Regulation of the cell division cycle by ubiquitin and ubiquitin-like modifications in yeast

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Abstract

The ability of a cell to regulate its cell cycle in response to external stimuli, such as oxidative stress, is important to maintain viability by preventing damage and allowing time for repair. However, the underlying sensing and signalling mechanisms behind cell cycle regulation in response to oxidative stress remain largely unclear. Ubiquitin and ubiquitin-like (Ubl) proteins are a family of highly conserved protein modifiers with a role in many cellular processes including cell cycle regulation. The use of catalytic cysteine residues in the conjugation pathways of ubiquitin and Ubls suggest a mechanism by which these modifiers can be redox-regulated. Thus the aim of this project was to investigate the regulation of the cell division cycle by ubiquitin and Ubls in response to two conditions previously observed to lead to G1 phase cell cycle arrest in *S. cerevisiae*, treatment with the oxidising agent diamide and glutathione depletion. We find that in response to diamide the ubiquitin E2, Cdc34 is particularly sensitive to oxidation compared to the other E2s examined. Oxidation of Cdc34 was shown to lead to an increase in the stability of the Cdc34 substrate Sic1, coincident with G1 phase arrest. We also find that the Rub1 Ubl modifier is essential for regulation of the cell cycle in response to diamide. Interestingly, we find that Rub1 is also required to prevent budding in response to glutathione depletion. Importantly, here we reveal that *SIC1* is essential to maintain viability by preventing replication-induced DNA damage following glutathione depletion. Our studies demonstrate that G1 phase cell cycle arrest in response to diamide and glutathione depletion is multifaceted, involving many of the same proteins but that these proteins are regulated differently in response to the two conditions.

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Abbreviations

BSA- bovine serum albumin

C. albicans – Candida albicans

C. elegans – Caenorhabditis elegans

CKI- cyclin dependent kinase inhibitor

DMSO- dimethyl sulfoxide

DTT- dithiothreitol

E. coli – Escherichia coli

EDTA**-** Ethylenediaminetetraacetic acid

EMM- Edinburgh minimal medium

H_2O_2- hydrogen peroxide

HRP- horseradish peroxidase

MMS – methyl methanesulfonate

PCR – polymerase chain reaction

PEG- polyethylene glycol

PMSF- phenylmethanesulfonyl fluoride

qPCR – quantitative polymerase chain reaction

S. cerevisiae – Saccharomyces cerevisiae

SD – synthetic dextrose

SDS- sodium dodecyl sulphate

S. pombe – Schizosaccharomyces pombe

tBOOH- tert-Butyl hydroperoxide

TCA- trichloroacetic acid

TE- Tris EDTA

UV- ultraviolet

YE5S- yeast extract 5 supplements

YPD – yeast extract-peptone-dextrose

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Chapter 1: Introduction

1.1 Ubiquitin and Ubls

Post-translational modification of proteins can alter the physical and chemical properties of the target protein thus allowing it to respond appropriately to changes in the cellular environment. Most post-translational modifications such as methylation, phosphorylation and acetylation, involve the addition of a small group to a protein. However, the addition of a whole protein can greatly increase the functional diversity of the target protein and provides greater scope for changes in protein conformation and interactions. The classical example of the addition of a whole protein as a modification is ubiquitin. Ubiquitin is highly conserved among eukaryotes although missing from bacteria and archaea, and is best known for its role in targeting proteins for degradation by the 26S proteasome. Since the initial discovery of ubiquitin a number of ubiquitin-like (Ubl) modifiers have been identified. While not necessarily sharing high levels of homology at the level of their primary sequence these Ubls share a common three-dimensional β-grasp fold structure and their conjugation pathways display similar features. The attachment of these modifiers to substrates has been implicated in a wide range of cellular processes and the dysregulation of these modifiers has been shown in a number of disease states. Indeed ubiquitin and Ubls are essential in many organisms demonstrating their importance in a wide range of vital cellular processes.

