS sequence, Figure 2.3B). Therefore these antibodies may recognise H3S10ph alone or in addition to H3S28ph in yeast histone preparations. We tested the antibodies in a h3S10A strain along with a h3S28A strain to eliminate this problem (Figure 2.4C). No band which is specific to H3S28 phosphorylation was detected indicating that if H3S28 phosphorylation is present in S. cerevisiae this modification occurs at low stoichiometry levels.

In vitro phosphorylation of recombinant histone H3 by Aurora B demonstrated that only one antibody can unambiguously distinguish between H3S28ph and H3S10ph. Sigma-H3S28ph antibody detects phosphorylation in the h3S10A but not in the h3S28A single or h3S10A,S28A double mutant indicating this antibody is specific to H3S28ph alone, whereas the Ni-H3S28ph antibody or Abcam-H3S28ph antibody are not specific to H3S28ph alone. Sigma-H3S28ph antibody was also tested on histone preparations but again similar to Ni-H3S28 antibody it could not detect a specific band corresponding to H3S28 phosphorylation.

As a last attempt to identify H3S28ph in vivo, we used mass spectrometry analysis as a sensitive method of detecting histone modifications from purified
histones in vivo. Unfortunately, we failed to detect H3S28 phosphorylation from yeast histone preparations by mass spectrometry analysis despite using cell growth conditions which might increase the probability of H3S28 phosphorylation to occur.

Independent studies have reported conflicting evidence on the role of H3S28 and its response to DNA damage (Dai et al., 2008; Matsubara et al., 2007; Nakanishi et al., 2008; Seol et al., 2008). In studies from the Boeke and Shilatifard laboratories using a systematic mutation of all of the residues of the four core histones in S. cerevisiae, no phenotype for h3S28A was reported. Another study which generated core histone point mutants and tested viability in a range of DNA damage assays did not report any phenotype for a h3S28A mutant either (Matsubara et al., 2007). In contrast to this, one study reported a double h3S28,30A mutant being sensitive to hydroxyurea (HU) and methylmethane sulphonate (MMS) (Seol et al., 2008). Interestingly, Dai et al., did report differences in phenotypes due to mutants being generated in different strain backgrounds (Dai et al., 2008). Therefore the phenotypes we see in our h3S28A strain may be due to strain background differences with strains generated in Boeke or Shilatifard laboratories. This does not explain why we cannot detect H3S28 phosphorylation when the absence of this presumptive mark is causing cells to be sensitive to DNA damaging agents. We decided to regenerate the h3S28A strain and test independent colonies for sensitivity to DNA damaging agents. To our surprise a newly generated h3S28A is not sensitive to DNA damaging agents (Supplementary figure 1). The h3S28A strain which is highly sensitive to DNA damaging agents (Figure 2.2C) has two additional mutations present, glycine at position 33 mutated to a serine and glutamate at position 50 is mutated to a glycine (h3S28,G33S,E50G). It has been reported that H3E50 when mutated to alanine is sensitive to DNA damaging agents (Seol et al., 2008) so we anticipated that the phenotypes we saw in our h3S28A strain (h3S28A,G33S,E50G) were due to H3E50 being mutated to a glycine. We generated a de novo h3E50G strain and saw it had all the phenotypes of the triple h3S28A,G33S,E50G strain (Supplementary figure 1) (Discussed in chapter 3). This mutant was further studied and this analysis is described in the following chapter.
2.6. Conclusion

In this chapter we aimed to determine the phosphorylation status of H3S28 in budding yeast. *In vitro* assays determined that yeast recombinant protein can be phosphorylated by aurora B kinase and that this kinase phosphorylates both H3S10 and H3S28. However we failed to find evidence for H3S28 phosphorylation *in vivo*. Our results suggest that H3S28 is not phosphorylated in *S. cerevisiae*. We cannot rule out the possibility that this mark does exist but is beyond the limits of the detection methods we used.
2.7. Materials and Methods

2.7.1. Media and growth conditions
Yeast cells were grown in YPD liquid (1% (w/v) yeast extract (Difco), 2% (w/v) bactopeptone (Difco), 2% (w/v) glucose (Difco)) or YPD solid media containing 2% (w/v) agar (Difco). Cells were grown in conical flasks with liquid media at 30°C in a shaker incubator at 170 r.p.m. The volume of culture did not exceed one third of the nominal flask volume to ensure adequate aeration. Cells that were grown in plates of YPD solid media were incubated at 30°C and depending on strain growth were left grow for 2-3 days.

2.7.2. Selective media and genotoxic treatments
The genotoxic treatment for sensitivity analysis was performed by the drop test methodology using media with different genotoxic agents 1) MMS (Sigma-Aldrich) was added to YPD media to a final concentration of 0.03%. 2) CPT (Sigma) was added to YPD final concentration of 10µM. 3) HU (Sigma) was used at final concentrations of 5mM and 100mM. 4) Cisplatin (Sigma) was used at a final concentration of 500µg/ml. 5) Bleocin (Calbiochem) was used at a final concentration of 0.25 µg/ml. 6) Zeocin (Invitrogen) was used at a final concentration of 5 µg/ml. 7) γ-irradiation (400 Gy) was carried out using a 137 Cs at a dose-rate of 12.10 Gy/min (Mainance Engineering, UK). 8) The UV irradiation rate (J/m²/s) from the lamp was measured before each irradiation, using a UV dosimeter, and the time of exposure was calculated (time = desired dose (80J) / UV intensity (J/m²/s)). DNA damage sensitivity analysis was performed by spotting five-fold serial dilutions of exponentially growing cultures (5x10⁶) of the indicated strains on plates containing the indicated genotoxic agents. Plates were incubated in the 30°C incubator unless otherwise specified for two days before scanning.

2.7.3. Yeast strains and Plasmids
All yeast strains used in this study are made in W303-1a background unless otherwise stated and are listed in table S2.2. Yeast strain and plasmid constructions are described below. Plasmids and oligonucleotides used in this study are in Tables S2.3 and S2.4 respectively.
2.7.3.1. Plasmid shuffle system

A one step PCR mediated approach (Longtine et al., 1998) was used to generate the parental plasmid shuffle system strain. Firstly, genomic HHT2-HHT4 was replaced with HIS5 from pML3 (pFA6a-His3MX6). These cells were then transformed with Ycp50-copyII (HHT2-HHF2) (rescued from yeast strain DY5377, kindly provided by Alain Verreault), a plasmid bearing wild-type H3-H4-copyII alleles with their natural promoter and aURA3 selectable marker. Then, genomic HHT1-HHF1 was replaced with KANMX from pML1 (pFA6a-KANMX6) (Thomas Costelloe Thesis 2009). For the plasmid shuffle of histone alleles, wild-type or mutated histone alleles on TRP1-marked plasmids were introduced into the parental plasmid shuffle strain carrying wild-type histones on the URA3-marked Ycp50-copyII plasmid. Cells containing both the wild-type (Ura+ and the mutant (Trp+ plasmids were selected by growth on selective media. These cells were then plated onto minimal media containing 5-FOA (1mg/ml) to select for cells that had lost the wild-type (URA3) plasmid. At each step of this strain construction, diagnostic PCR on selective media was carried out to confirm selection of the correct cells (Thomas Costelloe Thesis 2009). All yeast strains in this study are listed in table S2.1.

2.7.3.2. DNA mutagenesis

Point mutations in histone H3 and H4 were generated using the Stratagene Quickchange site-directed mutagenesis kit, and performed as per manufacturers guidelines. Primers were designed approximately 30bp in length, containing the mutation of interest in the middle of the sequence (Thomas Costelloe Thesis 2009). The reverse primer was the complementary sequence of the forward sequence. Mutagenesis was carried out directly on the yeast centromeric plasmid Ycp[HHT2-HHF2(TRP1)] and following confirmation by DNA sequencing, a BseR1-BamH1 fragment containing the mutation of interest was ligated back into the parental plasmid. After sequencing these plasmids were transformed into yeast using the standard Lithium Acetate method.

2.7.3.3. Recombinant histone H3 mutants

Site directed mutagenesis was carried out on a pET3a vector containing S. cerevisiae histone H3 (Martin Browne, Dr. Andrew Flaus Laboratory). Histones
were expressed in inclusion bodies from (*E. coli*) Rosetta 2 (DEB)plys cells upon induction with 0.5 mM IPTG (Isopropyl-β-D-Thiogalactopyranoside). Inclusion bodies were purified by several washes and centrifugation steps before being further purified in denaturing conditions by Ion exchange chromatography (SP Sepharose) (Martin Browne, Dr. Andrew Flaus Laboratory).

### 2.7.4. Acid extracted histones

1.5 litres of cells were grown to a concentration of 1-2x10⁷ cell/ml. Cells were treated with CPT, nocodazole or untreated. Wild-type cells were treated for 90 minutes while *h3S28A* mutant cells were treated for 120 minutes. Acid extracted histone preparations were prepared from a protocol by the Dent laboratory, MD Anderson Cancer centre ([http://www.mdanderson.org/education-and-research/departments-programs-and-labs/labs/dent-laboratory/protocols/yeast-histone-prep-1.html](http://www.mdanderson.org/education-and-research/departments-programs-and-labs/labs/dent-laboratory/protocols/yeast-histone-prep-1.html)). 20 mg Zymolyase (Seikagaku biobusiness corperation) was used to digest the cell wall. Okadaic acid (Ascent Scientific) was an additional phosphatase inhibitor used at 100 nM throughout the protocol. Protease inhibitor cocktail (2.8 µM leupeptin, 8 µM pepstatin A, 4 µM PMSF, 8 µM benzamidine, 8 µM antiapin, 4 µM chymostatin in ethanol) and phosphatase inhibitor cocktail (2 mM sodium fluoride, 1.2 mM β-glycerophosphpate, 0.04 µM sodium vanadate, 2 mM EGTA, 10 mM sodium pyrophosphate), were also used throughout the protocol.

### 2.7.5. Western blotting and antibodies

Western blotting was performed using standard molecular biology techniques. Histones were prepared by histone preparation protocol and histones were resolved in a 15% (36:1) acrylamide/bis-acrylamide (Sigma), SDS-PAGE gel and either probed with Comassie Brilliant Blue R-250 (Sigma) after gel running or transferred to nitrocellulose membrane and probed with various H3S28ph antibodies at 1:500 dilution in 5% BSA in TBS containing 0.01% Tween-20 overnight at 4°C. Membranes were blocked prior to incubation with antibody in 5% BSA for 1 hour at room temperature. Total H3 (Abcam) antibody was used at 1:10,000 dilution in 4% milk in 0.01% PBS-Tween-20 overnight at 4°C. The following day, secondary anti-goat was used at 1:10,000 dilution in 0.01% TBS-
Tween for the membrane which had been probed with Sigma-H3S28ph antibody. Secondary anti-rabbit or antigoat was used at 1:10,000 dilution. Super signal WestPico chemiluminescent substrate from Thermo Scientific (Product no. 34080) was used to detect the proteins.

2.7.5.1. Generation of antibodies
Peptide design and production of a phospho specific antibody was carried out by an antibody service (Eurogentec, Brussels). A polyclonal H3S28ph antibody was produced in rabbits using a synthetic peptide with the following sequence of histone H3: KAARKS(PO$_3^2$-)APSTGGC-CONH$_2$. The peptide was coupled to Keyhole Limpet Hemocyanin (KLH). A total of four immunizations were carried out. Crude sera and affinity purified antibody (affinity purification carried out by Eurogenetec) was received. Affinity purified serum was used in all experiments.

2.7.5.2. Peptide design and dot blotting:
Unphosphorylated and phosphorylated peptides were synthesized by Pepeceuticals, UK. Unphosphorylated peptide; Biotin-SGS-LASKAARKSAPSTGGVK-COOH Phosphorylated peptide; Biotin-SGS-LASKAARK[pS]APSTGGVK-COOH Unphosphorylated and phosphorylated H3S28 peptide were diluted in ddH$_2$O to a concentration of 2 mg/ml. Serial dilutions were dotted across a nitrocellulose membrane. Membranes were let dry and then blocked in 5% TBS-Tween for 60 minutes at room temperature. Membranes were incubated with primary antibody over night in the cold room. A 1:500 dilution of the various H3S28 antibodies in 5% BSA in TBS-Tween were used in dot blotting experiments. Membranes were washed in TBS-Tween and incubated with the appropriate secondary antibody 1:10,000 dilution for 45 minutes at room temperature. Membranes were incubated with antibody plus or minus unphosphorylated or phosphorylated H3S28/H3S28ph peptide (50µg/ml peptide was used for peptide competition). ECL and autoradiography was then performed.

2.7.6. Recombinant histone mutants
A kinase assay using commercial Aurora B (Abcam ab51435) was performed in vitro on 100ng of recombinant yeast and human H3 (Flaus Laboratory) in the presence or absence of 1 mM ATP (+/-). The 30 minute reaction was stopped by
the addition of SDS buffer and samples were loaded on an SDS page gel. Nl-H3S28ph antibody, Abcam H3S28 antibody, Sigma-H3S28ph antibody, H3S10 antibody were used at 1:1000 dilution in 5% BSA in TBS containing 0.01% Tween-20 overnight at 4°C. Total H3 (Abcam) antibody was used at 1:10,000 dilution in 4% milk in 0.01% PBS-Tween-20 overnight at 4°C. The following day, secondary anti-goat or anti-rabbit was used at 1:10,000 dilution

2.7.7. Mass spectrometry

Histones preparations were digested with trypsin and phosphopeptides were enriched using a titanium dioxide column (Jensen and Larsen, 2007). Peptides were analysed by LC-MS/MS and parent ions were fragmented using either electron transfer dissociation (ETD) or collision-induced dissociation (CID) (Mikesh et al., 2006). This approach identified H2A and H2B phosphopeptides in the same samples that were analysed to detect H3 serine phosphorylation.
2.8. Supplementary Data

2.8.1. Supplementary Figures:

Figures S2.1:

Supplementary Figure S2.1: **h3S28A mutant contains two extra mutations.** The h3S28A mutant strain which we had been studying has two extra mutations present in H3. Glycine 33 is mutated to a serine and glutamate 50 is mutated to a glycine. We predict the CPT sensitivty of this strain is due to the H3E50G mutation.

2.8.2. Supplementary Tables:

Table S2.1: Antibodies used in this study:

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*Table S2.1: Antibodies used in this study:* All antibodies are Anti-phospho-Histone H3 (Ser28) antibodies
S2.2: Yeast strains used in this study

All strains used in this study are in the W303a background and are MATa, unless otherwise stated.

<table>
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<th>Strain name</th>
<th>Genotype</th>
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<td>From Dr. Corrado Santoncale</td>
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S2.3 Plasmids used in this study
(Note all plasmids used to generate H3 and H4 mutants are listed in Thomas Costelloe, PhD Thesis 2009)

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<td>pWZ414-F13</td>
<td>pRS414, digested with SmaI/AflIII, ligated with 1.8kb SpeI fragment containing HHT2-HHF2 from YCp50copyII, TRP1 selectable marker. Used for site directed mutagenesis</td>
<td>Zhang et al 1998 EMBO J, 17:11, 3155-3167</td>
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<td>F13h3S28</td>
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S2.4 Primers used in this study

All primers used to generate Histone Mutant collection are listed in Thomas Costelloe, PhD Thesis 2009

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2.9. References


CHAPTER 3

A role for H3E50 in the DNA Damage Response
3.1. Summary
DNA is replicated and passed on to daughter cells in an error-free fashion to maintain genomic integrity. If damage to a cell is detected the cell responds by activating several DNA repair mechanisms. The DNA damage response (DDR) is the name given to the cellular response to DNA damage, which is conserved from yeast to human. It allows the damaged DNA to be repaired efficiently. Failure to do so can lead to the development of cancer. Like other processes involving DNA such as transcription or replication, DNA repair occurs in a chromatin environment. Chromatin is the DNA-protein packaging of chromosomes in all eukaryote cells. The main components are the histone proteins which wrap DNA in structures called nucleosomes. Specific amino-acids on histones have been shown to be involved in specific DNA-dependent processes.

In a screen performed in our laboratory the single site mutant $h3E50G$ was highly sensitive to MMS and CPT. H3E50 is part of the H3 αN helix located at the DNA entry and exit site of the nucleosome. Mutations in the H3 αN helix are frequently lethal or highly sensitive to DNA damaging agents, highlighting the importance of this region in DNA damage responses. For this reason we investigated the role that H3E50 plays in the DDR.

3.2. Highlights
- H3E50 mutations to glutamate, glycine, aspartate, alanine, glutamine and lysine were generated. All mutants displayed doubling times which were 1x-2x times longer than the isogenic WT
- All mutants generated are highly sensitive to a range of DNA damaging agents which activate the intra S phase
- $h3E50G$ and $h3E50A$ cells do not recover efficiently from an intra S phase checkpoint response
- H3E50 is not required for 5′-3′ DNA resection
- $h3E50G$ and $h3E50A$ mutant cells form persistent Rad52 foci after exposure to genotoxic agents
3.3. Introduction

Like other processes involving DNA such as transcription or replication, occurs in a chromatin environment. The fundamental subunit of chromatin is the nucleosome, (Luger et al., 1997a) which consists of 147bp DNA wrapped around a histone octamer comprised of two molecules each of histone H2A, H2B, H3 and H4. Histones consist of similar secondary structures with each histone comprising of three α-helices and 2 loops (Luger et al., 1997a) and both H3 and H2B have an extra αN and αC helix respectively. Each histone has an N and C terminas tail and most tails lie outside the core globular domain. A remarkable feature of histones, and particularly of their tails, is the large number of post translational modifications targeted to them (Kouzarides, 2007).

Site directed mutagenesis has become a powerful tool for studying and characterising the roles that histone residues play in transcription and the DDR. Extensive histone mutant screens involving site directed mutagenesis on histone residues have been completed in S. cerevisiae (Dai et al., 2008; Hyland et al., 2005; Matsubara et al., 2007). These screens have generated much data on the effect of mutating tail and core histone residues. One common and surprising finding between the screens carried out was the low number of non viable mutants. One study generated a library of 320 individually mutated residues on histones H2A, H2B, H3 and H4 (Matsubara et al., 2007). Remarkably 312 mutants were viable with only 8 point mutations showing lethality. Another study generated a versatile library consisting of 486 systematic H3 and H4 substitution deletion mutants in S. cerevisiae (Dai et al., 2008). They reported at least one phenotype for 83% of H3 and 87.2% of H4 residues.

In these studies the library of individual mutants was screened against a range of agents which included sensitivity to DNA damaging agents such as CPT, MMS, HU and UV radiation. Many mutants tested were found to be sensitive to a range of DNA damaging agents. Overall these screens were comparable to each other and also data generated from other screens (Dai et al., 2008; Hyland et al., 2005; Matsubara et al., 2007; Seol et al., 2008). Dai et al reported that mutation of residues in the tails of histones had less growth defects, presumably due to the
fact they are outside the globular histone core domain. Conversely, mutants within the globular core domain were more likely to be lethal or display growth defects. This observation may be explained by mutations in the core being more likely to affect the overall stability of the nucleosome, whereas mutations in the histone tail would not affect the overall stability of the nucleosome.