1.1.1 Conjugation

In general ubiquitin and Ubls share a common mechanism of conjugation involving E1, E2 and E3 enzymes (Figure 1.1). Ubiquitin and Ubls are initially inactive and must first be processed by specific proteases to expose a C-terminal glycine carboxylate. The processed ubiquitin /Ubl is then activated by the activating E1 enzyme which adenylates ubiquitin/Ubl forming a high energy Ubiquitin/Ubl-AMP intermediate. The intermediate is then attacked by the catalytic cysteine of the E1 leading to the formation of a thioester bond between ubiquitin/Ubl and this cysteine residue of the E1. In the next step, ubiquitin/Ubl is transferred to the active site cysteine of the E2 conjugating enzyme. In the final step, ubiquitin/Ubl is transferred to the target lysine residue of the

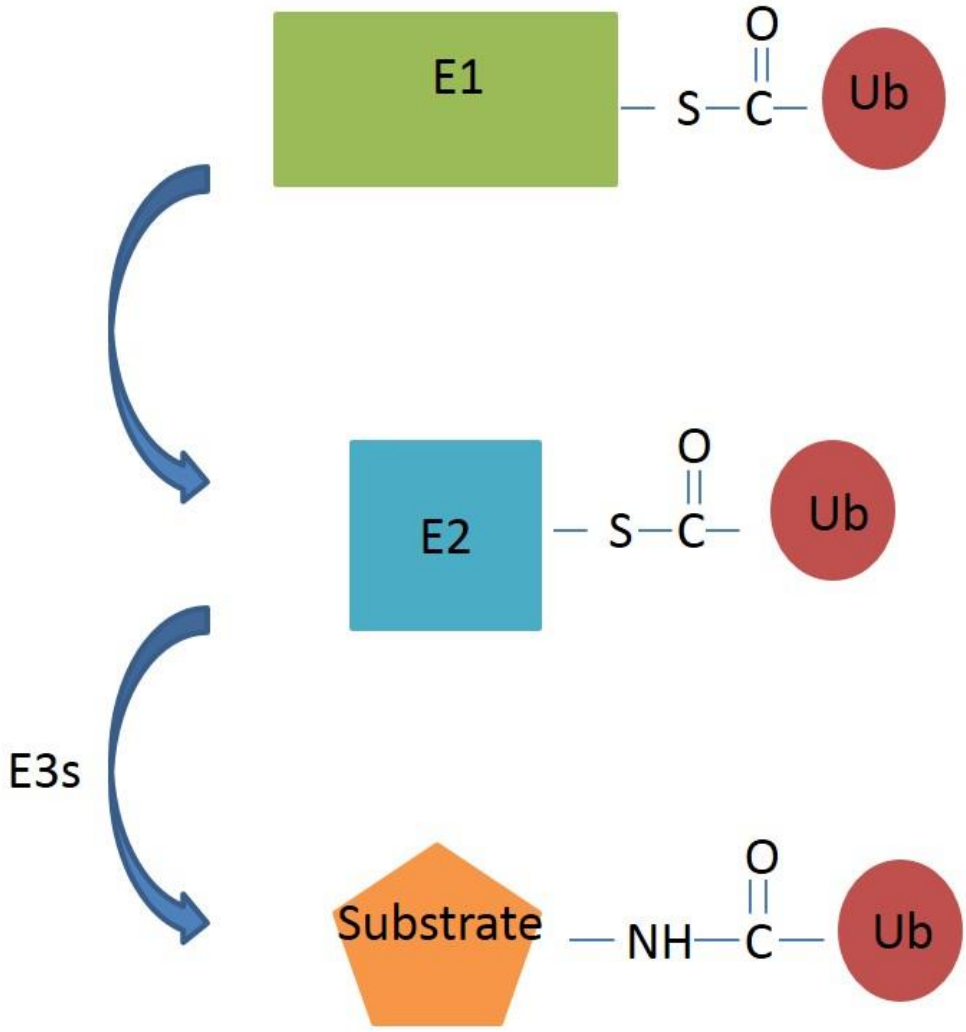


Figure 1.1 Ubiquitin and Ubl proteins share a common conjugation pathway. Ubiquitin and most Ubl proteins are conjugated to their target substrate through a common conjugation pathway involving an E1, activating enzyme, an E2 conjugating and an E3 ligase.

substrate forming an isopeptide bond. This final step often, but not always, requires an E3 ligase enzyme thought to aid the reaction either through stabilising the interaction between the E2 and the substrate or by binding to ubiquitin/Ubl itself before transferring it to the substrate lysine (Hochstrasser, 2000).

Protein modification by ubiquitin/Ubls is not irreversible. The modifier can be removed by deconjugating enzymes allowing for reversibility of the modification and the creation of a free pool of ubiquitin/Ubl that can rapidly be conjugated to other substrates.

1.1.2 Substrate specificity

Substrate specificity for ubiquitin/Ubls is achieved in a number of ways dependent on the conjugation pathway. The ubiquitin conjugation pathway for example contains many different E2 and E3 enzymes that direct substrate specificity (Figure 1.2). Substrate specificity is primarily directed by E3 enzymes using domains distinct from their catalytic domain to recognise substrates with or without the aid of a binding partner. Recognition can also occur through degron elements located within the substrates themselves which can aid the interactions between the substrate and the E3 enzyme (Mattiroli and Sixma, 2014).

The specificity of the target lysine for modification varies depending on the substrate and the role of the conjugated ubiquitin/Ubl. Non-selective lysine modification by ubiquitin often occurs on substrates that undergo polyubiquitination signalling their degradation by the proteasome (Mattiroli and Sixma, 2014). For these substrates it is the polyubiquitin chain that acts as the signal and thus the specificity of the lysine is not important. However, specific lysines are modified in some substrates such as PCNA (Hoege *et al.*, 2002). PCNA has a role in several S-phase processes such as DNA replication and DNA repair and has been shown to undergo modification by both SUMO and ubiquitin on lysine 164 (lys164). A mutation of the lys 164 of PCNA in *S. cerevisiae* that prevents its modification by SUMO and ubiquitin leads to cells that are highly sensitive to the DNA damaging agents MMS and UV light. Modification of PCNA lys 164 by SUMO occurs during S-phase and inhibits the role of PCNA in DNA repair suggesting a role for PCNA in DNA replication. Ubiquitin modification of PCNA is induced after exposure to DNA damaging agents and has been linked with DNA repair.