One region on histone H3 which had a high incidence of non viable mutants was the H3 αN helix. This helix maps from residue proline 43 to residue glutamine 55 (Figure 1.5B). Of the 8 non viable histone point mutations identified, three are located in H3 and all of these are within the αN helix (Matsubara et al., 2007). These three residues are H3L48, H3I51 and H3Q55. H3R49 and H3E50 were viable when mutated to alanine but are sensitive to MMS and HU (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). One report reported a triple H3-REI49-51A mutant that displayed no sensitivity to DNA damaging agents, whereas a single h3E50A mutant did display sensitivity to MMS, CPT and HU, corresponding to earlier reports published (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). This suggests that H3E50 plays an important role in the DDR in response to MMS, CPT and HU treatment.

Many mutants generated in the αN helix are sensitive to a range of DNA damaging agents indicating that this region plays an important role in inducing distinct DNA damage responses. It is important to note that there are some discrepancies reported in the literature for non viable mutants corresponding to the H3 αN helix residues. For example some reports differ in non viable mutants at H3 αN helix (Dai et al., 2008; Hyland et al., 2005; Matsubara et al., 2007; Seol et al., 2008). Residues H3V46 and H3R52 when mutated individually an alanines was non viable in one study but viable in another (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). These inconsistencies may be due to strain variations. Two residues, H3T45 and H3K56, which are present on either side of the αN helix are reported to be involved in the DDR (Baker et al., 2010; Celic et al., 2006; Hyland et al., 2005; Maas et al., 2006; Masumoto et al., 2005; Millar and Russell, 1992; Recht et al., 2006; Wurtele et al., 2012). H3K56, when mutated to an arginine (h3K56R) is highly sensitive to DNA damaging agents
In *S. cerevisiae* H3K56 acetylation has been reported to be present in vast majority of new H3 molecules deposited behind replicating forks (Celic et al., 2006; Masumoto et al., 2005).

Systematic mutations of residues of the four core histones in *S. cerevisiae* have been performed in the Boeke, Horikoshi’s and Shilatifard laboratories (Dai et al., 2008; Hyland et al., 2005; Matsubara et al., 2007; Nakanishi et al., 2008). Many histone residues when mutated to alanines were sensitive to DNA damaging agents, although some discrepancies were reported between the studies. Dai et al., reported differences in phenotypes due to mutants being generated in different background strains. To further understand nucleosome structure and function, a histone mutant screen using site directed mutagenesis in a W303 *RAD5*+ strain was performed in the laboratory to identify novel histone marks which play a role in the DNA damage response (Costelloe, PhD Thesis 2009).

Two histone mutants generated showed sensitivity to CPT. H3 glutamate 50 when mutated to a glycine (*h3E50G*) and H3 lysine 56 when mutated to an argingine (*h3K56R*) are highly sensitive to CPT. *h3E50A* has been reported to be sensitive to CPT, MMS and HU (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008) but no known role has been attributed to this residue. Subsequent to this screen H3K56 has been well characterised. H3K56 is acetylated in yeast (Masumoto et al., 2005) and H3K56ac defective cells are sensitive to genotoxic agents that damage during replication (Chen et al., 2008; Driscoll et al., 2007; Han et al., 2007b; Hyland et al., 2005; Masumoto et al., 2005; Ozdemir et al., 2005; Recht et al., 2006; Wurtele et al., 2012). Additionally, in response to genotoxic damage, *h3K56R* cells formed persistent Rad52 foci following a transient exposure to MMS and CPT (Wurtele et al., 2012) which indicates H3K56ac is important for cells to complete the repair of DNA lesions which occur during replication (Wurtele et al., 2012). One similarity between H3E50 and H3K56 is that both residues are located in the H3 αN helix, a region of histone H3 which is important for cell viability and nucleosome stability (Dai et al., 2008; Ferreira et al., 2007; Matsubara et al., 2007; Seol et al., 2008).
H3E50 is located in the middle of the H3 αN helix. Residues close to or beside H3E50 (H3L48 and H3I51) are lethal when mutated to alanine and most point mutations in this area are sensitive to DNA damaging agents (Matsubara et al., 2007). H3E50 is located at the nucleosome entry site of DNA, therefore explaining how this residue may be particularly important for nucleosome stability (Figure 3.1A). h3E50A cells are sensitive to MMS, CPT and HU (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). We wished to determine the role played by H3E50 in the DDR. We assessed the phenotype of different substitution mutations (Figure 3.1B) at this residue to determine how important this region is in cellular progression and repair of DNA damage during S phase.
Figure 3.1: Nucleosome core particle and Amino acid substitutions. (A) Nucleosome core particle: Ribbons representing the structure of the nucleosome, showing DNA in brown and eight histone protein chains in yellow: H3; blue: H4; cyan: H2A; grey: H2B. The view is down the DNA superhelix axis. The right particle is zoomed in on the area of the nucleosome containing the H3 $\alpha$-N helix and the H3E50 residue. The H3E50 residue located on the H3 $\alpha$-N helix, represented on the nucleosome by CPK colouring, is located at the DNA entry and exist site of the nucleosome. (Nucleosome core particle schematics courtesy of Dr. Andrew Flaus) (B) Schematic of amino acid residue glutamate. (C) Schematics of amino acid residues of H3E50 mutation targets.
3.4. Results

3.4.1. Generation of histone H3 glutamate 50 mutants

As h3E50G mutant cells are sensitive to genotoxic agents, we investigated how other more subtle and radical amino acid substitutions in this area at the H3E50 residue would affect the nucleosomes ability to function in cell cycle progression in a perturbed and unperturbed manner. Glycine is an uncharged amino acid. In comparison glutamate is a common negatively charged amino acid found in α helices (Chou and Fasman, 1974). A glutamate to glycine amino acid substitution may alter the nucleosome environment at the H3 αN helix, and this may affect the structure and stability of the H3 αN helix. We predicted certain amino acid changes at residue glutamate 50 on H3 would affect the structure of the αhelix leading to alterations in the structure of the nucleosome which in turn causes problems for the cell. Therefore we wanted to investigate the effect of various amino acid substitutions at H3E50 in the H3 αN helix.

To approach this we generated five different H3E50 mutations: h3E50G, h3E50D, h3E50A, h3E50Q and h3E50K. h3E50G and h3E50A cells are both are sensitive to CPT and MMS (Figure 3.2B). This confirms previous observations made in different yeast strains (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). In h3E50D, cells the introduced aspartate residue is identical in charge and similar in structure to glutamate. In h3E50Q cells, the introduced glutamine residue has a similar side chain in length to glutamate, both contain 3 carbon atoms in their side chain, however unlike the negatively charged glutamate, glutamine is uncharged. In h3E50K cells, the introduced lysine has an opposite charge to the WT glutamate residue (Figure 3.1B and C).

To further characterise the mutants generated we analysed the cell cycle progression in unperturbed conditions of all five H3E50 mutants generated (Figure 3.2A). Cell density of asynchronous cycling cells were counted at various intervals and doubling times of each mutant determined. We hypothesised that some mutants generated would incur problems in cellular proliferation due to the changes in their chromatin environment by introducing
different amino acids. For example lysine is oppositely charged to glutamate therefore h3E50K is likely to incur problems in cellular proliferation. All mutants generated are slow growing compared to WT (Figure 3.2A). h3E50D, h3E50Q and h3E50A cells are slightly slow growing compared to WT cells, whereas h3E50G cells, similar to h3K56R cells were very slow growing. h3E50K cells were the slowest growing of all H3E50 mutants generated displaying a doubling time 3X longer than isogenic WT (Data summarised in table 3.1).

After identifying that all H3E50 mutants generated have perturbed cellular progressions we set out to define the role played by this residue in the DDR in the W303-1a RAD5+ genetic background. h3E50A cells were previously assessed to be sensitive to CPT, MMS and HU in other genetic backgrounds (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). We find h3E50A cells are sensitive to CPT, MMS but not to acute doses of IR or UV (Figure 3.2B). MMS, CPT and cisplatin all generate lesions during replication whereas HU slows down replication fork progression. Bleocin similar to IR induces single and DSBs (Basu and Krishnamurthy, 2010; Chen and Stubbe, 2005; Frankenberg-Schwager et al., 2005; Pommier, 2006; Xiao et al., 1996). To analyse the role of H3E50 in maintaining genomic integrity, H3E50 mutants were spotted on YPD plates containing CPT, MMS, HU, cisplatin and bleocin. Cells were also spotted on YPD plates and treated with IR and UV (Figure 3.2B). We characterised the responses of all H3E50 mutants generated to these DNA damaging agents. h3K56R a known mutant sensitive to genotoxic agents which activates an intra S phase checkpoint was used as a control.

Chronic exposure to IR and UV did not impair colony formation in h3E50D, h3E50Q, h3E50A and h3E50G cells with all mutants being comparable to WT and h3K56R. Cells lacking H3K56ac are reported sensitive to a number of genotoxic agents that cause damage during replication but not to acute doses of IR (Driscoll et al., 2007; Masumoto et al., 2005). In contrast these genotoxic agents strongly impaired the formation of colonies of h3E50K cells (Figure 3.2B). h3E50D and h3E50Q cells are mildly sensitive to bleocin, CPT, MMS and cisplatin (Figure 3.2B). h3E50A cells are moderately sensitive to these genotoxic agents, whereas exposure to these drugs strongly impaired the formation of
colonies of h3E50G and h3E50K cells (Figure 3.2B). In response to HU, all mutant strains appeared mildly to severely sensitive to HU damage after exposure for 3 days on plates containing a high dose of HU (Figure 3.2C). However prolonged incubation of these plates led to the emergence of slow growing colonies (Figure 3.2C). After prolonged exposure a high concentration of HU did not result in severe loss of viability of colonies of h3E50D, h3E50Q and h3E50A cells (Figure 3.2C). h3E50K cells were very sensitive to HU exposure, whereas h3E50G cells were only moderately sensitive to HU treatment (Figure 3.2C). To confirm these results, we next determined the survival of asynchronous h3E50G and h3E50A cells after transient exposure to MMS and HU.

h3E50G and h3E50A mutant cells were selected for further characterisation. Apart from h3E50K cells, which have a severe growth phenotype in asynchronous cultures, h3E50G cells are the most sensitive to genotoxic agents. h3E50A cells are moderately sensitive to bleocin, CPT, MMS and cisplatin damage and display an intermediate phenotype between h3E50D and h3E50Q cells which are mildly sensitive, and h3E50G and h3E50K cells which are highly sensitive to these genotoxic agents.

In viability assays performed (Figure 3.2D) asynchronous cells were collected before damage (0 hours) and after damage treatment for 4 hours (4 hours). A low concentration of MMS for four hours led to a loss of viability in h3E50G and h3E50A cells. In response to MMS damage, h3E50A cells have 75-80% survival compared to WT. This is stark contrast to h3E50G cells which had 10% survival compared to WT cells after MMS damage. As previously reported h3K56R cells resulted in severe loss of viability in response to MMS (Figure 3.2D; (Wurtele et al., 2012). mec1Δsml1Δ cells which were used as a control were unable to form colonies after exposure to MMS.

In response to HU treatment no significant loss of viability was observed for h3E50G or h3E50A cells (Figure 3.2D). h3E50A and h3E50G cells, both had survival rates of 85-90% compared to WT following HU treatment. As has been previously reported, h3K56R cells did not result in any loss of viability in response to HU treatment (Figure 3.2D; (Wurtele et al., 2012). A
mec1Δsml1Δ mutant which was used as a control resulted in complete loss of viability in response to HU. meclΔsml1Δ cells are incapable of forming colonies on HU-containing plates due to the essential function of Mec1 in inhibiting late origin firing in an early S phase checkpoint response (Santocanale and Diffley, 1998).

These results for h3E50G cells are comparable to cells lacking H3K56ac, which had dramatic losses of viability to MMS and CPT but not to HU (Wurtele et al., 2012). These results show that h3E50G and h3E50A cells do not loose viability after exposure to HU which is known to strongly activate the intra-S-phase DNA damage checkpoint. Our results suggest that like H3K56ac deficient cells h3E50G and h3E50A cells can recover from HU-induced checkpoint activation but not from an MMS or CPT-induced checkpoint activation.

This data indicates that H3 glutamate 50 is important for cellular progression in a perturbed and unperturbed cell cycle. A variety of H3E50 mutants generated resulted in mildly to severely sensitive phenotypes in response to DNA damaging agents indicating that this residue plays an important role in regulating the DNA damage response. These phenotypes observed are unlikely to be due to defects in checkpoint kinetics as h3E50G cells are not sensitive to high doses of HU which induces a checkpoint response.
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. (A) Growth analysis of five H3E50 mutants generated by analysing cell division. Mutants differ in their doubling times taking just over 120 mins for h3E50D, the most comparable mutant to WT in comparison to over 6 hours for h3E50K to double. h3K56R resembled h3E50G in taking under 180 mins to double. (DT is strain doubling time) (B) Preliminary characterisation of the five H3E50 mutants by drop test. h3E50G, resembling h3K56R, is highly sensitive to DNA damaging agents causing damage in S phase. (C) Prolonged incubation of cells on HU plate shows h3E50G mutant cells are slow growing rather than sensitive to high doses of HU. (D) Viability assays revealed h3E50G cells are as sensitive to MMS as are h3K56R mutant cells but the h3E50A mutant is only slightly sensitive to MMS. h3E50G and h3E50A, similar to h3K56R are not sensitive to high doses of HU.
3.4.2. *h3E50G* and *h3E50A* cells do not recover efficiently from an intra S phase checkpoint

Upon detecting damage, coordinated responses are regulated by the DDR. The DSB-induced response in *S. cerevisiae* leads to activation by phosphorylation of Rad9 and Rad53, both of which are widely used markers of DNA damage checkpoint activation. As *h3E50G* and *h3E50A* cells are highly sensitive to agents which cause damage in S phase, although not to HU, we investigated whether H3E50 is required for an efficient checkpoint response. We hypothesised that *h3E50G* and *h3E50A* cells would be competent in activating and deactivating the DNA damage checkpoint and that loss of viability of *h3E50G* and *h3E50A* cells would therefore be due to other factors other than checkpoint kinetics.

First we analysed Rad9 and Rad53 phosphorylation in *h3E50G* and *h3E50A* cells which were arrested in G1 and and released into S phase in the absence of genotoxic agents (Figure 3.3A). In the absence of damage no checkpoint activation is observed, as analysed by the absence of DNA damage dependent Rad9 and Rad53 hyperphosphorylation, in WT or mutant strains (Figure 3.3B).
Figure 3.3: DNA damage checkpoint and recovery analysis of h3E50G and h3E50A mutants. (A) Schematic of experiment layout. Cells were arrested in G1 before being released synchronously into S phase. (B) Checkpoint analysis by monitoring Rad9 and Rad53 cell cycle and DNA damage dependent phosphorylation forms. No checkpoint activation is detected in the absence of DNA damage. (Note for Rad9 phosphorylation forms: C-Rad9 denotes the cell cycle-dependent forms of rad9 and D-Rad9 denotes the DNA damage-dependent hyperphosphorylated Rad9).
We next investigated checkpoint kinetics in h3E50G and h3E50A cells after MMS, CPT and HU treatments (Figure 3.4A and D). Activation and deactivation of the DNA damage checkpoint was monitored in WT, h3E50G and h3E50A cells after a transient exposure to MMS, CPT and HU. Cells were arrested in G1 and released into the cell cycle in the presence of MMS, CPT or HU (Figure 3.4A and D). The DNA damage checkpoint is strongly activated in MMS damaged cells as monitored by Rad9 and Rad53 hyperphosphorylation forms. In contrast to WT cells, where Rad53 phosphorylation is back to basal levels 60 minutes after the end of MMS treatment, h3E50G and h3E50A cells show persistent Rad53 phosphorylation up to 180 to 240 minutes after the end of the DNA damage treatment (Figure 3.4B).

Transient exposure to CPT did not induce a strong checkpoint response in WT cells, as seen by lack of Rad53 and Rad9 damage dependent phosphorylation (Figure 3.4C). In contrast, CPT induced a strong checkpoint response in h3E50G and h3E50A cells. Rad9 is hyperphosphorylated in h3E50G and h3E50A cells up to 180 minutes after damage by CPT. Rad53 hyperphosphorylation also starts to decrease at 180 minutes in h3E50G and h3E50A cells. h3E50G and h3E50A cells are more sensitive to damage by CPT as seen by a strong checkpoint response compared to no obvious checkpoint response in WT cells. This indicates that histone residue H3E50 is fundamental to an appropriate DNA damage response after CPT treatment.

In response to HU damage, we predicted h3E50G and h3E50A cells would have proficient checkpoint kinetics as these mutant cells did not display loss of viability when exposed to high HU doses in previous experiments (Figure 3.2D). As expected in response to 200 mM HU treatment, the intra S phase checkpoint is immediately activated after removal of HU in WT cells, although treatment did not activate a strong checkpoint response in any of the strains. Rad53 quickly becomes dephosphorylated with no phosphorylation visible at 60 minutes in WT or h3E50A cells (Figure 3.4E). No Rad53 phosphorylation is evident from western blotting analysis in h3E50G cells after the removal of HU but this may be due to the specificity of the Rad53 antibody used as it is raised to the hypophosphorylated forms of Rad53. Mrcl is the mediator protein, not Rad9 for
the S phase checkpoint response which is activated upon HU treatment (Finn et al., 2012). Therefore no Rad9 hyperphosphorylation is present after HU treatment.

These results demonstrate that $h3E50G$ and $h3E50A$ cells recover efficiently from a HU induced checkpoint response but are delayed in checkpoint recovery as judged by prolonged Rad53 phosphorylation compared to WT in response to MMS and CPT damage. These results indicate that sensitivity of $h3E50G$ or $h3E50A$ cells to genotoxic agents is not due to their inability to deactivate the S phase checkpoint but may be due to another function in the DDR cascade which requires an intact H3E50 residue.
Figure 3.4: *h3E50G* and *h3E50A* cells have S phase recovery defects in response to MMS and CPT damage. (A) Experimental layout of checkpoint kinetics experiment. Cells were arrested in G1, damaged for 45 mins and released into the cell cycle. (B and C) *h3E50G* and *h3E50A* cells have a prolonged checkpoint activation after MMS and CPT damage compared to WT. (D) Experimental layout of checkpoint kinetics experiment in response to HU damage. (E) *h3E50G* and *h3E50A* cells in response to HU treatment deactivate the cell cycle checkpoint with similar kinetics to WT cells.
3.4.3. **H3E50 is not required for 5’-3’ DNA end resection**

Following a DSB, resection takes place in a 5’-3’ direction producing ssDNA which is rapidly coated by replication protein A (RPA). Coating of ssDNA by RPA is an early event in the DNA damage response which promotes the recruitment of further downstream proteins. *h3E50G* and *h3E50A* cells are highly sensitive to DNA damaging agents CPT and MMS and they maintain an activated DNA damage checkpoint for longer compared to WT cells. In *h3E50G* cells the DNA damage checkpoint is activated with similar kinetics to WT (Figure 3.4 B and C) indicating that these phenotypes may be due to factors downstream of checkpoint activation such as repair.

To investigate the requirement of H3E50 in DNA end-resection, we performed Chromatin Immunoprecipitation (ChIP) coupled to quantitative PCR (qPCR) to monitor the recruitment of the ssDNA-binding replication protein A, RPA, to a single, site-specific DSB (Figure 3.5). We used the GAL::HO yeast system, as previously described (Lee et al., 1998). Briefly, these strains contain a galactose-inducible HO gene, as well as a single, defined HO cut site introduced at the MAT locus. Furthermore, these strains are deleted for both HML and HMR donor sequences, and as a result repair of the HO-induced DSB at the MAT locus by gene conversion is prevented. After induction of the HO-DSB at MAT, RPA recruitment was monitored at time 0 and 4 hours. Following ChIP, qPCR using primer pairs that anneal specifically at sites of increasing distance from the break site was used to monitor spreading of RPA (van Attikum et al., 2007; Zhu et al., 2008). We observed that the recruitment of RPA was comparable in both a *h3E50G* mutant and the isogenic WT (Figure 3.5B).