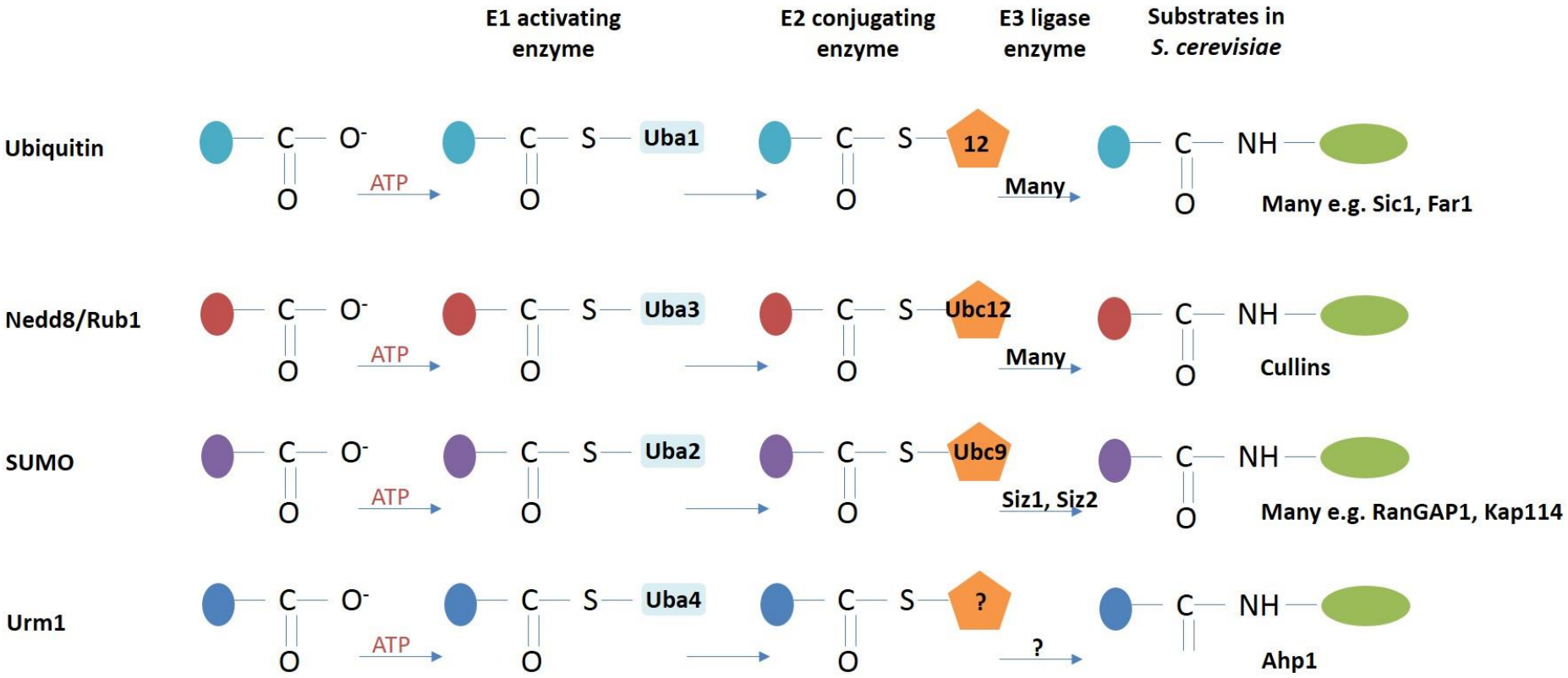


Figure 1.2 The conjugation pathways of the ubiquitin, Nedd8/Rub1, SUMO and Urm1 pathways of *S. cerevisiae*. The ubiquitin conjugation pathway has been shown to contain a single E1 enzyme, Uba1 which works with multiple E2 enzymes (12 have been identified in *S. cerevisiae*) and E3 enzymes to direct the modifier to specific substrates. In contrast, a single E1 and E2 enzyme have been identified in the Nedd8/Rub1 (Uba3 and Ubc12) and SUMO (Uba2 and Ubc9) conjugation pathways. An E1 enzyme, Uba4 has been characterised for the Urm1 pathway in *S. cerevisiae* but currently no E2 or E3 enzymes have been identified.

Unlike the ubiquitin conjugation pathway which has many E2 enzymes (12 in *S. cerevisiae*) to direct specificity of the modifier, the SUMO conjugation pathway has only one identified E2. A consensus motif of ψ-K-X-D/E (where ψ is a hydrophobic residue, K is the lysine that undergoes SUMO modification, X is any amino acid, D/E represents an acidic amino acid) has been identified in many SUMO substrates (Bernier-Villamor *et al.*, 2002). Although E3 enzymes are thought to ease the interactions between Ubc9 and the substrate, E3s are dispensable in SUMO conjugation pathways and SUMO conjugation has been shown to occur *in vitro* with only the E1 (Uba2), E2 (Ubc9), mature SUMO and ATP. Thus, it is the E2, Ubc9 that is able to recognise and interact with the consensus motif in the substrate directing the conjugation of the modifier to the target lysine.

1.1.3 Roles and regulation of ubiquitin and Ubls

Many Ubls have been identified which have roles in diverse cellular processes. Discussion of all of these modifiers is beyond the scope of this introduction however many reviews are available on this subject (van der Veen and Ploegh, 2012). The remainder of this section will focus on the roles and regulation of the ubiquitin, SUMO, Rub1/Nedd8 and Urm1 conjugation pathways.

1.1.3.1 Urm1

The β-grasp fold and carboxy-terminal glycine found in ubiquitin/Ubl proteins are also found in the prokaryotic sulphur carrier proteins suggesting an evolutionary relationship between these pathways (Figure 1.3). Indeed, the Ubl modifier, Urm1 was originally identified through its similarity to the prokaryotic sulphur carrier proteins MoaD and ThiS rather than previously identified Ubls and has dual functions as a sulphur donor in tRNA thiolation and as a protein modifier (Furukawa *et al.*, 2000).

1.1.3.1.1 Urm1 conjugation and deconjugation

In contrast to other ubiquitin/Ubl pathways, the mechanism underlying Urm1 conjugation is still largely uncharacterised. An E1 enzyme has been identified in both mammalian cells (MOCS3) and *S. cerevisiae* (Uba4) however no E2, E3 or deconjugating enzymes have been discovered to date. Furthermore, the basis of substrate specificity is not understood. The Urm1 conjugation pathway shows similarity to both the ubiquitin pathway and the prokaryotic sulphur carrier proteins MoaD and ThiS which act as

sulphur donors in the synthesis of molybdopterin and thiamine respectively (Figure 1.3) (Wang *et al.*, 2011). The MoaD/ThiS, ubiquitin and Urm1 pathways all share a common first step in their conjugation pathway where the modifier undergoes activation of its C-terminal glycine residue by an E1-like enzyme. However, in contrast to ubiquitin which forms a thioester bond with its E1 enzyme, Urm1 forms an acyl-disulphide with Uba4 and becomes thiocarboxylated similar to MoaD/ThiS. Thiocarboxylation of Urm1 provides sulphur which can be used by Urm1 in its sulphur carrier role however, thiocarboxylation of Urm1 is also required for its role as a protein modifier (Van der Veen *et al.*, 2011). To date no E2 or E3 enzymes have been identified for Urm1 conjugation, however it has been suggested that a thioester intermediate is required for Urm1 conjugation to occur. In common with ubiquitin, Urm1 is conjugated to lysine residues on its target substrate by an isopeptide bond.

1.1.3.1.2 Targets and functions of Urmylation

Urm1 was originally identified as a sulphur donor in the thiocarboxylation of tRNA (Huang *et al.*, 2008a). Codon families are made up of codons which encode the same but differ in their third nucleotide (Fonseca *et al.*, 2012). Typically there is only one tRNA for each codon family therefore tRNA must form a wobble base (non Watson-Crick base pairing) with at least one of the nucleotides to recognise all family members. Post-translational modifications, for example the wobble base modification of uridine, are able to stabilise the interaction between the tRNA and mRNA at these bases. The wobble base modification of uridine is a two-step process. In the first step, the oxygen at position 2 in uridine is replaced by sulphur donated by Urm1 and in the second step, position 5 of uridine is modified to methoxy-carbonyl-methyl by the elongator protein complex (Wang *et al.*, 2011).

In addition to its role as a sulphur donor, Urm1 modification has been implicated in a number of cellular processes. Loss of either the *URM1* or *UBA4* gene in *S. cerevisiae* renders cells sensitive to rapamycin and unable to invade agar under starvation conditions, suggesting a role for Urm1 in nutrient sensing and survival in response to nutrient deprivation (Goehring *et al.*, 2003). Loss of *URM1* in *S. cerevisiae* also leads to loss of the nitrogen-dependent regulation of *GAP1* and *CIT2* which are regulated by the