Based on these data, we conclude that H3E50 is not required for DNA end resection following an HO-induced DSB. Therefore sensitivity of *h3E50G* cells to genotoxic agents is not due to defects in the initial DNA damage response, but may be due to failure to recover following repair/failure to signal completion of DNA repair.
3.4.4. *h3E50G* and *h3E50A* mutant cells form persistent Rad52 foci after exposure to genotoxic agents

After MMS and CPT damage the DNA damage checkpoint remains activated for longer in *h3E50G* and *h3E50A* cells compared to WT. Following checkpoint activation, signals to downstream effectors activate the major biological responses to DNA damage, such as DNA repair. Homology dependent processes are involved in the repair of DNA damage at replication forks. HR is active in S
and G2 phases of the cell cycle in which genetic information is exchanged between DNA sequences that share homology. Rad52 is an important HR protein which forms foci at the sites of DNA damage (Lisby et al., 2001). Persistence of Rad52 foci may signify problems in repairing the damage induced and ultimately problems in completing homology dependent processes. We hypothesised that \( h3E50G \) and \( h3E50A \) cells maintain an activated checkpoint due to the persistence of unrepaired damage.

We investigated the kinetics of Rad52 foci formation in \( h3E50G \) and \( h3E50A \) cells. Firstly, we analysed Rad52 foci formation in the absence of genotoxic agents (Figure 3.3A). \( h3E50G \) and \( h3E50A \) cells have slower doubling times compared to WT, so we anticipated these mutant cells may have higher instances of endogenous damage compared to WT cells. To investigate this possibility, cells were arrested in G1 and released into fresh media after arrest. Cells were examined for Rad52 foci 1hr to 6hrs after release. \( h3E50G \) and \( h3E50A \) cells accumulated DSBs in the absence of damage as measured by Rad52 foci occurring spontaneously compared to WT cells (Figure 3.5A and B). WT cells have approximately 5% of Rad52-YFP foci in undamaged cycling cells whereas \( h3E50G \) and \( h3E50A \) cells have approximately 20% Rad52-YFP foci.
We next investigated Rad52 foci formation in cells treated with MMS. Homology dependent processes are reported to repair MMS damage in S phase of the cell cycle (Nikolova et al., 2010). Cells were arrested in G1 and released into fresh media containing 0.03% MMS. Cells were damaged for 45 mins after which the drug was removed and cells released into fresh media (Figure 3.4.A). In response to MMS, h3E50G and h3E50A cells have persistent Rad52 foci up to 360 minutes after damage. After MMS damage WT cells had 20% Rad52 foci after 60 minutes which peaked at 120 minutes with approximately 30% foci. In h3E50G and h3E50A cells, damage was much greater with 60-80% foci.

Figure 3.6: Rad52 foci occur spontaneously in h3E50G and h3E50A cells. (A) DIC and YFP fluorescence images of Rad52-YFP foci in the absence of damage in WT, h3E50G and h3E50A cells. Examples of foci are shown in each strain with a yellow arrow. (B) Rad52-YFP foci occur spontaneously in h3E50G and h3E50A cells demonstrating that endogenous damage is occurring in these cells.
occurring after 60 minutes and which remained high throughout the duration of the experiment (Figure 3.7A and B). \textit{h3E50A} cells had higher incidences of Rad52 foci at 60 and 120 minutes compared to \textit{h3E50G} cells. Whereas \textit{h3E50G} cells had greater Rad52 foci at 180 minutes up to 360 minutes. This difference may be due to \textit{h3E50G} cells being slower to progress through the cell cycle than \textit{h3E50A} cells.

In addition to MMS, \textit{h3E50G} and \textit{h3E50A} cells are sensitive to CPT. CPT damage is different to damage induced by MMS. CPT is a TopI poison which induces DSBs in S phase by preventing the transient cleavage and religation of DNA Top1, thus leading to the formation of DSBs during replication (Pommier, 2006). We investigated the kinetics of Rad52 foci formation after transient exposure to CPT. Cells were arrested in G1 and released into fresh media containing 25 $\mu$M CPT. Cells were exposed to the genotoxic agent for 45 minutes after which the drug was removed and cells released into fresh media.

In response to CPT damage immediately after removal of the drug, WT cells had approximately 20% Rad52-YFP foci compared to 75% Rad52-YFP foci for \textit{h3E50G} and \textit{h3E50A} cells. Rad52 foci formation in WT cells peaked at 30% at 120 minutes. In contrast, \textit{h3E50G} and \textit{h3E50A} cells had over a 3 fold increase of Rad52-YFP foci occurring compared to WT (Figure 3.7C and D). After CPT exposure, \textit{h3E50G} cells suffered a 50% loss of viability, compared with only 90% and 85% for \textit{h3E50A} and WT cells respectively (Figure 3.7E). Similar to MMS treatment, in response to CPT treatment, Rad52-YFP foci were transitory in WT cells where these Rad52-YFP containing structures persisted for longer and remained significantly higher in \textit{h3E50G} and \textit{h3E50A} cells compared to WT.
Figure 3.7: Rad52 foci persist in h3E50G and h3E50A cells after treatment with genotoxic agents. (A) DIC images and YFP fluorescence images of Rad52-YFP foci after MMS treatment. Examples of foci indicated with yellow arrow. (B) After transient exposure to MMS Rad52-YFP foci persist in h3E50G and h3E50A cells. (C) DIC images and YFP fluorescence images of Rad52-YFP foci after CPT treatment. Examples of foci indicated with yellow arrow. (D) After transient exposure to CPT, Rad52-YFP foci persist in h3E50G and h3E50A cells. (E) After CPT exposure h3E50G cells suffered a 50% loss of viability compared to WT.
As seen in earlier experiments, \textit{h3E50G} and \textit{h3E50A} cells are sensitive to genotoxic agents but not to treatment with HU, which slows down replication fork progression in S phase but does not generate damage. Therefore we analysed Rad52 foci formation in cells after treatment with transient exposure to HU. Cells were arrested in G1 and released into fresh media containing 200 mM HU. Cells were damaged for 90 minutes after which the drug was removed and cells released into fresh media (Figure 3.4D).

A significant number of Rad52-YFP foci occurred in \textit{h3E50G} and \textit{h3E50A} cells. Wild-type cells had a maximum amount of Rad52-YFP foci of 10% at 60 minutes post HU treatment, which then decreased to below 10% for the duration of the experiment. In contrast to this \textit{h3E50G} and \textit{h3E50A} cells both had 20% Rad52-YFP foci present at 60 minutes which increased to 40% at 180 minutes for \textit{h3E50G} and 30% for \textit{h3E50A} cells at this time also. \textit{h3E50G} cells have a slightly higher occurrence of Rad52 foci after 120 minutes until the end of the experiment compared to \textit{h3E50A} (Figure 3.8A and B). There was no loss of viability in \textit{h3E50G} or \textit{h3E50A} cells after HU exposure. \textit{h3E50G} and \textit{h3E50A} cells showed 70-80% viability compared to 60% survival for WT cells (Figure 3.8C).

Although a notable amount of Rad52-YFP foci occurred in \textit{h3E50G} and \textit{h3E50A} cells after transient exposure to HU, the incidence of Rad52 foci formation is much lower than what is seen after MMS or CPT damage. H2A-S128 phosphorylation is a mark rapidly induced after formation of a DSB. Cells lacking H3K56ac displayed H2A-S128 phosphorylation after transient exposure to HU suggesting that HU causes DNA damage in a fraction of these cells (Wurtele et al., 2012). In addition, similar to \textit{h3E50G} and \textit{h3E50A} cells, \textit{h3K56R} cells had persistent Rad52 foci after HU damage (Wurtele et al., 2012). This suggests, that like \textit{h3K56R} cells, HU causes DNA damage in a fraction of \textit{h3E50G} and \textit{h3E50A} cells. HU leads to some persistent Rad52 foci in \textit{h3E50G} and \textit{h3E50A} cells but most foci formed after HU are short lived.

In summary, we first conclude that mutating the H3E50 residue to G or A leads to an accumulation of DSBs during normal cellular proliferation as seen by
increased Rad52 foci in undamaged \textit{h3E50G} and \textit{h3E50A} cells (Figure 3.6B). Secondly we conclude that transient exposure to HU during S phase results in some persistent Rad52 foci in \textit{h3E50G} and \textit{h3E50A} cells. However, seeing as \textit{h3E50G} and \textit{h3E50A} cells survive after a transient exposure to HU (Figure 3.8C), it is likely these persistent Rad52 lesions are efficiently resolved. Finally, transient exposure to either MMS or CPT results in persistent homologous Rad52 lesions during replication in \textit{h3E50G} and \textit{h3E50A} cells which indicates these cells encounter problems in repairing damage induced during replication.

![Figure 3.8](image)

**Figure 3.8: Rad52 foci persist in \textit{h3E50G} and \textit{h3E50A} cells after treatment with HU.**

(A) DIC images and YFP fluorescence images of Rad52-YFP foci after HU treatment. Examples of foci are indicated with yellow arrows in WT images. (B) After transient exposure to HU Rad52-YFP foci occur at a higher rate than WT cells. (C) There is no loss of viability in \textit{h3E50G} or \textit{h3E50A} cells after transient exposure to HU.
3.5 Discussion

H3E50 is located near the centre of the H3 αN helix. Based on the nucleosome structure, this region could be important for nucleosome assembly and disassembly as it is located at the DNA entry and exit site of the nucleosome (Figure 3.1A; (Luger et al., 1997a). Acetylation of H3K56 located in the C-terminal of the H3 αN helix has been implicated in the regulation of nucleosome assembly during DNA replication and repair, and in nucleosome assembly during gene transcription (Chen et al., 2008; Das et al., 2009; Kim and Haber, 2009; Li et al., 2008; Masumoto et al., 2005; Tjeertes et al., 2009; Williams et al., 2008; Xie et al., 2009). The H3 αN helix is also reported to be important for cell viability and nucleosome stability (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). Studies where H3E50 had been mutated to an alanine have found this mutant to be sensitive to MMS, CPT and HU (Ferreira et al., 2007; Matsubara et al., 2007; Seol et al., 2008). However, the molecular mechanism of action of H3E50 is unknown. We provide evidence that H3E50 is an important residue for cellular proliferation and is vital for survival after an S phase DNA damage response.

Five H3E50 mutants had varied and detrimental effects for cellular proliferation and survival after damage. H3E50 was mutated to aspartate, glutamine, alanine, glycine and lysine to generate h3E50D, h3E50Q, h3E50A, h3E50G and h3E50K respectively. These mutants allow investigation of how charge and structure affect the local environment at residue 50 in the H3 α-N helix. Mutation to glutamine or lysine provides information about the importance of the negative charge of the glutamate sidechain. Mutation to alanine or aspartate provides information on how the side chain length contributes to the role of the glutamate. Finally, mutation to a glycine is informative about the role of H3 αN helix stability.

Cell cycle progression was delayed in all mutants generated (Figure 3.2A and summarised in Table 3.1) and all mutants were sensitive to CPT and MMS (Figure 3.2B). These results show that an intact H3E50 residue is important for cellular function both in the absence and presence of exogenous genotoxic
agents. The slow growth rates observed for each individual mutant also corresponded to their sensitivity to genotoxic agents (Figure 3.2A and B and Table 3.1) For example, \textit{h3E50D} and \textit{h3E50Q} cells are slightly slower growing than WT cells and these mutants are only very mildly sensitive to DNA damaging agents. In contrast \textit{h3E50K} cells are the slowest growing mutants and take over 360 minutes to double with very pronounced sensitivity to the genotoxic agents tested.

<table>
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\textbf{Table 3.1: H3E50 mutants show slow growth phenotypes in the presence and absence of damage.} In all panels, WT is represented by +++ with symbols less than this signifying the rate of sensitivity of these cells to damaging agents in all panels except the last. The last panel represents growth of these mutants under normal conditions.

Aspartate (D) is similar to glutamate (E) in possessing a side chain with a negatively charged carboxyl group. The aspartate and glutamate side chains differ by one methylene group (CH$_2$) with aspartate having a shorter side chain. Introducing an aspartate residue at H3E50 was possibly the least disruptive amino acid change we could introduce and we expected \textit{h3E50D} cells to behave similarly to WT cells. Intriguingly, \textit{h3E50D} cells had slightly slower doubling times (120 mins compared to 110 mins for WT). This slight difference correlates with \textit{h3E50D} cells being slightly more sensitive to DNA damaging agents. This suggests that even the smallest alteration of H3E50 residue results in
unfavourable changes in chromatin structure which in turn affects cellular function.

Chou and Fasman designed an empirical secondary structure propensity scale for amino acids that has been more recently updated (Table 3.2; Fujiwara et al., 2012). Alanine and glutamate have the highest $\alpha$ helical propensity with glutamine in the next highest group. In contrast, glycine (and proline) have the lowest propensity.

Glutamine (Q) is equivalent in size and helical propensity to glutamate but has a neutral charge (Figure 3.1C). We predict that both $h3E50D$ and $h3E50Q$ cells should not distort the H3 $\alpha$-helix significantly compared to other H3E50 mutants generated (Figure 3.2B). $h3E50Q$ cells were similar to $h3E50D$ cells in that they were slightly sensitive to DNA damaging agents and slightly slower growing than WT.

The sidechain of alanine consists of a single methyl group (CH$_3$) and removes the negative charge normally introduced by glutamate. Alanine has the highest alpha helical propensity so should not affect the structure of the $\alpha$N helix itself. The $h3E50A$ cells had doubling times similar to $h3E50D$ and $h3E50Q$ but slower than WT. These cells were somewhat more sensitive to DNA damaging agents than the $h3E50Q$ and $h3E50D$ mutants suggesting that sidechain volume does contribute to stability (Figure 3.2A and B).

Glycine is the simplest amino acid and lacks any functional group as sidechain. We predict that substitutions of glycine at residue 50 would result in increased backbone flexibility and potentially severe disruption of the $\alpha$N helix. Indeed, $h3E50G$, has highly increased doubling time equal to the $h3K56R$ mutant and is also almost completely unable to remove DNA damaging agents.
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Table 3.2 Amino acid residue structure and propensity scale. Data taken from (Creighton TE, 1992) and (Fujiwara et al., 2012)
Lysine (K) introduces a positive charge in place of the negative charge found in the WT protein and would be predicted to have significant effect on the local environment at the H3 αN helix. We predicted that inverting the charge at H3E50 would have major consequences.

H3E50 when mutated to a lysine was the most severe of mutations that we introduced. Specifically, H3E50 is surrounded by several positive charges likely to be balanced by glutamate in WT. Arginine is a positively charged residue and interestingly four arginine residues on histone H4 lie in close proximity to H3E50. These residues which are H4R35, H4R36, H4R39 and H4R40, all lie within 7 angstrom distance of H3E50 (Figure 3.9A) with two of these residues, which are the only residues present, (Figure 3.9B) within 5 angstrom distance of H3E50. This is significant as H3E50 being a negatively charged residue is likely to contribute to maintaining a balance of charges within this area of H3 and H4. h3E50K cells were extremely slow growing and highly sensitive to DNA damaging agents. By adding another positive charge in this area of H3 which lies in close proximity to H4 we believe we disrupted this delicate balance of charges which leads to catastrophic implications for the cells, as seen by severe slow growth and the highly sensitive nature of h3E50K cells to DNA damaging agents.

We believe due to this mutating H3E50 to uncharged or positively charged residues had significant implications for the cells as this may have disrupted the balance of charges in this area and resulted in changes to the structure of the chromatin and subsequently interactions with the surrounding DNA. This data suggests that the negative charge present at H3E50 in WT cells may be vital in maintaining the structure of the chromatin at this site of the nucleosome.
Figure 3.9 H3/H4 tetramer illustrating positive charges surrounding H3E50.
(A) H3/H4 tetramer structure showing H3E50 surrounded by four arginines on H4. Arginines are shown by blue arrows and H3E50 by red arrow. (Distance within 7 angstrom). (B) H3E50 lies in close proximity to two arginines which are present on H4 (Distance within 5 angstrom). (Acknowledgements to David Pinto in helping to capture images)
Previously \textit{h3E50A} cells have been reported to be sensitive to CPT, MMS and HU (Dai et al., 2008; Matsubara et al., 2007; Seol et al., 2008). We report that transient exposure to CPT and MMS, but not to HU, results in moderate loss of viability in \textit{h3E50A} and severe loss of viability in \textit{h3E50G} cells (Figure 3.2B, C and D). Our data shows that \textit{h3E50G} and \textit{h3E50A} cells are competent in checkpoint activation but exhibit defects in completing S phase replication after exposure to MMS and CPT. These defects are not due to an inability to deactivate the DNA damage checkpoint as \textit{h3E50G} and \textit{h3E50A} cells deactivate the checkpoint with similar kinetics to WT after exposure to 200 mM HU (Figure 3.4E). Our results suggest that loss of cell viability in \textit{h3E50G} and \textit{h3E50A} cells after exposure to MMS or CPT is not due to the failure of these cells to deactivate the DNA damage checkpoint after DNA repair but because these genotoxic agents generate persistent DNA lesions in \textit{h3E50G} and \textit{h3E50A} cells.

HR is the major form of DSB repair in \textit{S. cerevisiae} cells. \textit{h3E50G} and \textit{h3E50A} cells are proficient in 5’-3’ DNA end resection as measured by accumulation of RPA at the DSB (Figure 3.5). Thus, histone H3 residue E50 is not required for the early stages of the DNA damage response. Therefore, we anticipated that \textit{h3E50G} and \textit{h3E50A} cells would be non-viable after exposure to cytotoxic agents due to defective repair in these cells. We also saw increased Rad52 foci in undamaged \textit{h3E50G} and \textit{h3E50A} cells indicating that repair is ongoing in these cells in the absence of exogenous damage (Figure 3.6). This suggests that in the absence of the normal glutamate residue at position 50 of histone H3 endogenous damage may be increased, which in turn would suggest that H3E50 is fundamentally important for cellular proliferation and preventing genomic instability.

We see an accumulation of Rad52 foci after exposure to CPT or MMS and to a lesser extent HU, demonstrating the persistence of lesions which can not be resolved in \textit{h3E50G} or \textit{h3E50A} cells (Figure 3.7 and figure 3.8). Evidence exists for MMS generating DSBs during S phase which are repaired by the HR pathway (Nikolova et al., 2010). CPT cytotoxicity results largely from covalent bonds between DNA topoisomerase I and single strand nicks located
ahead of replisomes, which in turn can lead to DSBs during replication (Pommier, 2009). Although MMS and CPT generate different lesions both result in the accumulation of persistent Rad52 HR structures in \textit{h3E50G} and \textit{h3E50A} cells. Intriguingly, HU also results in persistent, although less numerous, Rad52 HR structures in \textit{h3E50G} and \textit{h3E50A} cells. These results suggest that regardless of the nature of damage, DNA damage during replication leads to the formation of persistent Rad52 foci in a certain fraction of \textit{h3E50G} and \textit{h3E50A} cells.