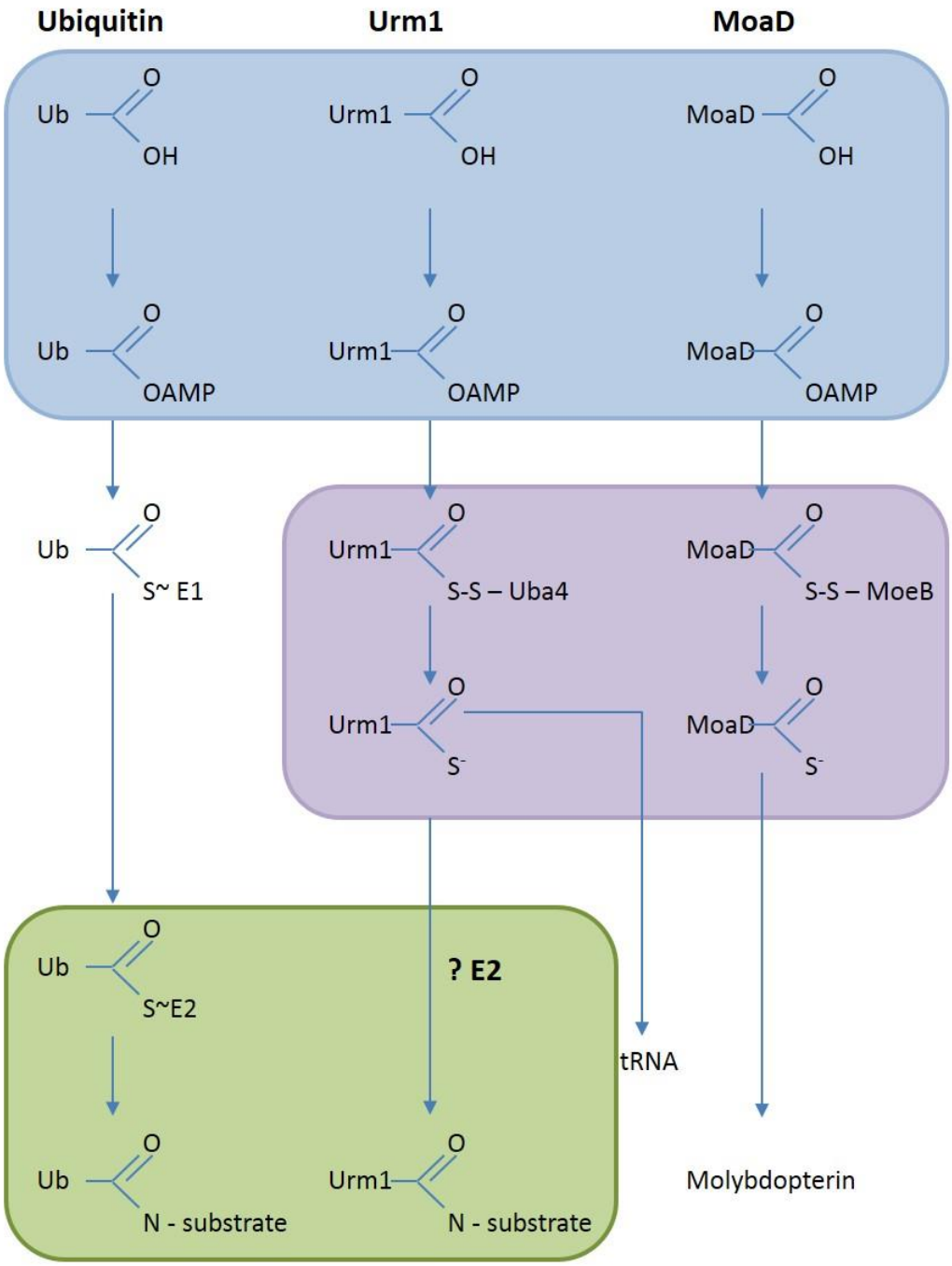


Figure 1.3 The Urm1 conjugation pathway shows similarity to ubiquitin-like modifiers and the prokaryotic sulphur carrier MoaD. Shaded areas indicate features which are shared between the Urm1 conjugation pathway and that of ubiquitin and/or MoaD. Similar to MoaD, Urm1 forms an acyl disulphide bond with its E1, Uba4 instead to the thioester bond formed between ubiquitin and its E1. Although no E2 or E3 enzymes have been identified for the Urm1 conjugation pathway, Urm1 has been shown to modify a number of substrates in both mammalian and *S. cerevisiae* cells. Adapted from Petroski ,2011.

transcription factors Gln3 and Nil1 (Rubio-Texeira, 2007). Gln3 and Nil1 in turn are regulated both in response to the nitrogen source the cells are grown in and their nuclear localisation. For example, in the presence of glutamine, Gln3 is repressed by a physical interaction with Ure2 which sequesters the transcription factor in the cytosol (Kulkarni *et al.*, 2001). In the absence of *URM1*, cells lose the nitrogen-dependent control of the intracellular localisation of Gln3 and Nil1 (Rubio-Texeira, 2007). However, the mechanism behind the Urm1-dependent regulation of Gln3 and Nil1 remains unknown as Urm1 does not immunoprecipitate with either of these proteins suggesting they are not directly targeted by the modifier.