We conclude that changing the nature of the chromatin environment by modifying the H3E50 residue significantly delays the completion of DNA replication and also leads to the formation of persistent lesions that undergo processing by the Rad52 homologous recombination protein. Therefore an intact H3E50 residue is vital for survival after exposure to MMS or CPT damage. \textit{h3E50G} and \textit{h3E50A} cells behaved similarly to \textit{h3K56R} cells in sensitivity to DNA damaging agents, delayed checkpoint recovery and persistent Rad52 homologous structures after damage during replication (Table 3.2 and Table 3.3; Wurtele et al., 2012). We predict that H3E50 functions in the same pathway as H3K56ac and that an intact H3E50 residue is vital for a functioning H3K56ac residue.

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</table>

Table 3.3: \textit{h3E50G}, \textit{h3E50A} and \textit{h3K56R} cells are defective in S phase checkpoint recovery after MMS and CPT damage as measured by persistent Rad53. \textit{h3E50G}, \textit{h3E50A} and \textit{h3K56R} cells are defective in S phase checkpoint recovery. Comparison of \textit{h3E50G}, \textit{h3E50A} and \textit{h3K56R} cells strains in response to untreated, MMS, CPT and HU treatment. Note data for \textit{h3K56R} taken from Wurtele et al., 2012.
In summary, our results provide evidence that an intact H3E50 residue is vital to the overall structure of the H3 αN helix, for cellular proliferation and for an efficient recovery from cytotoxic damage during replication.

### 3.6 Conclusion and Future work

We provide evidence that H3E50 is an important residue for cellular progression and for survival after damage. Mutating H3 glutamate residue 50 to various amino acids revealed that this residues contribution to charge and maintaining the structure of the H3 αN helix is vital for the function of the nucleosome. The sensitivity profiles observed in h3E50G are similar to h3K56R cells, whereas h3E50A cells display a milder sensitivity profile to damagaiing agents as observed by drop test analysis. On further analysis we observed that both h3E50G and h3E50A are defective in repair, similar to h3K56R cells (Chapter 3; Wurtele et al., 2012). Similarly h3E50G, h3E50A and H3K56ac deficient cells display sensitivity to S phase genotoxic agents and they also form persistent Rad52 homologous structures after MMS and CPT damage (Figure 3.2 and 3.7; Wurtele et al., 2012). h3E50A was lethal when combined with asf1Δ suggesting

<table>
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Table 3.4: Formation of persistent Rad52 homologous structures after MMS, CPT and HU treatment. h3E50G, h3E50A and h3K56R cells all form persistent Rad52 homologous structures during replication after treatment with MMS, CPT and HU. Note data for h3K56R taken from (Wurtele et al., 2012).
that a normal H3 α-N helix is critical when Asf1 activity is compromised (Seol et al., 2008). Asf1 is the histone chaperone for H3K56 acetylation. Therefore, we believe that H3E50 is important in a pathway that involves H3K56ac.

Mms22, Mms1, Rtt101, Rtt109 and Asf1 are required for MMS induced HR. However, Mms22 and Mms1 are not required for HR when DSBs are induced by IR and these proteins are specifically required for HR involving blocked replisomes (Duro et al., 2008; Vaisica et al., 2011). Mms22 and H3K56ac work in the same genetic pathway in response to DNA damage (Wurtele and Verreault, 2006). Therefore we propose that an intact H3E50 residue is important for this genetic pathway and mutation of the H3E50 residue reduces the ability of this Mms22-H3K56ac pathway to carry out efficient repair when cells are exposed to genotoxic agents during replication. To determine if H3K56 and H3E50 work in the same pathway we need to determine the epistatic relationship between h3E50G and h3K56R.

Glycine is an extremely unfavorable residue in an α-helix (Chou and Fasman, 1974), and is likely to cause flexible hinge-like behaviour in the middle of the H3 αN helix. This could affect that ability of Mms22 to regulate repair via H3K56ac. This structural change may prevent H3K56ac being recognised by Mms22 or recruitment of other factors in this pathway may be inhibited due to the aberrant H3 αN helix structure. To test this possibility we will have to investigate H3K56ac in our h3E50A and h3E50G mutant cells to identify if H3E50 contributes to the acetylation of H3K56. H3E50 may be vital in maintaining an intact H3 αN helix structure and when this structure is compromised H3K56 is unable to become acetylated. If H3K56 is acetylated in h3E50A and h3E50G cells, H3E50 may be important for maintaining an intact Mms22 and H3K56ac pathway. A distorted H3 αN helix structure could affect the recruitment of proteins involved in a H3K56ac dependent pathway.

Another hypothesis is that H3E50 is involved in more than just facilitating H3K56 acetylation. Preliminary results obtained from our collaborators (Jerome Poli; Phillipe Pasero; Montpellier) suggest that h3E50G cells are competent in
replication fork restart after MMS damage. This is in contrast to h3K56R cells which are reported to have delayed replication and fork restart after MMS damage (Wurtele et al., 2012). This data suggests that H3E50 is contributing to more than just facilitating H3K56 acetylation. h3E50A mutation has been shown to reduce the stability of histone octamers (Ferreira et al., 2007; Somers and Owen-Hughes, 2009). H3E50 mutations may affect overall nucleosome structure and dynamics and we could investigate the possibilities of analysing nucleosome structure and stability in vitro.

Overall mutation of H3E50 results in detrimental effects for the cell. Although we have identified that H3E50 is an important residue for cellular proliferation and is vital for survival after an S phase DNA damage response the precise molecular mechanism remains to be determined.
3.7. Materials and Methods

3.7.1. Media and growth conditions

Yeast cells were grown in YPD liquid (1% (w/v) yeast extract (Difco), 2% (w/v) bactopeptone (Difco), 2% (w/v) glucose (Difco)) or -Leu minimal liquid medium (Formedium). Yeast cells grown on solid plates were grown on YPD media containing a plus of 2% (w/v) agar (Difco). Solid selective media was YNB (yeast nitrogen base) with 2% agar and the required amino acids (W303 requires histidine, leucine, tryptophan, uracil and adenine) with 2% glucose as the carbon source.

Cells were grown in conical flasks with liquid media at 30°C in a shaker incubator at 170 r.p.m. The volume of culture did not exceed one third of the nominal flask volume to ensure adequate aeration. Cells that were grown in plates of YPD solid media were incubated at 30°C and depending on strain growth were left grow for 2-3 days.

3.7.2. Cellular growth and genotoxic treatments

Asynchronous cultures were diluted to a concentration of 1x10^6 cell/ml and left cycling for 8 hours. Samples were taken at the indicated times to determine strain doubling time.

The genotoxic treatment for sensitivity analysis was performed by the drop test methodology using media with different genotoxic agents 1) MMS (Sigma-Aldrich) was added to YPD media to a final concentration of 0.1%. 2) CPT (Sigma) was added to YPD final concentration of 10µM. 3) HU (Sigma) was used at final concentrations of 100mM. 4) Cisplatin (Sigma) was used at a final concentration of 150µg/ml. 5) Bleocin (Calbiochem) was used at a final concentration of 0.15 ug/ml. 6) γ-irradiation (400 Gy) was carried out using a 137 Cs at a dose-rate of 12.10 Gy/min (Mainance Engineering, UK). 7) The UV irradiation rate (J/m^2/s) from the lamp was measured before each irradiation, using a UV dosimeter, and the time of exposure was calculated (time = desired dose (50J) / UV intensity (J/m^2/s)). DNA damage sensitivity analysis was performed by spotting five-fold serial dilutions of exponentially growing (5x10^6) cultures of the indicated strains on plates containing the indicated genotoxic
agents. Plates were incubated in the 30°C incubator unless otherwise specified for two days before scanning.

For survival assays asynchronous cells were incubated in liquid medium containing MMS or HU. Cells were then plated onto YPD before (0hrs) or four hours after genotoxic treatment (4hrs). YPD plates were incubated for 5 days at 30°C and then colonies counted.

### 3.7.3. Yeast strains and Plasmids

All yeast strains used in this study are made in W303-1a background unless otherwise stated and are listed in table S3.1. Yeast strain and plasmid constructions are described below. Plasmids and oligonucleotides used in this study are in Tables S3.2 and S3.3 respectively. All constructs were sequenced to confirm the presence and absence of the specific mutations.

#### 3.7.3.1. Plasmid Shuffle strain

A one-step PCR mediated approach (Longtine et al., 1998) was used to generate the parental plasmid shuffle system strain (Thomas Costelloe Thesis 2009). Firstly, genomic *HHT2-HHF2* was replaced with *HIS5* from pML3 (pFA6a-His3MX6). These cells were then transformed with YCp50-copyII (*HHT2-HHF2*) (rescued from yeast strain DY5733, kindly provided by Alain Verreault), a plasmid bearing wild-type H3-H4-copyII alleles with their natural promoter and a *URA3* selectable marker. Then, genomic *HHT1-HHF1* was replaced with *KANMX* from pML1 (pFA6a-KANMX6). For the plasmid shuffle of histone alleles, wild-type or mutated histone alleles on *TRP1*-marked plasmids were introduced into the parental plasmid shuffle strain carrying wild-type histones on the *URA3*-marked YCp50-copyII plasmid. Cells containing both the wild-type (Ura+) and mutant (Trp+) plasmids were selected by growth on selective media. These cells were then plated onto minimal media containing 5-FOA (1mg/ml) to select for cells that had lost the wild-type (*URA3*) plasmid (Thomas Costelloe Thesis 2009).
3.7.3.2. H3E50 mutant strains construction

Point mutations in histone H3 at residue 50 was generated by site-directed mutagenesis (Protocol from Dr. Andrew Flaus Laboratory). Primers were designed approximately 45bp in length, containing the mutation of interest in the middle of the sequence (Primers Supplemental table S3.3). The reverse primer was the complementary sequence of the forward sequence. Mutagenesis was carried out directly on the yeast centromeric plasmid Ycp[HHT2-HHF2(TRP1)] and following conformation by DNA sequencing, a BseR1-BamHI fragment containing the mutation of interest was ligated back into the parental plasmid. After sequencing these plasmids were transformed into yeast using the standard Lithium Acetate method.

3.7.3.3. Strains for detection of Rad52 foci

WT, h3E50G and h3E50A strains were transformed with a centromeric plasmid, pWJ1344-Rad52-YFP-LEU, harboring Rad52 protein tagged with YFP protein using the standard lithium Acetate protocol. Cells were selected on –Leu plates to select cells with pWJ1344-Rad52-YFP-LEU plasmid present. Cells were grown on –Leu minimal media plates to maintain the plasmid.

3.7.4. G1 arrest and release experiment.

Asynchronous, exponentially growing cells (5x10^6 cells/ml) were arrested in G1 by addition of α-factor (7.5 µg/ml final concentration) for 120 minutes. Following arrest, cells were washed with pre-warmed 0.9% saline, followed by YPD, and then resuspended in fresh media in the presence or absence of drug treatment. MMS or CPT was added at a concentration of 0.03% or 25µM respectively for 45 minutes. HU was added at a concentration of 200mM for 90 minutes. Cells were again washed with pre-warmed 0.9% saline, followed by YPD, and then resuspended in fresh media and let cycle for the duration of the experiment. Samples were taking for western blot and FACS as previously described (O'Shaughnessy et al., 2006). Samples were also taken for microscopy analysis described below.
3.7.5. Immunoblotting
Immunoblotting was performed as previously described (O'Shaughnessy et al., 2006; Vialard et al., 1998). Yeast whole cell extracts were prepared as described in (Kushnirov, 2000). Rad9 and Rad53 were resolved in 6.5%, 80:1 acrylamided/bis-acrylamide, SDS-PAGE gel and probed with NLO5 and NLO16 (O'Shaughnessy et al., 2006; Vialard et al., 1998) at 1:10000 dilution. HRP conjugated anti-rabbit secondary antibody (from pierce) and super signal WestPico chemiluminescent substrate from Thermo Scientific (Product no. 34080) was used to detect the proteins.

3.7.6. Chromatin Immunoprecipitation
ChIP was performed as previously described (van Attikum et al., 2007). In brief, cells were grown overnight in YPAD, diluted in YPLGg and grown to mid-log phase. Glucose was added to a fraction of cells to repress HO, whereas galactose was added to the remainder of the cells to induce HO. Cells were fixed with formaldehyde and collected at 0 and 4 hours after addition of galactose (cells grown in the presence of glucose were collected at the 2-h time point). Extracts were prepared and subjected to ChIP using rabbit anti-RPA (Agrisera). For each time point and site, the normalized input ChIP signals were normalised to the input DNA signals. The h3E50G strain is slightly slower growing compared to WT therefore (cleavage efficiency in WT is ~95% at 4 hours whereas cleavage efficiency in h3E50G is closer to 80% at 4 hours), taking this into account all strains were normalised to WT cleavage efficiency.

3.7.7. Microscopy analysis to detect Rad52-YFP foci
Strains used for microscopy experiments were grown in -Leu minimal liquid medium (Formedium) overnight to have at a concentration of 3-4x10^6 cells/ml in the morning. Asynchronous cultures were pelleted and resuspended in YPD media and let cycle for 30 minutes. Asynchronous cultures were then prepared as described in G1 arrest and release experiment above. For microscopy analysis 5x10^6 cell/ml were pelleted and washed twice in sterile H_2O. Cells were resuspended in 10μl H_2O with 5μl of 2% low melting point agrose added to the tube. 5μl of cells were mounted on a slide. Live cell images were captured using
an an Olympus Fluoview 300 system (Centre for microscopy and imaging, NUIG). Images were captured with an inverted microscope enclosed in a temperature-controlled box at 30ºC. 100X oil objective was used to image yeast. For each field of cells, DIC and fluorescent images were taken. On average 15-25 Z-positions at 0.25 µm intervals were captured. Fluorescence illumination was at set at emission 543 nm for imaging YFP. To minimize the effect of lamp variability on the measurements, experiments involving wild-type and mutant strains were performed at the same time. Images were taken with Flouview software and all foci in images were counted manually. On average 150 cells were counted per time point per strain.

3.7.8. **Fluorescence Activated Cell Sorting (FACS)**

Approximately 1.5 – 4x10⁷ cells were harvested and resuspended in 2ml of cold 70% ethanol (70%). The pellet was resuspended and the samples were sonicated for 3-4 sec (20% amplitude). The cells were pelleted and washed twice with 50mM Tris 7.5. For the first wash, the cells were allowed to rehydrate by leaving them in Tris 7.5 for 30 min. After the second wash the cells were pelleted again and resuspended in 1ml Tris 7.5 containing 200mg/ml RNase A (RNase is boiled for 10 min to ensure that it does not contain DNAse). The samples were incubated at room temperature overnight on a rotor. Subsequently, the cells were spun down and the pellet was resuspended in 500µl of HCl containing pepsin (55mM HCl, 5mg/ml pepsin) and incubated at 37ºC for 30 min. The pellet following centrifugation was washed once with FACS buffer (200mM Tris pH 7.5, 211nM NaCl, 78mM MgCl₂) and resuspended in 500µl FACS buffer containing 0.055mg/ml Propidium Iodide). The cells can be stored frozen for weeks. Prior to analysis by FACS, the cells were defrosted and 50µl is added to 1ml of 50mM Tris pH 7.5 and the sample sonicated briefly. Buffers are filter sterilised. Samples were processed on a Becton Dickinson FACSCanto flow cytometer. Samples were analysed with FlowJo software.
3.8 Supplementary Data:

3.8.1. Supplementary Tables

Table S3.1 Strains used in this study

All strains used in this study are in the W303a background and are MATα, unless otherwise stated.

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Table S3.2: Plasmids used in this study

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<td>From Alain verreault</td>
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<td>Zhang et al 1998 EMBO J, 17:11, 3155-3167</td>
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### Table S3.3: Primers used in this study

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<tr>
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<tr>
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3.9 References


Han, J., Zhou, H., Li, Z., Xu, R.-M. and Zhang, Z. (2007). Acetylation of Lysine 56 of Histone H3 Catalyzed by RTT109 and Regulated by ASF1 Is


CHAPTER 4

A role for Rad9 CDK sites S494 and S618 in the DNA Damage Response
4.1. Summary

When damage to DNA is detected, a cell responds by arresting its cell cycle and carrying out repair processes. Failure to do this results in genomic instability, which can lead to the development of cancer. Cell cycle checkpoints monitor the genome for DNA damage to prevent the replication or segregation of damaged chromosomes. In *S. cerevisiae*, the checkpoint protein Rad9 plays a key role in the DNA damage response. Similar Rad9-like proteins are involved in the regulation of genomic integrity in human cells. Rad9 is phosphorylated in response to DNA damage in all phases of the cell cycle and is concomitant to Rad9 recruitment on damaged DNA. It is known that Rad9 is phosphorylated in a normal cell cycle mainly in the S, G2 and M phases by Clb forms of Cdc28 (Abreu et al., 2013; Vialard et al., 1998).

The Rad9 protein contains 20 putative (cyclin-dependent kinase) CDK phosphorylation sites. We have identified two of these sites to play an important role in the recovery of cells from the G2/M checkpoint. Here we show that CDK sites S494 and S618 are important for Rad9 cell cycle phosphorylation and when mutated, induce a significant delay in resuming from the DNA damage induced G2 cell cycle. Our study suggests that these two sites might be important for Rad9 function in Non Homologous End Joining (NHEJ).

4.2. Highlights

- Mutation of Rad9 CDK sites S494 and S618 to alanines results in abrogated Rad9 cell cycle phosphorylation.
- Rad9 CDK sites S494 and S618 are required for recovery from the G2/M checkpoint.
- rad9<sup>S494A</sup>, rad9<sup>S618A</sup>, rad9<sup>S494A+S618A</sup> cells survive after exposure to UV and IR.
- rad9<sup>S494A+S618A</sup> cells are not defective in resection of a HO induced DSB demonstrating these sites are not required for DNA resection.
- CDK sites S494 and S618 are required for efficient NHEJ repair.
4.3. Introduction

Rad9 is the prototypical checkpoint gene and was first identified by the pioneering genetic studies of Weinert and Hartwell in 1988 in *Saccharomyces cerevisiae*. Rad9 is a checkpoint protein involved in cell cycle arrest via a role in signal transduction of the checkpoint signal to downstream effector kinases. Structural and functional homologues of Rad9 include the *S. pombe* protein Crb2 and the human proteins BRCA1, 53BP1 and MDC1 (Mochan et al., 2004; Saka et al., 1997; Scully et al., 2004; Stucki and Jackson, 2004; Willson et al., 1997). Similar to Rad9, these proteins are phosphorylated during cell cycle and hyper-phosphorylated after DNA damage.

Rad9 is phosphorylated in the absence of DNA damage in a cell-cycle dependent manner. Rad9 is both cell cycle phosphorylated and hyper-phosphorylated in response to DNA damage (Vialard et al., 1998). Western blot analysis demonstrated that Rad9 is cyclically phosphorylated during cell cycle progression (Figure 4.1A; Abreu et al., 2013). Cells arrested in G1, S or G2/M phase of the cell cycle by α-factor, HU and nocodazole respectively, demonstrated that Rad9 is phosphorylated in S phase and G2/M phase cells as seen by the presence of slower migrating forms of Rad9 compared to faster migrating forms of Rad9 seen in G1 arrested cells (Figure 4.1B; Vialard et al., 1998). Treatment of these extracts with λ protein phosphatase resulted in the disappearance of these faster migrating forms of Rad9 detected in S and G2/M cells demonstrating that they are phospho-forms of Rad9 (Vialard et al., 1998).

This cell cycle phosphorylation is dependent on the cyclin dependent kinase, Cdc28 (Abreu et al., 2013). Cdc28 is a proline-directed serine/threonine kinase responsible for phosphorylating numerous substrates (Holt et al., 2009; Ubersax et al., 2003) which function in DNA replication, transcription, mitosis and the DNA damage response (Enserink and Kolodner, 2010). Cdc28 preferentially phosphorylates the strict consensus sequence [S/T]-P-X-[R/K] (X represents any amino acid), but it can also phosphorylate the minimal consensus sequence [S/T]-P (Endicott et al., 1999; Songyang et al., 1994). Rad9 contains 20 putative Cdk sites, of which 9 conform to the strict consensus. Of the 20 putative CDK
sites, mass spectrometry has identified phosphorylation of 16 CDK sites \textit{in vivo} (Abreu et al., 2013; Albuquerque et al., 2008; Holt et al., 2009; Smolka et al., 2005)\textsuperscript{126}Karen Finn Thesis, 2010). The number of potential CDK phosphorylation sites suggests important functions for these sites that remain to be fully determined.

Rad9 and its structural and functional homologues are known CDK targets which contain multiple CDK sites. Several studies have implicated specific CDK

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.1.png}
\caption{Rad9 is phosphorylated in an unperturbed cell cycle.}
(A) Asynchronous cells were arrested in G1 and released into the cell cycle. Rad9 phosphorylation was analysed by Western blot (Abreu et al., 2013). (B) Asynchronously growing cells were arrested in G1 with α-factor, S-phase with HU, or G2/M with nocodazole and the phosphorylation profile of Rad9 was analysed by western blot (Vialard et al., 1998) (C) Schematic of Rad9 showing the position and phosphorylation sites of the 20 putative CDK sites. (Note site 16 is ambiguous due to clustering of multiple S/T residues)
\end{figure}

Rad9 and its structural and functional homologues are known CDK targets which contain multiple CDK sites. Several studies have implicated specific CDK
sites in regulating distinct protein functions. In *S. pombe*, an N-terminal CDK site (T215) in fission yeast Crb2, has been implicated in checkpoint recovery (Esashi and Yanagida, 1999), checkpoint maintenance (Nakamura et al., 2005), DNA repair (Caspari et al., 2002) and the recruitment of Crb2 to DNA damage induce foci (Du et al., 2006).

In higher cells, 53BP1 protein is extensively phosphorylated by CDK (Linding et al., 2007). A study in human cells found that CDK1-dependent phosphorylation of 53BP1 S380 is required for efficient checkpoint recovery following IR-induced checkpoint activation (van Vugt et al., 2010). BRCA1 protein is also phosphorylated in a CDK dependent manner. CDK dependent phosphorylation on BRCA1 at sites S1497 and S1189/S1197 is important for efficient BRCA1 foci formation following cisplatin-induced damage (Johnson et al., 2009).

Studies performed in the Lowndes laboratory have investigated the role of CDK sites on Rad9 in cell cycle progression. One study generated a Rad9 mutant, *rad9*12CDK, in which 12 Rad9 CDK (Sites: S11A, S56A, S83A, T125A, T218A, T348A, S494A, S584A, S618A, S720A, S937A, S1136A) sites were mutated to non phosphorylatable alanine residues (Karen Finn Thesis 2010). This study revealed that although Rad9 cell cycle phosphorylation was almost completely abolished in this *rad9*12CDK mutant, these 12 CDK sites on Rad9 were not required for normal cell cycle progression. However, in response to genotoxic agents CDK-dependent phosphorylation of CDK S937 (CDK19) was required for efficient survival following MMS-induced damage (Karen Finn Thesis 2010).

A study from the Toczyski laboratory generated a *rad9*-18A mutant in which the 18 N terminal CDK sites on Rad9 were mutated to alanines at the RAD locus (Bonilla et al., 2008). Rad9 cell cycle phosphorylation was abrogated in the *rad9*-18A mutant but no cellular defects in cellular proliferation were reported (Bonilla et al., 2008).

Furthermore, the G2/M checkpoint is proficient in the *rad9*12CDK mutant as measured by Rad9 and Rad53 hyperphosphorylation, indicating that CDK-dependent phosphorylation of Rad9 might not be absolutely required for G2/M
checkpoint activation (Karen Finn Thesis 2010). Interestingly, the \textit{rad9-18A} mutant failed to hyperphosphorylate Rad9 and Rad53 after low doses of Zeocin but higher zeocin concentrations did result in hyperphosphorylation of Rad9 and Rad53 (Bonilla et al., 2008). However the \textit{rad9-18A} mutant exhibits significantly reduced protein levels, suggesting that these multiple mutations compromised protein stability and therefore may account for a defective checkpoint in \textit{rad9-18A} cells.

Another study conducted in the Lowndes laboratory concentrated on CDK sites located in the N terminus of Rad9, a region named the Chk1 Activation Domain (CAD) (Blankley and Lydall, 2004). This study identified T125 and T143 as 2 CDK sites on the CAD region of Rad9, which are important residues for Rad9 and Chk1 to interact (Ramesh Kumar Thesis 2012). Phosphorylation of T143 is the most important feature promoting Rad9/Chk1 interaction, while the much more abundant phosphorylation of the neighbouring T125 residue impedes the Rad9/Chk1 interaction (Abreu et al., 2013).

To date despite the knowledge of the existence of Rad9 CDK sites, the biological roles that these CDK sites are involved in are still not very well characterised. Our preliminary data on two consensus CDK sites in the central portion of Rad9, S494 and S618, indicate that these two sites function specifically in checkpoint recovery. Both CDK sites S494 and S618 on Rad9 are reported phosphorylated \textit{in vivo} (Albuquerque et al., 2008; Smolka et al., 2005). Although Rad9 cells mutated at both CDK sites S494 and S618 to alanines (\textit{rad9}^{S494A+S618A}) progress normally through the cell cycle, they are defective in checkpoint recovery as judged by persistent Rad9 and Rad53 hyperphosphorylation. This recovery defect is unlikely to be due to defective DNA repair as \textit{rad9}^{S494A+S618A} cells are not sensitive to UV and IR. In this chapter we investigate the role played by these two CDK sites in unperturbed and perturbed cell cycle conditions.
4.4. Results

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

The importance of CDK sites S494 and S618 in Rad9 cell cycle phosphorylation was identified by previous work in the Lowndes laboratory using strains overexpressing \textit{RAD9} from the \textit{GAL1} promoter on a centromeric plasmid and mutated for CDK consensus sites. Initially 9 CDK sites at the N terminus of the protein were eliminated by deletion of the first 223 amino acids of Rad9 generating a truncated Rad9 protein, \textit{Rad9\textsuperscript{ANT}}. \textit{Rad9\textsuperscript{ANT}} maintained a complex cell cycle pattern of phosphorylation indicating the presence of extensive Clb-Cdc28 phosphorylation. When four full consensus CDK sites, S494, S618, S720 and S1136, which are located in the middle and end of Rad9 were mutated to alanines and introduced, Rad9 cell cycle phosphorylation was almost completely abrogated (Figure 4.2A and B). To elucidate which of these CDK sites are involved in Rad9 cell cycle phosphorylation, single and combination mutations of these four CDK sites were introduced on the truncated Rad9 protein generating; \textit{Rad9\textsuperscript{S494\textsuperscript{A} NT}}, \textit{Rad9\textsuperscript{S618\textsuperscript{A} NT}}, \textit{Rad9\textsuperscript{S720\textsuperscript{A} NT}}, \textit{Rad9\textsuperscript{S1136\textsuperscript{A} NT}}, \textit{Rad9\textsuperscript{S494+S618\textsuperscript{A} NT}}, \textit{Rad9\textsuperscript{S494+S618+S720\textsuperscript{A} NT}} and \textit{Rad9\textsuperscript{S494+S618+S720+S1136\textsuperscript{A} NT}} strains.

WT G2 arrested cells overexpressing \textit{Rad9\textsuperscript{S494\textsuperscript{A} NT}} or \textit{Rad9\textsuperscript{S618\textsuperscript{A} NT}} from the \textit{GAL1} promoter on centromeric plasmids displayed defects in the pattern of Rad9 cell cycle phosphorylation that was not significantly exacerbated by mutating sites S720 and S1136 (Figure 4.2B). To eliminate the possibility that the defects we see are due to overexpression of Rad9 protein or due to a combined effect of the deletion of sites S494 and S618 along with deletion of N terminal CDK sites, residues S494 and S618 were mutated to alanines at the Rad9 locus, resulting in full length Rad9 mutated for both residues S494A and S618A and normally expressed from the \textit{RAD9} endogenous promoter.
Rad9 is cyclically phosphorylated in an unperturbed cell cycle (Figure 4.1A). (Note: Rad9 cell cycle phosphorylation is termed C-Rad9 in western blot figures, whereas DNA damage induced Rad9 phosphorylation is termed D-Rad9). A truncated N terminal Rad9 protein including mutations at CDK sites S494 and/or S618 showed abrogated Rad9 cell cycle phosphorylation in G2/M phase of the cell cycle. Therefore we hypothesised that Rad9 cell cycle phosphorylation would be slightly abrogated in G2/M phase of the cell cycle and possibly the other stages of the cell cycle where Rad9, mutated at sites S494 and/or S618

Figure 4.2: CDK sites S494 and S618 are required for Rad9 cell cycle phosphorylation in G2. (A) Schematic representing Rad9 CDK sites and the N terminal region, comprising of the first 223 amino acids which was eliminated. (B) Cells were transformed with yCplF16-Rad9 plasmid that contained Rad9 under the control of the galactose promoter. The Rad9 protein was deleted for the first 223 amino acids (NTRAD9). This was combined with single and combination mutations of four CDK sites. Work carried out by Jean Soulier.

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to alanines, is expressed from its endogenous promoter. Mutant strains where CDK sites 494 and S618 alone or together were mutated to alanines at the RAD9 locus were generated; and rad9S494A, rad9S618A and rad9S494A+S618A. To investigate our hypothesis, WT, rad9S494A, rad9S618A and rad9S494A+S618A cells were asynchronous or arrested in G1, S or G2/M phase of the cell cycle with α-factor, hydroxyurea (HU) or nocodazole respectively.

In asynchronous cells all mutants displayed subtle defects in Rad9 cell cycle phosphorylation (Figure 4.3B) (Note: lanes in figure 4.3B have been numbered 1-16 to refer to the specific lanes mentioned), with rad9S494A and rad9S494+S618A cells (lane 2 and 4) having slightly more abrogated modified Rad9 phosphorylation than WT or rad9S618A cells (lane 1 and 3). As expected, the defect in Rad9 phosphorylation is subtle indicating that more CDK sites along with S494 and S618 are involved in Rad9 cell cycle phosphorylation. Rad9 is not heavily phosphorylated in G1 arrested cells (Vialard et al., 1998). However phosphorylation has been observed at least in Rad9 N-terminus region in G1 arrested cells (Abreu et al., 2013). In both rad9S494A and rad9S494+S618 cells (lane 6 and 8), the Rad9 band appeared to be more compact than WT or rad9S618A cells (lane 5 and 7) (Figure 4.3B). This suggests that Rad9 is modified to some degree in G1 cells and that this modification is partially dependent on CDK site S494. In cells delayed in S phase (HU treated), rad9S494A cells (lane 10) displayed negligible differences in Rad9 phosphorylation compared to WT cells. Whereas in rad9S618A and rad9S494A+S618A cells (lane 11 and 12) the proportion of the fast to the slower migrating bands appeared lower compared to WT (Figure 4.3B). This indicates that both sites S494 and S618 are involved in Rad9 phosphorylation in S phase. In G2 arrested cells a more prominent faster migrating band was evident in rad9S494A and rad9S494A+S618A cells (lanes 14 and 16) relative to the pattern observed in WT and rad9S618A cells (lane 13). This illustrates the significant defect in Rad9 cell cycle phosphorylation in G2 arrested cells that is primarily dependent upon phosphorylation of CDK site S494. Rad9 phosphorylation was not dramatically affected as would be expected due to the large number of predicted CDK sites on Rad9. But these results show that mutation of S494 to an alanine affected the pattern of Rad9 phosphorylation observed in G1 and G2/M arrested cells.
We see Rad9 phosphorylation is slightly perturbed in rad9\textsuperscript{S494A} and rad9\textsuperscript{S494+S618A} cells arrested in different phases of the cell cycle. HU and nocodazole activate the replication and spindle assembly checkpoints, respectively (Koc et al., 2004; Wang and Burke, 1995) and Rad9 modifications which are observed in nocodazole arrested cells but not during an unperturbed cell cycle can be attributed to some extent to being a consequence of nocodazole-induced activation of the spindle assembly checkpoint (Clemenson and Marsolier-Kergoat, 2006). Therefore to study cell cycle phosphorylation in G2/M in the absence of nocodazole, asynchronous cells were arrested in G1 with \(\alpha\)-factor and released synchronously into the cell cycle (Figure 4.3C) (Lanes for figure 4.3C are labelled 1-16 and will be referred to in the following text). We predicted similar to cells arrested, we would also see defects in Rad9 phosphorylation during a normal unperturbed cell cycle. Similar to figure 4.1, wild-type cells in G1 displayed a compact Rad9 band by western blotting analysis (Figure 4.3C lanes 2-5 of WT). Cells in S phase display different phospho forms of Rad9 comprising of a slower migrating phospho form of Rad9 that displays stronger intensity with time and a faster less intense form of Rad9 (Figure 4.3C lanes 6 and 7 of WT). As cells passed through the G2/M phase this slower migrating form of Rad9 disappeared indicating that this form of Rad9 is dephosphorylated as cell exit mitosis. A similar Rad9 phosphorylation profile was observed in the subsequent cell cycle (Figure 4.3C lanes 8, 9 and 10 of WT).

Single mutations of S494 and S618 to alanines did not abrogate Rad9 cell cycle phosphorylation dramatically but strongly suggested that these sites have a role in Rad9 cell cycle phosphorylation induced mobility. Although the kinetics of appearance and disappearance of the cell cycle phosphorylation forms of Rad9 are similar in rad9\textsuperscript{S494A} cells, rad9\textsuperscript{S618A} cells and WT cells there some subtle differences in Rad9 cell cycle phosphorylation in these mutant strains. rad9\textsuperscript{S494A} cells are less extensively phosphorylated in G2/M compared to WT (Figure 4.3C). In particular, the two distinct forms of Rad9 observed in WT cells could not be observed in rad9\textsuperscript{S494A} cells but rather an intermediate Rad9 phospho form was detected (compare lanes 6 and 7). The phosphorylation pattern observed in rad9\textsuperscript{S618A} cells is most similar to WT cells but differences in the Rad9 phosphorylation pattern could be observed (compare lanes 6 and 7).
Interestingly, an obvious defect in Rad9 cell cycle phosphorylation is evident when both CDK sites, S494 and S618, are mutated (\(rad9^{S494A+S618A}\)) (Figure 4.3C). In G1 phase, \(rad9^{S494A+S618A}\) cells display a more compact Rad9 banding pattern compared to WT. This form of Rad9 persisted until 40 minutes after release from G1 block compared to 30 minutes for WT (lanes 1-6), indicating that cell cycle phosphorylation in these cells is delayed as they exit the G1 phase. FACS analysis confirmed a delay of \(rad9^{S494A+S618A}\) cells entering S phase. At time 30 minutes WT cells enter S phase where this is delayed by 10 minutes in \(rad9^{S494A+S618A}\) cells (Figure 4.3D). In \(rad9^{S494A+S618A}\) cells, Rad9 was phosphorylated to a lesser extent than in WT cells over two successive cell cycles, indicating that these cells have a significant defect in cell cycle dependent phosphorylation of Rad9 (Compare lane 7-9 and 10-16 in WT and \(rad9^{S494A+S618A}\) cells).

Rad9 consists of 20 putative CDK sites. When 12 or 18 of these CDK sites were mutated to alanines, Rad9 cell cycle phosphorylation was practically abolished (Karen Finn Thesis 2010; Bonilla et al., 2008). We identified just two sites that when mutated reduces the ability of Rad9 to carry out efficient cell cycle phosphorylation. One site S494 when mutated to an alanine subtly affects Rad9 phosphorylation. A double mutation of S494 and S618 to alanines notably perturbs Rad9 cell cycle progression and these cells are delayed on entering S phase, indicating these two sites are important for timely and extensive Rad9 phosphorylation during an unperturbed cell cycle.
Figure 4.3: Mutation of Rad9 CDK sites S494 and S618 mildly affects cell cycle progression.

(A) Rad9 CDK sites S494 and S618 were mutated to alanines to investigate if they are required Rad9 cell cycle phosphorylation. (B) Cells were arrested in the different phases of the cell cycle. Arrow in nocodazole arrested cells indicates the faster migrating Rad9 band. (C) Cells were arrested in G1 and released into the cell cycle. Rad9 cell cycle phosphorylation is mildly perturbed in rad9\textsuperscript{S494A} and rad9\textsuperscript{S494A+S618A} cells. (D) FACS analysis confirmed a delay in rad9\textsuperscript{S494A+S618A} cells on entering S phase.

<table>
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<tr>
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<th>G1</th>
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<th>G2/M</th>
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<tr>
<td>rad9\textsuperscript{S494}</td>
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<tr>
<td>rad9\textsuperscript{S618}</td>
<td>+ + +</td>
<td>+ + +</td>
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<td>+ + +</td>
</tr>
<tr>
<td>rad9\textsuperscript{S494A+S618A}</td>
<td>+ ±</td>
<td>+ ±</td>
<td>+ + ±</td>
<td>+</td>
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</table>

Table 4.1: Rad9 phosphorylation is perturbed in rad9\textsuperscript{S494}, rad9\textsuperscript{S618} and rad9\textsuperscript{S494A+S618} cells. In all panels, WT is represented by + + + with symbols less than this signifying the lack of Rad9 phosphorylation as cells are asynchronous and as they progress through the different stages of the cell cycle.
4.4.2. Rad9 CDK sites S494 and S618 are required for recovery from the G2/M checkpoint

Recovery from the G2 checkpoint is an important component of the DDR that reflects the ability of cells to delay mitotic entry in the presence of unrepaired DNA lesions (reviewed in Bartek and Lukas, 2007). In addition to their role in Rad9 cell cycle phosphorylation and cellular proliferation, phosphorylation on S494 and S618 may also be required for activation of and recovery from the DNA damage checkpoint. To investigate this we performed a checkpoint recovery assay. Asynchronous cells were arrested in G2 with nocodazole and either mock-treated or irradiated with 400Gy or treated with bleocin. IR and bleocin primarily cause DNA double strand breaks. Following irradiation cells were released into fresh media containing nocodazole and α-factor, to prevent them entering the next cell cycle (Figure 4.4A). Rad9 and Rad53 were analysed by western blotting. In WT cells Rad9 and Rad53 were hyperphosphorylated 15 minutes after damage with IR (Note: Rad9 hyperphosphorylation represented by D-Rad9 and phosphorylated Rad53 represented by P-Rad53) (Figure 4.4B). Rad9 hyperphosphorylation in these cells appeared to persist for slightly longer compared to WT although this was most prominent in rad9S494A+S618A cells (Figure 4.4B). Rad53 hyperphosphorylation in the single rad9S494A and rad9S618A mutants was similar to WT cells with Rad53 largely dephosphorylated by 45-60 minutes after damage. In the double rad9S494A+S618A mutant, Rad53 phosphorylation persisted for longer and remained phosphorylated for the duration of the experiment (Figure 4.4B). Thus rad9S494A+S618A cells exhibit a significant delay in the dephosphorylation of Rad53, suggesting that phosphorylation on both S494 and S618 is required before cells can exist the G2/M checkpoint arrest.

In all rad9S494A, rad9S618A and rad9S494A+S618A cells the DNA damage checkpoint was activated with similar kinetics to WT cells (Figure 4.4B). Rad9 hyperphosphorylation in these cells appeared to persist for slightly longer compared to WT although this was most prominent in rad9S494A+S618A cells (Figure 4.4B). Rad53 hyperphosphorylation in the single rad9S494A and rad9S618A mutants was similar to WT cells with Rad53 largely dephosphorylated by 45-60 minutes after damage. In the double rad9S494A+S618A mutant, Rad53 phosphorylation persisted for longer and remained phosphorylated for the duration of the experiment (Figure 4.4B). Thus rad9S494A+S618A cells exhibit a significant delay in the dephosphorylation of Rad53, suggesting that phosphorylation on both S494 and S618 is required before cells can exist the G2/M checkpoint arrest.
We also investigated checkpoint kinetics after bleocin treatment, a DNA damaging agent which induces single and Double strand break similar to IR (Chen and Stubbe, 2005). We observed that in response to bleocin damage, Rad53 was phosphorylated in WT and rad9^{S494A+S618A} cells 30 minutes after damage (Figure 4.4C). In WT cells Rad53 remained phosphorylated until 60 minutes post bleocin treatment, with the faster migrating form of Rad53 appearing at 90 minutes after damage, thus indicating deactivation of the DNA damage checkpoint. In contrast to WT, Rad53 remained phosphorylated in rad9^{S494A+S618A} cells until the end of the experiment (Figure 4.3C) indicating that phosphorylation on both S494 and S618 is required for checkpoint recovery in response to DSB damage.

Rad53 and Chk1 both act in two distinct, additive and parallel pathways (Sanchez et al., 1999). Rad9 is the activator of both of these downstream kinases (Abreu et al., 2013; Blankley and Lydall, 2004; Gilbert et al., 2001; Sanchez et al., 1999; Schwartz et al., 2002; Sun et al., 1996; Sweeney et al., 2005; Vialard et al., 1998). Chk1 and Rad53 function in a parallel pathway through Pds1 and Cdc5, respectively to prevent anaphase entry and mitotic exit after DNA damage (Agarwal et al., 2003; Cheng et al., 1998; Sanchez et al., 1999). Chk1 phosphorylation promotes proteasome destruction of Pds1, preventing passage through mitosis (Agarwal et al., 2003; Sanchez et al., 1999). In contrast, Rad53 maintains CDK activity during the checkpoint response by inhibiting Polo-like kinase Cdc5 (Sanchez et al., 1999).

The N terminus of Rad9 comprises of the Chk1 Activation Domain (CAD), corresponding to the first 231 amino acids of the protein (Blankley and Lydall, 2004). The CAD region is required for Rad9-dependent activation of Chk1 (Blankley and Lydall, 2004) and specific CDK sites on Rad9 are necessary for Chk1 activation in response to DNA damage (Abreu et al., 2013). We wanted to investigate Chk1 phosphorylation in rad9^{S494A+S618A} cells to determine if CDK sites S494A and S618A are specifically defective in Rad53 molecular mechanisms or if these mutations are also defective in Chk1 molecular mechanisms. If rad9^{S494A+S618A} cells were defective in both Chk1 and Rad53
checkpoint mechanisms this would argue against these sites being specifically required for a Rad53 checkpoint molecular mechanism.

To investigate if \textit{rad9}^{S494A+S618A} cells are defective in both Chk1 and Rad53 activation, Chk1 was tagged with a 3-HA tag at the C terminus to investigate if Chk1 phosphorylation is abrogated in \textit{rad9}^{S494A+S618A} cells. In WT cells Chk1 is phosphorylated following bleocin treatment and remains phosphorylated up to 30 minutes post bleocin treatment. At 60 minutes residual Chk1 phosphorylation is evident but at 90 minutes Chk1 phosphorylation is back to basal levels (Figure 4.4D). Chk1 is activated in \textit{rad9}^{S494A+S618A} cells immediately after bleocin, although Chk1 activation does not appear as strongly activated in \textit{rad9}^{S494A+S618A} cells as in WT cells. Chk1 remains phosphorylated until 45 to 60 minutes after bleocin with some residual Chk1 phosphorylation being still present at 90 and 120 minutes after damage (Figure 4.4D). Chk1 is not dephosphorylated as quickly as WT indicating a Chk1 G2/M checkpoint recovery defect exists after bleocin. Taken together, these results indicate that phosphorylation is required on S494 and S618 for a general checkpoint recovery mechanism and not a specific Rad53 checkpoint molecular mechanism.
**Figure 4.4:** *rad9<sup>S494A+S618A</sup>* cells have a G2/M checkpoint recovery defect.

(A) Experimental layout of checkpoint kinetics experiment. Cells were arrested in G2/M and damaged with IR or bleocin and released into fresh media containing nocodazole and α-factor. (B) *rad9<sup>S494A+S618A</sup>* cells activate the DNA damage checkpoint but have prolonged checkpoint activation as measured by Rad9 and Rad53 phosphorylation after IR and bleocin treatment compared to WT. (D) Rad53 and its parallel kinase, Chk1 both have prolonged checkpoint activation after bleocin treatment.
4.4.3. *rad9<sup>S494A+S618A</sup>* cells are proficient in repair and DNA resection.

Homologous recombination (HR) and non-homologous end joining (NHEJ) are two principal mechanisms of repair which evolved to deal with DSBs and hence maintain the genomic integrity of the cell. DSBs generated in G1 are not repaired by HR but by NHEJ (Aylon et al., 2004), whereas HR is active in S and G2 phases of the cell cycle. Clb-CDK activity is essential for recombinational repair of DSBs and the choice of repair pathway is determined by CDKs (Aylon et al., 2004; Ira et al., 2004a). Cells in which Cdc28 activity is inhibited failed to resect the DNA ends efficiently due to an inefficient HR repair process but instead channelled repair of the DSB into the NHEJ pathway, promoting a G1-like response CDKs (Aylon et al., 2004; Ira et al., 2004b). Therefore we investigated if CDK sites S494 and S618 are required for efficient HR.

Drop test analysis was performed to determine if CDK sites S494 and S618 are important for DNA repair after IR and UV damage. WT cells formed colonies on YPD plates exposed to UV and IR. *rad51Δ* cells which are defective in HR, failed to form colonies on YPD plates after chronic exposure to IR and UV. *yku70Δ* cells which are defective in NHEJ formed colonies after acute doses of IR, but colony formation of cells was greatly impaired after exposure to UV. This is consistent reports that NHEJ mutants are not sensitive to IR that induces DSB (Manolis et al., 2001; Siede et al., 1996) and that a *yku70Δ* mutant experiences increased IR sensitivity only in the absence of a functional HR pathway (Boulton and Jackson, 1996b). Rad9 and Mec1 are two DNA damage checkpoint proteins whose deletion results in sensitivity to acute doses of both IR and UV. All three mutants *rad9<sup>S494A</sup>, rad9<sup>S618A</sup> and *rad9<sup>S494A+S618A</sup>* behaved similarly to WT after exposure to IR and UV damage (Figure 4.5A). *rad9<sup>S494A</sup>, rad9<sup>S618A</sup> and *rad9<sup>S494A+S618A</sup>* do not exhibit the IR and/or UV sensitivity observed in any of the control strains, indicating that they are not defective in DNA repair after exposure to IR or UV. This would suggest that CDK sites S494 and S618 are not required for HR, as HR is the primary repair mechanism for IR induced damage.
The endonuclease Sae2 has been identified as a CDK target (Clerici et al., 2005; Lengsfeld et al., 2007) whose CDK-dependent phosphorylation promotes DNA-end resection, thus channelling repair into HR (Huertas et al., 2008). CDK-dependent phosphorylation on site S267 of Sae2 during S and G2 phase is required for proper DNA-end resection and HR (Huertas et al., 2008). Rad9 also functions in resection by inhibiting the accumulation of ssDNA at DSBs at uncapped telomeres (Booth et al., 2001; Jia et al., 2004; Lazzaro et al., 2008; Lydall and Weinert, 1995). Therefore, we investigated if resection was the reason for a prolonged checkpoint activation in rad9<sup>S494A+S618A</sup> cells. rad9<sup>S494A+S618A</sup> cells exhibit a G2/M recovery defect therefore the checkpoint may be activated for longer if DNA end resection is delayed in these cells.

To investigate if sites S494 and S618 are important for resection we investigated the kinetics of ssDNA formation after a single unrepairable DSB in rad9<sup>S494A+S618A</sup> cells, using an inducible HO endonuclease. Plasmids harbouring wild-type RAD9 and rad9<sup>S494A+S618A</sup> were transformed separately into a rad9Δ Galactose inducible HO endonuclease conditional strain to produce rad9Δ<pRAD9> and rad9Δ<rad9<sup>S494A+S618A</sup> > strains respectively. Cells were arrested in G2 with nocodazole to prevent cell cycle dependent effects on resection and samples collected at various time points after induction of the HO endonuclease break. rad9Δ<rad9<sup>S494A+S618A</sup> > (rad9<sup>S494A+S618A</sup>) cells are not defective in resection of an HO induced DSB, the kinetics of DSB resection are similar in WT (rad9Δ<pRAD9>) and mutant cells (rad9Δ<rad9<sup>S494A+S618A</sup> >) (Figure 4.5B). This indicates that the checkpoint recovery phenotype in rad9<sup>S494A+S618A</sup> cells is not associated with the persistence of unresected DNA.
Figure 4.5: CDK sites S494 and S618 are not required for survival after DNA damage or DNA resection. (A) Asynchronous cells were arrested in G2 with nocodazole. *rad9*<sup>S494</sup>, *rad9*<sup>S618</sup>, *rad9*<sup>S494+618</sup> cells all formed colonies after IR and UV damage and are comparable to WT. (B) Mutations of S494 and S618 to alanines has no effect on resection of a HO induced DSB.
4.4.4. Rad9 CDK sites S494 and S618 contribute to an efficient NHEJ repair process.

DNA resection is proficient in rad9\textsuperscript{S494A+S618A} cells but these mutant cells are unable to recover from a G2/M checkpoint response. Rad9 is reported to play a role in promoting efficient repair of DSBs via the NHEJ pathway (de la Torre-Ruiz and Lowndes, 2000). Therefore we proceeded to investigate if NHEJ repair, is proficient in rad9\textsuperscript{S494A+S618A} cells. HR is the principal repair mechanism in S. cerevisiae but NHEJ is active in the absence of proteins catalysing HR or in the absence of homology between linear plasmid molecules and the host genome (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; Siede et al., 1996). We used an in vivo plasmid re-joining assay to assess the role of CDK sites S494 and S618 in NHEJ repair (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; de la Torre-Ruiz and Lowndes, 2000). To do this, an S. cerevisiae-E. coli shuttle plasmid (pRS315) was linearised with BamHI enzyme to produce 5’–3’ overhangs. The linearised and supercoiled versions of the plasmid were transformed into WT, yku80Δ and mutant strains. Linearised plasmids can only propagate in S. cerevisiae after they have been re-circularised and ligated. Therefore by counting the number of transformants obtained with the digested plasmid normalised to the number of transformants recovered with the undigested plasmid we can measure the efficiency of ligation and NHEJ can be measured (Figure 4.6A; Boulton and Jackson, 1996b).

yku80Δ cells and rad9Δ cells were used as a control. Deletion of YKU80, which encodes a protein required for NHEJ (Boulton and Jackson, 1996a) resulted in 8.4% NHEJ repair occurring. Deletion of RAD9, which is reported to have a 4–5 fold decrease in NHEJ repair, (de la Torre-Ruiz and Lowndes, 2000) resulted in 41% NHEJ occurring (Figure 4.6B). Differences in results presented here and previous reports published may be due to variations in growth conditions, but overall the same pattern of defective NHEJ in yku80Δ cells and rad9Δ cells exist. In rad9\textsuperscript{S494A+S618A} cells 75% NHEJ occurred significantly higher than rad9Δ cells but still not as efficient as WT cells (Figure 4.6B). Intriguingly, a rad9\textsuperscript{CDK12A} mutant where 12 CDK sites (including sites S494 and S618) on Rad9 have been mutated to alanines (Karen Finn Thesis 2010) resulted in 91.5% of NHEJ
occurring which is significantly higher than the \textit{rad9}^{S494A+S618A} cells, suggesting that some level of redundancy exists between the CDK sites on Rad9. Overall \textit{rad9}^{S494A+S618A} cells are slightly defective in NHEJ repair but not as defective as \textit{rad9}\Delta cells suggesting more CDK sites are involved in NHEJ repair process.

\textbf{Figure 4.6: Partial decreased efficiency of non homologous end joining in \textit{rad9}^{S494A+S618A} cells.} (A) Schematic of assay used to measure NHEJ in WT and mutant cells. (B) Mutation of CDK sites S494 and S618 on Rad9 results in partial NHEJ defects.

\textbf{4.5. Discussion}

Rad9 is a critical component of the DNA damage response, particularly during the G2/M phase of the cell cycle when CDK activity is high (Ira et al., 2004a; Weinert and Hartwell, 1988). Studies have implicated Rad9 in several DNA repair pathways, including homologous recombination, non-homologous end joining, nucleotide excision repair and post-replication repair (Aboussekhra et al., 1996; Al-Moghrabi et al., 2009; Barbour et al., 2006; Conde et al., 2009; de la Torre-Ruiz and Lowndes, 2000; Lazzaro et al., 2008; Murakami-Sekimata et al., 2010). A single DNA DSB in yeast is sufficient to induce a DNA damage response in a Rad9-dependent manner, illustrating how highly sensitive this signal transduction system is (Harrison and Haber, 2006). The exact mechanisms
of how Rad9 mediates these checkpoint responses remains to be elucidated. Rad9 and its structural and functional homologues are CDK targets (Albuquerque et al., 2008; Esashi and Yanagida, 1999; Linding et al., 2007). Rad9 is extensively phosphorylated in S and G2/M phases of the cell cycle and this cell cycle phosphorylation is dependent upon CDK-Clb activity (Abreu et al., 2013; Vialard et al., 1998). The extent of the roles played by these CDK sites in cell cycle regulation of Rad9 is not fully understood.

We identified two CDK sites on Rad9, S494 and S618, which are required for efficient Rad9 cell cycle phosphorylation. Deletions of multiple CDK sites on Rad9 have abolished Rad9 cell cycle phosphorylation, but these studies mutated over half of the 20 putative CDK sites on Rad9 which will account for a significant loss of CDK phosphorylation (12 CDK sites mutated; Karen Finn Thesis 2010) or (18 CDK sites mutated; Bonilla et al., 2008). Rad9 cell cycle phosphorylation was significantly abrogated when just two CDK sites, S494 and S618 were mutated to alanines together (Figure 4.2B). Either deletion did not result in a considerable defect in Rad9 cell cycle phosphorylation (Figure 4.2C). This would indicate although additional CDK sites to S494 and S618 are important for Rad9 cell cycle phosphorylation, these two sites are significant in maintaining CDK dependent regulation of Rad9 during a normal cell cycle. No defects in cellular proliferation in rad9S494A+S618A cells exist although this is not surprising as RAD9 null cells are viable without noticeable growth defects. Additionally cell cycle progression was not perturbed in a rad912CDK mutant or a rad9-18A mutant (Karen Finn Thesis 2010; Bonilla et al., 2008). These observations show sites S494 and S618 are important in the phosphorylation of Rad9 during the cell cycle. They also support data that CDK activity does not play a role in the regulation of Rad9 function during an unperturbed cell cycle.

Studies have been performed on the roles of CDK sites on Rad9 in the DDR but our understanding of the full extent that these sites play in the DDR remains to be fully elucidated (Abreu et al., 2013; Bonilla et al., 2008; Wang et al., 2012). Rad9 contains 20 putative CDK sites and it is believed that a great deal of redundancy exists between the CDK sites. However, some studies have identified certain sites on Rad9 that are important for Rad9 regulation. Rad9
CDK site S937 was found to be required for efficient survival following MMS-induced damage (Karen Finn Thesis 2010). CDK sites located on Rad9 promote Dpb11-dependent recruitment of Rad9 to sites of damage leading to its PIKK-dependent phosphorylation and CDK-dependent phosphorylation of S11 on Rad9 plays an important role in regulating this Dpb11 and Rad9 interaction (Granata et al., 2010). In addition to this single CDK sites in the CAD region of Rad9 are important for Rad9 and Chk1 interaction after DNA damage (Abreu et al., 2013).

In this study we identified two CDK sites out of the 20 putative sites on Rad9 which mediate exit from the G2/M checkpoint. Rad53 and Chk1 remained phosphorylated for longer in rad9^{S494A+S618A} cells compared to WT after IR and Bleocin damage (Figure 4.4B and C). Rad53 kinase plays an essential role in the regulation of the G2/M checkpoint by phosphorylating various substrates involved in cell cycle progression and DNA damage repair. Rad53 phosphorylation is sufficient to arrest the cell cycle and dephosphorylation of this kinase leads to recovery after genotoxic stress (Keogh et al., 2006; Leroy et al., 2003; Tercero and Diffley, 2001). rad9^{S494A+S618A} cells exhibit a significant delay in the dephosphorylation of Rad53 suggesting that phosphorylation on both sites is required before cells can exist the G2/M checkpoint arrest.

Chk1, the parallel kinase to Rad53 was phosphorylated immediately after bleocin damage but to a lesser extent than WT cells (Figure 4.4C and D). Chk1 phosphorylation is evident until the end of the experiment signifying that rad9^{S494A+S618A} cells are defective in both Chk1 and Rad53 checkpoint mechanisms. This data indicates that sites S494 and S618 are not just involved in a Rad53 molecular mechanism but rather a general checkpoint recovery mechanism. The persistent checkpoint activation suggests that phosphorylation on both serines is necessary to allow the timely resumption of the cell cycle following the DNA damage induced checkpoint.

Checkpoint activation is not affected in rad9^{S494A+S618A} cells but cells fail to turn off the checkpoint as seen by persistent Rad53 phosphorylation (Figure 4.4B and C). Downstream of checkpoint activation repair occurs to maintain genomic integrity and allows cells re enter the cell cycle. When rad9^{S494A+S618A} cells were
exposed to IR and UV, repair occurred with similar kinetics to WT as tested by drop test analysis, indicating delays in checkpoint deactivation are not due to defects in repair (Figure 4.5A). Rad9 functions in resection by inhibiting the accumulation of ssDNA at DSBs at uncapped telomeres (Booth et al., 2001; Jia et al., 2004; Lazzaro et al., 2008; Lydall and Weinert, 1995). Therefore we investigated if resection was the reason for a prolonged checkpoint activation in rad9S494A+S618A cells. However rad9S494A+S618A cells are not defective in resection (Figure 4.5B), indicating the defect in checkpoint recovery after DSBs is not likely associated with the persistence of unresected DNA. These results suggested sites S494 and S618 play a role in another principal pathway, NHEJ. Rad9 is required for an efficient NHEJ response (de la Torre-Ruiz and Lowndes, 2000), so we investigated NHEJ repair in rad9S494A+S618A cells. NHEJ is slightly defective in rad9S494A+S618A although not as defective as RAD9 null mutants (Figure 4.6B). Phosphorylation of sites on S494 and S618 may be required for an efficient NHEJ process but it is clear that other factors are involved and that these sites may play a redundant role. We have found that the defect observed in rad9S494A+S618A cells for NHEJ it is not sufficient alone to account for the defects we observed in deactivation of the DNA damage checkpoint.

Our evidence suggests that phosphorylation on sites S494 and S618 possibly by Cdc28 is required to deactivate the DNA damage checkpoint. If phosphorylation on these sites is absent repair of the damage is not sufficient for cells to resume cycling. These phenotypes may be due to modifications of sites S494 and S618 or rather these mutations may have disrupted a surface of Rad9 protein that may be involved in checkpoint recovery. Altogether the process of phosphorylation of certain CDK sites and the roles they play in the DNA damage response remains complex and remains to be determined the exact roles played by Rad9 CDK sites.

4.6. Conclusion and Future work
Rad9 is phosphorylated during the normal cell cycle and this modification is dependent on Clb forms of Cdc28 (Abreu et al., 2013). We find two sites (S494 and S618) on Rad9 are important in cell cycle regulation and in checkpoint regulation. Cell cycle progression is only subtly affected when sites S494 and
S618 are mutated to alanines and this does not cause considerable defects in cell cycle progression.

Checkpoint recovery is defective in rad9^{S494A+S618A} cells as measured by persistent Rad53 and Chk1 phosphorylation. This illustrates sites S494 and S618 are required for a general checkpoint recovery response and not one that is specific to a Rad53 molecular mechanism. Although defective in turning off the G2/M checkpoint, rad9^{S494A+S618A} cells appear to be proficient in checkpoint activation and repair. rad9^{S494A+S618A} cells are not defective for DNA resection and they survive on YPD plates after exposure to IR and UV. If rad9^{S494A+S618A} cells are repaired efficiently another possibility is that phosphorylation on S494 and S618 by Cdc28 is required to turn off the checkpoint and repair of the damage is not sufficient for cells to resume cycling. Following completion of DNA repair, Cdc28 may phosphorylate Rad9 on S494 and S618. This may reduce Rad53 activity, alleviating the Rad53-dependent checkpoint signal and leading to exit from the G2/M checkpoint.

Additionally, we cannot rule out that sites S494 and S618 function in adaptation. A process related to, but conceptually distinct from, checkpoint recovery is checkpoint adaptation. Adaptation is a process where cells divide following a sustained checkpoint imposed cell cycle arrest despite the presence of persistent DNA damage (Bartek and Lukas, 2007). One kinase which is known to function in adaptation is Cdc5 (Toczyski et al., 1997) We cannot rule out the possibility that sites S494 and S618 are modified by another kinase other than Cdc28, such as Cdc5.

Different DNA damaging agents produce various types of lesions. Survival of a Rad9 CDK mutant rad9^{S937A} (CDK site S937 mutated to an alanine) was comparable to WT following IR and UV induced damage (Karen Finn Thesis 2010). But this mutant, rad9^{S937A}, was highly sensitive to MMS damage (Karen Finn Thesis 2010). Therefore we need to investigate if rad9^{S494A+S618A} cells are sensitive to DNA damaging agents that generate other types of lesions rather than lesions produced by IR. This would indicate if sites S494 and S618 are
required for just a G2/M checkpoint response of if they are required in other processes such as repair during replication.

Another possibility for prolonged checkpoint activation in rad9\textsuperscript{S494A+S618A} cells, is the persistence of lesions after damage is induced resulting in the G2/M checkpoint being activated for longer. We would need to take time points after 120 minutes to investigate if and when the G2/M checkpoint is deactivated in rad9\textsuperscript{S494A+S618A} cells. Cells exposed to damage and spotted on drop tests may be experiencing delays in repair but this may not be evident if the delay is for an intermediate time after which the damage is resolved and the DNA damage checkpoint deactivated. One approach to this would be to investigate Rad52 foci in rad9\textsuperscript{S494A+S618A} cells after damage. Persistence of Rad52 foci would indicate the persistence of HR lesions that are not resolved as timely as WT cells.

We believe that sites S494 and S618 are involved in a regulatory mechanism that involves these CDK sites of Rad9. Rad9 contains 20 putative CDK sites therefore it is likely extensive phosphorylation of a critical number of sites is needed for certain cellular functions. However we provide evidence that just two sites out of the 20 putative CDK sites on Rad9 are involved in a particular cellular function which is required for cells to recover from a G2/M induced DNA damage checkpoint. Altogether the process of phosphorylation on certain CDK sites and the roles they play in the DNA damage response remains complex and much remains to be determined of the exact roles played by Rad9 CDK sites.

4.7. Materials and Methods

4.7.1. Media and growth conditions

Yeast cells were grown in YPD liquid (1% (w/v) yeast extract (Difco), 2% (w/v) bactopeptone (Difco), 2% (w/v) glucose (Difco)) or -Leu minimal liquid medium (Formedium). Yeast cells grown on solid plates were grown on YPD media containing a plus of 2% (w/v) agar (Difco). Solid selective media was YNB (yeast nitrogen base) with 2% agar and the required amino acids (W303 requires
histidine, leucine, tryptophan, uracil and adenine) with 2% glucose as the carbon source.

Cells were grown in conical flasks with liquid media at 30°C in a shaker incubator at 170 r.p.m. The volume of culture did not exceed one third of the nominal flask volume to ensure adequate aeration. Cells that were grown in plates of YPD solid media were incubated at 30°C and depending on strain growth were left grow for 2-3 days.

4.7.2. Genotoxic treatments
γ-irradiation (400 Gy) was carried out using a 137 Cs at a dose-rate of 12.10 Gy/min (Mainance Engineering, UK). The UV irradiation rate (J/m²/s) from the lamp was measured before each irradiation, using a UV dosimeter, and the time of exposure was calculated (time = desired dose (50J) / UV intensity (J/m²/s)). Bleocin (Calbiochem) was used at 2.5 μg/ml and 10 μg/ml.

4.7.3. Drop test methodology
DNA damage sensitivity analysis was performed by spotting five-fold serial dilutions of exponentially growing (5x10⁶) cultures of the indicated strains on plates containing the indicated genotoxic agents. Plates were incubated in the 30°C incubator unless otherwise specified for two days before scanning.

4.7.4. Strains used in this study
All yeast strains used in this study are made in W303-1a background unless otherwise stated and are listed in table S4.1. Yeast strain and plasmid constructions are described below. Plasmids and oligonucleotides used in this study are in Tables S4.2 and S4.3 respectively.

4.7.4.1. Chk1 tagged strains
To C terminally tag Chk1 with 3HA, Chk1-DiaF and Chk1-DiaR, primers were used to amplify 1.5Kb of CHK1-3HA-KIURA3 DNA cassette from genomic DNA of strain YNL1144 (Clerici et al., 2004). Wild-type and mutant yeast strains were transformed with the CHK1-3HA-KIURA3 cassette and selected for growth on plates lacking uracil. The putative transformants were screened for the
expression of Chk1-3HA by western blot analysis and the correct integration of the CHK1-3HA allele at the Chk1 locus was verified by diagnostic PCR.

4.7.4.2. Generation of rad9<sup>S494A</sup>, rad9<sup>S618A</sup> and rad9<sup>S494A+5618A</sup> strains

To generate point mutations in Rad9, the stratgene Quickchange site-directed mutagenesis kit (Catalog no. 200518) was used as per manufacturers recommendations. Primers designed were approximatley 30bp in length containing the mutation of interest in the middle of the sequence. The reverse primer was the complementary sequence of the forward sequence. Plasmids were checked by diagnostic digestion and mutations confirmed by sequencing prior to transforming yeast (Aisling O’Shaugnessy Thesis 2006). For integration following transformation, the plasmids were linearised with PshA1. Following integration, the URA3 marker was removed by streaking cells on FOA (1 mg/ml). Each step was checked by diagnostic PCR and sequencing (Aisling O’Shaugnessy Thesis 2006).

4.7.5. Cell cycle and checkpoint experiments

4.7.5.1 G1 arrest and release experiment.

Asynchronous, exponentially growing cells (5x10<sup>6</sup> cells/ml) were arrested in G1 by addition of α-factor (5 µg/ml final concentration) for 95 minutes. Following arrest, cells were washed with pre-warmed 0.9% saline, followed by YPD, and then resuspended in fresh media. Samples were taking for western blot and FACS as previously described (O’Shaughnessy et al., 2006).

4.7.5.2. G2 arrest and release.

G2/M checkpoint analysis was performed as previously reported (O’Shaughnessy et al., 2006). Asynchronous, exponentially growing cells (5x10<sup>6</sup> cells/ml) were arrested in G2 by addition of nocodazole (10 µg for 90 minutes). Following arrest cells were treated with genotoxic agent, cells were washed with pre-warmed 0.9% saline, followed by YPD, and then resuspended in fresh media containing nocodazole (10 µg/ml) and of α-factor (5 µg/ml final). Synchronised cells were either mock treated or treated with 400 Gy or bleocin and released into medium containing nocodazole (10 µg/ml final concentration) and α-factor (7.5 µg/ml final concentration) to trap cycling cells in the subsequent G1 phase.
Samples were taken for western blot and FACS as previously described (O'Shaughnessy et al., 2006).

4.7.6. Western blotting and antibody conditions
Immunoblotting was performed as previously described (O'Shaughnessy et al., 2006; Vialard et al., 1998). Yeast whole cell extracts were prepared as described in (Kushnirov, 2000). Rad9 and Rad53 proteins were resolved in 6.5%, 80/1 acrylamide/bis-acrylamide, SDS-PAGE gel and probed with NLO5 (O'Shaughnessy et al., 2006; Vialard et al., 1998) and NLO16 (O'Shaughnessy et al., 2006; Vialard et al., 1998) at 1:10000 dilution. Chk1-3HA was resolved in a 10%, 80/1 80/1 acrylamide/bis-acrylamide, SDS-PAGE gel and probed with anti HA (12CA5) 1:5000 dilution. HRP-conjugated anti-rabbit secondary antibody (from Pierce) or anti-mouse (from Pierce) and super signal WestPico chemiluminescent substrate from Thermo Scientific (Product no. 34080) was used to detect the proteins.

4.7.7. Non homologous End-joining ligation assay
Asynchronous cells growing in –Leu minimal medium were transformed in parallel with either 250 ng of the centromeric plasmid pRS315 digested to completion with BamH1 or with 250 ng of undigested pRS315. The values plotted as % repair efficiency correlate to the number of transformants obtained with the digested plasmid normalised to the number of transformants recovered with the undigested plasmid. This value was then normalised to the value obtained with WT which was then assigned a value of 100%. Six independent transformations were assayed.

4.7.8. DNA Resection assay
Plasmids harbouring wild-type RAD9 and rad9^{S494A+S618A} were transformed separately into a rad9Δ Galactose inducible HO-endonuclease conditional strain to produce rad9Δ<pRAD9> and rad9Δ< rad9^{S494A+S618A}> strains respectively. Cells were arrested in G2 with nocodazole to prevent cell cycle dependent effects on resection and samples were collected at various time points after induction of the HO endonuclease break.
Approximately $1.5 - 4 \times 10^7$ cells were harvested and resuspended in 2 ml of cold 70% ethanol (70%). The pellet was resuspended and the samples were sonicated for 3-4 sec (20% amplitude). The cells were pelleted and washed twice with 50 mM Tris pH 7.5. For the first wash, the cells were allowed to rehydrate by leaving them in Tris pH 7.5 for 30 min. After the second wash the cells were pelleted again and resuspended in 1 ml Tris 7.5 containing 200 mg/ml RNAse A (RNAse is boiled for 10 min to ensure that it does not contain DNAse). The samples were incubated at room temperature overnight on a rotor. Subsequently, the cells were spun down and the pellet was resuspended in 500 µl of HCl containing pepsin (55 mM HCl, 5mg/ml pepsin) and incubated at 37°C for 30 min. The pellet following centrifugation was washed once with FACS buffer (200 mM Tris pH 7.5, 211 nM NaCl, 78 mM MgCl$_2$) and resuspended in 500µl FACS buffer containing 0.055 mg/ml Propidium Iodide). The cells can be stored frozen for weeks. Prior to analysis by FACS, the cells were defrosted and 50 µl is added to 1 ml of 50 mM Tris pH 7.5 and the sample sonicated briefly. Buffers are filter sterilised. Samples were processed on a Becton Dickinson FACSsort cytometer equipped with CELLQuest software.
4.8 Supplementary Data:

4.8.1. Supplementary Tables

Table S4.1 Strains used in this study
All strains used in this study are in the W303a background and are MATα, unless otherwise stated

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<td>RAD5+</td>
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<td>RAD5 rad9S494A</td>
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<td>O’Shaughnessy Thesis 2006</td>
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<td>RAD5+ rad9D2CDK</td>
<td>RAD5 rad91,3,4,6,9,11,14,15,16,17,18,19,20A</td>
<td>Finn Thesis 2010</td>
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S4.2 Plasmids used in this study

<table>
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<th>Reference</th>
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<tr>
<td>pRS315</td>
<td>Used in NHEJ assay</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
</tbody>
</table>
4.9 Reference


Wang, Y. and Burke, D. J. (1995). Checkpoint genes required to delay cell division in response to nocodazole respond to impaired kinetochore function in the yeast Saccharomyces cerevisiae. Molecular and Cellular Biology 15, 6838-44.


CHAPTER 5

Conclusions and Future Directions
5.1 Chromatin and its vital role in the DDR

Chromatin structure and modifications play critical roles in the ability of eukaryotic cells to sense and carry out repair processes in response to chromosomal breaks. To maintain genomic integrity, cells execute a coordinated series of events that regulate cell cycle progression and repair in response to DNA lesions (Ciccia and Elledge, 2010; Harrison and Haber, 2006). The highly ordered packaging of chromatin hinders the ability of proteins to gain accesses to lesions so sophisticated mechanisms are required to modulate chromatin structure and increase the accessibility of repair machinery to lesions which occur in this chromatin environment.

The structure of chromatin can be altered by ATP-dependent chromatin remodeling complexes which use ATP hydrolysis to facilitate nucleosome sliding, nucleosome disruption and exchange of histone components. Initially these ATP-chromatin remodelling complexes were identified in transcriptional regulation but more recently some of these complexes have been implicated in DNA repair (Clapier and Cairns, 2009). Chromatin remodellers, such as SWI/SNF, RSC, SWR or INO80, have an important role in DNA metabolism and are essential to cellular mechanisms protecting the genome. The RSC chromatin remodelling complex is rapidly recruited sites of damage where it functions in both NHEJ and HR repair pathways (Chai et al., 2005; Shim et al., 2007). Early chromatin remodelling events mediated by RSC also facilitate the PIKK-dependent phosphorylation of H2A on S129 (Liang et al., 2007; Shim et al., 2007), one of the earliest modifications following a DSB (Downs et al., 2000). Following a DSB in S. cerevisiae, phosphorylation of H2A on S129 (γ-H2A) is rapid and covers about 50 kb of DNA around a single DSB (Shroff et al., 2004), where it promotes efficient DSB repair, through the recruitment of cohesin, histone modifiers and chromatin remodelling complexes (Huertas et al., 2009).

The INO80 complex in yeast is involved in regulating the mobility and exchange of histone variants and has been shown to be involved in DNA repair (Morrison et al., 2004a; van Attikum et al., 2004a). The INO80 complex in mammals is a
critical component of HR (Gospodinov et al., 2011; Wu et al., 2007), but in contrast to yeast, mammalian INO80 localises to DNA lesions independently of γ-H2AX (Kashiwaba et al., 2010). Overall, several families of protein are involved in chromatin modulation.

Recent reports in the literature are highlighting new proteins that are essential for regulating repair and checkpoint kinetics. *S. cerevisiae* Fun30 protein and its human counterpart SMARCAD1 have recently been reported as new proteins being directly involved in the DSB response (Costelloe et al., 2012). Fun30 is an ATPase which facilitates the transfer of H2A-H2B dimers and sliding of nucleosomes in vitro (Awad et al., 2010). Cells lacking Fun30 are viable but are hypersensitive to CPT (Neves-Costa et al., 2009). Fun30 physically associates with DSB ends and directly promotes both Exo- and Sgs1-dependent end resection through a mechanism that involves its ATPase activity (Costelloe et al., 2012). Fun30 promotes long range resection at DSBs (Costelloe et al., 2012; Eapen et al., 2012). In addition, loss of SMARCAD1 also resulted in impaired resection and impaired recombinational DSB repair, thus identifying an evolutionarily conserved role for Fun30 and SMARCAD1 chromatin remodellers in controlling DNA end resection (Costelloe et al., 2012). It is believed that Fun30 and SMARCAD1 weaken histone-DNA interactions in nucleosomes flanking DSBs, which facilitates ssDNA production by the Exo1- and Sgs1-Top3-Rm11 (STR)-Dna2 resection machineries respectively (Costelloe et al., 2012).

These results highlight the importance of chromatin remodellers in the DDR. We identified a residue located in the H3 αN helix which is vital for survival after exposure to genotoxic agents (Chapter 3). This along with reports mentioned above highlights the importance of an intact and stable chromatin structure to carry out efficient repair. Every amino acid residue in the nucleosome offers a side chain which affects the overall composition of the nucleosome and chromatin structure. We are increasingly discovering how these residues contribute to cellular processes either by modifications or structure as we highlighted by showing the importance of an intact H3E50 residue.
Components of chromatin remodelling complexes are mutated in cancer (Sulli et al., 2012). This highlights the importance of these systems in maintaining genomic integrity. Chromatin conformation is an important element for the activation of the DDR signalling and repair. We are constantly gaining insight into the complex role of chromatin and the DDR and it is likely that novel chromatin modifiers and histone modifications will be identified. Therefore a thorough understanding of the interaction between the DDR and chromatin modulators is necessary in leading to new avenues for improving cancer therapies.

The interaction between checkpoint proteins and the chromatin states is also an important aspect of the DDR to study. The Rad9 checkpoint protein plays an integral role in the cellular response to DNA damage. Rad9 is a chromatin protein and the regulation of its chromatin localisation in connection with its different DDR function is not well understood. Rad9 is among a list of checkpoint proteins, which are regulated by CDK activity (Enserink and Kolodner, 2010). Rad9 contains 20 putative CDK sites (discussed in Chapter 4) and although this protein was identified over 20 years ago (Weinert and Hartwell, 1988), a full understanding of how its functions are regulated by CDK is far from complete. Our laboratory has identified individual CDK sites on Rad9 that regulate cellular processes (Chapter 4; Karen Finn Thesis., 2010; Abreu et al., 2013; Granata et al., 2010). Unpublished preliminary data in our laboratory suggest that CDK could control Rad9 localisation on chromatin (Carla Abreu Thesis 2012). In chapter 4 we provide evidence that two sites on Rad9, S494 and S618 are important for recovery from a G2/M DNA damage checkpoint response. The exact mechanism, which these two sites regulate, remains to be elucidated and we can not exclude that the sites affect Rad9 localisation at the sites of DNA damage. The data generated on CDK-dependent regulation of Rad9 is complex and much more remains to be determined to fully elucidate the complex regulatory mechanisms that govern the Rad9 checkpoint protein in the DDR.
5. 2 References


APPENDIX A

Laboratory Protocols
YEAST HISTONE PREPARATION

(From the Dent laboratory website http://www.mdanderson.org/education-and-research/departments-programs-and-labs/labs/dent-laboratory/protocols/yeast-histone-prep-1.html I have added extra notes to all steps that I learned from Jessica Downs)

Day 1:
Inoculate 1-2 L of YPD with cells overnight to be at a density of 1-2x^7 cells/ml the following morning.

Day 2:
1. Spin cells at 3000g for 5 minutes.
   - Take some cells here ~100ul, for your whole cell fraction for running on a comassie.
2. Wash cells pellets in 200-400ml sterile water and centrifuge again.
   - Done in the conical 500 ml flasks we have for spinning.
3. Once washed transfer these cells to 50 ml falcoms and pellet again.
4. Resuspend cells in 50 ml DDT/Tris-HCL pH 9.5. Incubate with shaking at 30°C for 15 minutes. Spin as above.
   - Here split the 50 ml into two 50 ml falcoms to have 25 ml volume in each and put into a rack and into the 30°C yeast shaker for 15 minutes. When resuspending the cells here you can be quite harsh and vortex the tube to resuspend the pellet fully.
5. Resuspend the pellet in 50 ml/Sorbitol/Hepes buffer (Buffer 2). Re spin at room temperature.
   - For resuspension here you can use a vortex but not too harsly and use a 10 ml pipette to pipette the suspension up and down.

----Steps 6-15 are done in the presence of phosphatase and protease inhibitors----

6. Resuspend the pellet in 50 ml buffer 2, add 1-2 ml zymolyase (10mg/ml).
This step is very important! Split the cells into two 50 ml falcons to have 25 ml per tube. Add zymolyase powder directly to the cells, forget about having it dissolved. I used 20mg of Zymolyase per 50 ml. I weighed out 40 mg zymolyase and I added it to the 100mls of buffer 2 (doing two cell lines therefore the 100mls buffer) then I resuspended the cells in 50 ml of this. Then I split the 50 ml into two falcons to have 25mls per falcon for shaking the 30°C yeast shaker.

7. Incubate the tubes in the 30°C shaker for 45-60 mins (or until well ghosted).
- While incubating invert the tubes every so often just make sure its mixing. The incubator should be shaking fairly vigorously.

8. At 45-60 minutes (I normally waited for 60 minutes) check for the presence of ghosts.
- Take 10 ul of cells and put on a slide. On the slide have 10 ul cells with no SDS and 10 ul cells where you added 1 ul SDS. Get your cells with no SDS in focus. These cells will look clumped but do not look really like cells look more like smaller round balls clumped together. Then from this don’t adjust the focus and slide the objective over to the cells with SDS added. You should see what’s called ghosts. It will look like there is nothing on the slide but you will see shawdows of cells. Get someone who knows to show you.

-----Steps 9-15 must be done in the cold room. (speed is important, do these steps as quickly as you can)-----

9. Add 100 ml ice cold Buffer 3 (Sorbitol/Pipes/MgCl2). Spin at 3200g for 5 min at 4°C.
- On the protocol it says to spin at 3.5K (JA10 Rotor) for 5 min at 4°C. But I was wasting to much time balancing and using the centrifuge at the back, so I use our yeast centrifuge at the front and have it chilled to 4°C, I spin at 3200g and this pellets the cells. For this step I split the cells into 3 falcons for this wash and I just combined them again and pelleted to have in one 50 ml falcon for the next steps.

10. Resuspend the pellet in 50 ml ice cold NIB buffer and hold on ice for 20 minutes. Spin at 3200g for 5 minutes at 4°C.
-Note on washes: When you add the buffer into the tube you need to start your timer. The resuspending of the pellets can take 2-4 minutes so you are leaving the cells sitting on ice for about 16 minutes. Be careful resuspending the pellet you need to be gentle. Use 10 ml plastic pipettes and set the pipette bouy to about half speed. Then pipette up and down slowly but not to slow, just be careful not to blow out the cells from the peipette as you don’t want to create too much force. Make sure the cells are all resuspended. I put in about one third of the buffer to resuspend then I add the rest and invert the tube a few times then leave on ice for the time remaining. Bring the tubes to the centrifuge on ice and take out on ice. Keep everything really cold all the time.

11. Repeat step 10 two times. (These steps take just over an hour with the 20 minutes incubations and spinning for 5 minutes.)

12. Resuspend pellet in 50 ml Wash A and hold on ice for 15 minutes.
- Again start the timer once the buffer hits pellet and then leave on ice for the remainder of time once the pellet is resuspended.

13. Repeat A wash and spin

14. Resuspend pellet in 50 ml B wash and hold on ice for 5 min. Spin as above for 5 min.
- Here once the pellet is resuspended I leave it on ice for the full 5 mins, I start the timer once I have the pellet resuspended

15. Resuspend in 25 ml B wash and spin immediately.

16. Resuspend pellet in 10 ml cold 0.8M H₂SO₄ to extract histones. Hold on ice for 30 minutes, vortexing occasionally.
- Make up 0.8M H₂SO₄ in the morning so it is cold. Bring the vortex into the cold room along with your bucket of ice so you can leave on ice and vortex to keep it all cold. At this step you need to change tubes. Your next step is to spin at 10K for 10 minutes so you need to change to JA17 rotor tubes. You can resuspend in the falcoms but then pour this into the new tubes.

17. Spin at 10K for 10 minutes. **SAVE SUPERNATANT**, which contains the extracted histones. Measure volume with a pipette.
- Resuspend a bit of the pellet in SDS loading buffer for a western.

18. Add 100% TCA to a final concentration of 20%. A large precipitate should form almost immediately. Hold on ice for 30 minutes.
- Do this on your bench not in cold room. Do not chill the TCA. Have your volume measured from step 17 and add TCA to a final conc of 20%. It says a large precipitate will form but its quite hard to see this. Its kind of on the top, but untill you get used of seeing it it might be hard to notice at the start.

19. Spin at 12K for 30 minutes at 4°C. If supernatant looks cloudy, pour into a fresh tube and spin again.

- For this step it is important to orientate tubes so you know where the supernatant will be. Mark the tube so you know which side the pellet will be. The tubese are opaqe and the pellet is near impossible to see. Also at this step keep the supernatant incase you do not have your histones at the final step and you can come back to this fraction if you have lost them.

20. Wash pellet in 10 ml acetone and spin for 5 minutes at 10K at 4°C.

- No need to chill the acetone. Be very carefull pouring off the supernatant here. Pour it off slowly and then pipette of the last few ml, leave a bit at the bottom of the tube so you do not remove your histones.

21. Wash pellet in acetone again and spin for 5 minutes at 10K at 4°C.

- Again be careful as last step.

22. Pour off acetone and air-dry the pellet.

- Pipette off the acetone with a 1ml pipette and leave approx 1 ml at the end. Remove this then with a 200 ul pipette. You will see there is tiny white flakes instead of a stable pellet. Everytime you pipette you unsettle these and you need to be so carful pipetting off the acetone so as not to take any of your pellet. I pippetted of some then left it a few minutes to settle again and then took a few more microlitres. At the end for air drying you want to have a small amount of acetone to air dry off and this can take up to an hour or bit more to air dry. You need to make sure the pellet is completely air-dried beofre you resuspend. You might not see anything in the tube when you have the pellet completely air dried but do not worry your histones are there but you can’t see the pellet.

23. Resuspend the pellet in a total of 1ml of 10mM Tris-HCL pH8.0. If hard to resuspend, add more Tris buffer, 0.5ml at a time. Try to keep histones as concentrated as possible.

- I started resuspending in 600ul of Tris buffer and adding more if the pellet was hard to resuspend. You do not want to add much more than 1ml as you you’re your histones to be concentrated.
24. Aliquot histones once resuspended and store at -20°C.

- Once histones are resuspended aliquot out so as you are not freeze thawing the one fraction all the time. Keep some to run on a commassie and western and aliquot the rest into 50 or 100 ul aliquots.
**BUFFERS:**

<table>
<thead>
<tr>
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<th>Buffer 2</th>
<th>Buffer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DTT/Tris</strong></td>
<td>1.2M Sorbitol</td>
<td>1.2M Sorbitol</td>
</tr>
<tr>
<td>0.1mM Tris-HCL pH 9.4</td>
<td>20mM Hepes pH 7.4</td>
<td>20mM Pipes pH 6.8</td>
</tr>
<tr>
<td>10mM DTT</td>
<td>+ Inhibitors</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>A Wash</th>
<th>B Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NIB</strong></td>
<td>10 mM Tris</td>
<td>10 mM Tris-HCL pH 8.0</td>
</tr>
<tr>
<td>0.25M sucrose</td>
<td>0.5% NP-40</td>
<td>0.4 M NaCl</td>
</tr>
<tr>
<td>60mM KCL</td>
<td>75mM NaCl</td>
<td>1mM PMSF</td>
</tr>
<tr>
<td>15mM NaCl</td>
<td>1mM PMSF</td>
<td></td>
</tr>
<tr>
<td>5mM MgCl2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mM CaCl2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15mM MES, pH 6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mM PMSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8% Triton x-100</td>
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FLUORESCENCE MICROSCOPY (Looking at damaging agents in S phase)

1. Set up cultures O/N in –LEU minimal media
2. Cells should be 3.5–6x10⁶ cell/ml in the morning
3. Pellet cells and resuspend in YEP + glucose
   - All media and saline used needs to be pre warmed before using
4. Shake cells for 30 mins in yeast shaker
5. Arrest cells with 7.5µg/ml α-factor for 2h
   - Take an asynchronous sample for budding index, FCAS and western blot analysis
6. Once arrested wash out α-factor and resuspend in fresh YEP+G media
   - Pellet cells wash once through 0.9% saline and once through YEP media.
   - Resuspend in the same amount of media cells were previously in
   - Take samples here (G1) for budding index, FCAS and western blot and microscopy analysis.
7. Take 1ml media for microscopy sample
8. Add drug to your cultures and put back in the shaker for the appropriate time
9. Prepare your sample for imaging on the microscope
   - Spin down 1ml of culture
   - Wash once through water
   - Before you spin your water wash sonicate briefly 3 sec at 10% to disperse the cells so as not to be clumped when looking at under the microscope
   - Leave a few µl of water above the pellet
   - Add 5-10µl of 2% low melting point agarose. Amount you add is dependent on how much water and how big your pellet is but 5-10µl is generally what I added
   - The low melting point agarose should be left in a heating block at 40°C while not in use as once it goes below this it will solidify. If it is left on the bench for any more than 2-3 mins it will solidify. When making you need to heat it up to about 70°C to get it melt but then you must have it cooled before you add to your cells
   - Resuspend the pellet by flicking gently the bottom of the tube with your finger, do not vortex. Be quick the agarose will solidify very quickly when it is out of the heating block.
- When all resuspended add a few drops to a slide and varnish over your cover slip.

10. **Image on the microscope. Have temperature chamber set to 30°C**
- Settings will differ on microscopes therefore carry out preliminary experiments to optimise conditions for imaging
- Yeast cells bleach quite quickly
- Take stacks. Yeast can be on different plains therefore if you take one image you are losing data from other yeast cells. Amount of stacks you take depends on how well the yeast have settled on the slide.
- Stack size I took is 0.25µm.
- Be careful when counting that your background is not high as you will get spots that look like foci but are due to background.
**NaOH PROTEIN EXTRACTS (Kushnirov, 2000)**

1. Collect 2x10⁷ cells/ml.
2. Freeze the pellet on dry ice. Freeze at -80°C.
3. Do 4 extracts at one time. Any more will reduce the quality of the extract.
4. Let pellet thaw for a few seconds on bench.
5. Add 100µl ddH₂O, resuspend by pipetting gently.
6. Add 100µl 0.2M NaOH, invert tubes to mix.
7. Incubate for 5 mins at room temp.
8. Spin 14000 rpm for 2 min. Remove supernatant.
9. Resuspend in 50µl SDS buffer.
10. Boil for 3 mins at 95°C.
   - You need to be quick. Resuspend one pellet, make sure it is well resuspended. Then place in the heating block, start your timer once the first extract is in the heating block. Continue on with second resuspend and place in heat block. Same for third and last extract. Once 3 mins is up take out first extract then second third and fourth.
   - Here I take out the first and give the second a few more second and then remove and the same for the third and last extract.
11. Spin 14000 rpm for 2 min.
12. **Keep Supernatant.**
13. Freeze on dry ice immediately.

PEPTIDE PULL-DOWN (Ross Chapman protocol)

Design of peptides:
BIOTIN-SerGlySer( flexible linker)- [~15 amino acid residues of choice with phospho-Thr/Ser residue close to the centre (unless phospho-residue is N or C terminal)] –amide/COOH (depending if the amino acid residues are derived from internal or C-terminal sequence respectively)

Phosphorylated peptide:
Control: γ, phosphorylated H2AX peptide (Chapman and Jackson, 2008; Stucki et al., 2005)
BIOTIN-SGSTVGPKAPSGGKKATQA[pS]QEY-COOH

Phosphorylated H3S28 peptide (H3S28):
Biotin-SGS-LASKAARK[pS]APSTGGVK-COOH

Unphosphorylated peptide:
Control: γ, phosphorylated H2AX peptide (Chapman and Jackson, 2008; Stucki et al., 2005)
BIOTIN-SGSTVGPKAPSGGKKATQASQEY-COOH (H2AX pS139-binds MDC1)

As above but not phosphorylated:
Biotin-SGS-LASKAARKSAPSTGGVK-COOH

Peptides synthesized by Pepceuticals.
Once received store peptide as powder in -20°C in a parafilm sealed vial (peptides tend to be highly hydroscopic)

- Take 1-2mg of peptide solution (1mg/ml- approx 1mM for most peptides of the length above but you will need to check this) in Tris Buffered Saline (TBS) with 1mM DTT. This is frozen in aliquots at -80°C
- I found weighing this small amount hard therefore if I received ~8mg of peptide I resuspended in the bottle it came, aliquoted and froze at -80°C. The peptides are highly hydroscopic and can be a bit difficult to weigh very accurately.

**Reagents:**
Dynabeads M-280 Streptavidin, Prod. No. 112.05; by DYNAL Inc.
HeLa nuclear extract (8mg/ml) by CilBiotech, Belgium
Tris buffer saline +0.1% Tween20 (TBST), or equivalent Peptide pulldown buffer (150mM NaCl, 3mM KCL, 25mM Tris pH8.0, 10% glycerol, 1mM DTT, 1X protease inhibitors. Do not use sodium orthovanadate as a Ppase inhibitor as Ross Chapman found this to inhibit phospho-peptide binding.

**Protocol:**
1. Couple 10μl of 1mM peptide stock to 40μg beads for 30 minutes at room temperature. Put on a rotating wheel on your bench.
2. Wash beads X3 with TBS-T (including 1 change tube).
3. Dilute 1-4 mg of nuclear extract in an equal volume of peptide pull-down buffer (including inhibitor).
   - I used 4mg. I had 500μl of extract and dilutes in 500μl peptide pull-down buffer.
4. Do a pre-clarification step. Before you add the nuclear extract to the peptide coated beads, do a pre-clarification step to remove non-specific proteins. Pre-incubate protein extract from step 3 with TBS-T washed plain beads.
   - Wash X3 in TBS-T
   - 10μl beads/mg protein for 30 minutes at 4°C with agitation. (Put on a rotating wheel in the cold room)
   - Put tube in a magnetic rack and carefully remove extract.
5. Add the peptide coated beads to the diluted extract.
6. Incubate with agitation for 60-120 minutes on a rotating wheel.
7. Wash beads 3-4 times in peptide pulldown buffer. After second wash transfer beads to a fresh tube. (At this stage it gets very hard to see your beads but they should be there)

8. Remove all peptide pulldown buffer. Add loading buffer, boil and load 30 μl on a commassie gel.


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Wang, Y. and Burke, D. J. (1995). Checkpoint genes required to delay cell division in response to nocodazole respond to impaired kinetochore function in the yeast Saccharomyces cerevisiae. *Molecular and Cellular Biology* 15, 6838-44.


and Mms1 in budding yeast form a CUL4DDB1-like ubiquitin ligase that promotes replication through damaged DNA. *EMBO Rep* **9**, 1034-1040.


PHOSPHATASE AND PROTEASE INHIBITORS

50X phosphatase inhibitors (Ppis)

0.105g NaF  
0.540g β-glycerophosphate  
0.092g Na3VO4  
0.951g EGTA  
5.579g Sodium pyrophosphate

Made up in 50ml milliQ H2O
- Get 40ml milliQ  
- Weigh out ingredients  
- Add ingredients and then heat to 50℃ and stir  
- Make up to 50ml  
- Takes a few minutes for it to get into solution  
- Aliquot into 1mls in eppindorfs  
- Freeze every batch of 10 on dry ice to make sure it does not get a chance to get out of solution  
- Note: β-glycerophosphate falls out of solution and takes ages to get into solution, may need to break it up but make sure it is all in solution.

100X protease inhibitors

1.42mg leupeptin (-20℃)  
6.85mg pepstatin A (-4℃)  
0.85g PMSF (On shelf, wear mask)  
1.65g benzamidine (-4℃)  
6.25mg antipain (-20℃)  
4mg chymostatin (-20℃ Dissolves in DMSO, Weigh out and put in 500μl DMSO until it dissolves)

Make up in 100% to 50ml ethanol (Filter sterilise ethanol)  
- Again vortex well to dissolve until clear, it may take time but it will dissolve  
- Again aliquot quickly into 500μl in eppindorfs.
APPENDIX B

Authors Funding and Contributions
FUNDING:

PhD Scholarship award from IRCSET EMBARK scholarship

ORAL PRESENTATIONS

Oral presentation at the EU DNA repair meeting 2007, Lake Garda, Italy 5th-7th October 2007
Mary Walsh, Muriel Grenon and Noel F.Lowndes “Identification and characterisation of new proteins involved in the cell cycle specificity of DNA damage-dependent activation of Rad9 complex”

POSTER PRESENTATIONS

British Yeast Group Meeting, NUI Maynooth, Ireland, 18th-20th March 2008
Aisling O’Shaughnessy, Jean Soulier, Mary Walsh, Muriel Grenon, Noel Lowndes “Cell cycle phosphorylation of S. cerevisiae and it’s role in checkpoint recovery”

Irish Fungal meeting, NUI Galway, 3rd June 2008
Aisling O’Shaughnessy, Jean Soulier, Mary Walsh, Muriel Grenon, Noel Lowndes “Cell cycle phosphorylation of S. cerevisiae and it’s role in checkpoint recovery”

Irish Fungal meeting, university of Dublin, January 2009
Thomas Costelloe, Mary Walsh, Muriel Grenon, Noel Lowndes “Identification of a novel histone mark specifically involved in camptothecin resistance”

Irish Association for Cancer Research, Galway Ireland 3rd – 5th March 2010
Thomas Costelloe, Mary Walsh, Muriel Grenon, Noel Lowndes “Identification of a novel histone mark specifically involved in camptothecin resistance”

British Yeast group meeting, Oxford, UK, 17-19th March, 2010
Thomas Costelloe, Mary Walsh, Muriel Grenon, Noel Lowndes “Identification of a novel histone mark specifically involved in camptothecin resistance”