In mammalian cells no Urm1 conjugates are observed under steady state conditions while in *S. cerevisiae* there is a low level of Urm1 conjugates present under these conditions (Van der Veen *et al.*, 2011). However, in response to H_2O_2 and diamide, global Urm1 conjugation increases in both mammalian and *S. cerevisiae* cells. Interestingly, global Urm1 conjugates do not increase in response to other cellular stresses such as heat, ER stress or DNA damage suggesting a specific role for Urm1 in oxidative stress responses. Indeed, deletion of either *URM1* or *UBA4* causes an increased sensitivity to the thiol oxidising agent diamide and the first identified target of Urm1 modification was Ahp1, a peroxiredoxin involved in cellular antioxidant defences (Goehring *et al.*, 2003) (see Section 1.3.4.2.3). Removal of reactive oxygen species (ROS) by peroxiredoxins such as Ahp1 involves oxidation of their catalytic cysteine which must be reduced by thioredoxin (Trx) in order to restore enzymatic activity (Morano *et al.*, 2012). Urm1 modification of Ahp1 has been suggested to either decrease or abolish its activity by affecting its reduction by Trx (Lian *et al.*, 2012). Other substrates of Urm1 modification identified in mammalian cells following oxidative stress suggest a diverse role for Urm1 modification in a range of processes, for example, nuclear transport and RNA processing although how Urm1 modification affects these targets remains unclear (Van der Veen *et al.*, 2011).

1.1.3.2 Ubiquitin

Ubiquitin is best known for its role in targeting proteins for degradation by the proteasome. Ubiquitin itself contains seven lysine residues which can themselves be

ubiquitinated leading to the formation of different types of ubiquitin chains e.g. K6, K11, K27, K29, K33, K48, K63 which can alter the fate of the substrate.

1.1.3.2.1 Ubiquitin conjugation and deconjugation

The human genome encodes two ubiquitin E1 enzymes, 38 E2 enzymes and more than 600 E3 ubiquitin ligase enzymes. In *S. cerevisiae*, a single E1 Uba1 has been identified which along with twelve E2 enzymes (Table 1.1) and 60-100 E3 ligases catalyse the addition of ubiquitin to its target.

E2 conjugating enzymes are central in ubiquitin conjugation as they interact with both the E1 and E3 enzymes. Interestingly, studies have revealed that several mechanisms are in place to ensure that ubiquitin flows only one way through the pathway. In particular, the ubiquitin E1 enzyme shows a higher binding affinity for free, unconjugated E2 enzymes while E3 enzymes have a higher affinity to ubiquitin-bound E2 enzymes. In addition to these relative binding affinities, both the E1 and E3 enzymes share a binding site on the E2 ensuring unidirectional flow of ubiquitin through the pathway (Finley *et al.*, 2012). Interestingly, of the E2, conjugating enzymes identified in *S. cerevisiae* only the *CDC34* gene is essential for viability. Temperature sensitive *cdc34* mutants arrest at G1/S phase due to their inability to degrade the cyclin-dependent kinase inhibitor (CKI) Sic1 (Schwob *et al.*, 1994). Other ubiquitin E2 enzymes have been demonstrated to have roles in distinct processes in *S. cerevisiae* (Table 1.1). For example, Ubc4 and Ubc5 are functionally redundant in the cellular response to heat shock. Double *ubc4ubc5* mutants are inviable at elevated temperatures and their expression is induced in response to heat where their gene products are required for the degradation of abnormal cytosolic proteins (Seufert and Jentsch, 1990). Three other ubiquitin pathway E2 enzymes, Ubc1, Ubc6 and Ubc7 are required for degrading misfolded proteins from the ER in *S. cerevisiae* (Finley *et al.*, 2012). Several other E2 enzymes have also been demonstrated to work together in the polyubiquitination of a single substrate. For example, Ubc1 and Ubc4 act together, sequentially to ubiquitinate the anaphase promoting complex (APC/C) (Finley *et al.*, 2012). In this example, monoubiquitination of the APC/C is catalysed by Ubc4 while ubiquitin chain synthesis requires Ubc1.

E2	Examples of biological processes linked to the E2
Ubc1	Vesicle biogenesis, ERAD, E2 for APC/C
Ubc2/Rad6	DNA repair
Cdc34	Cell cycle, E2 for SCF ligases
Ubc4	Protein quality control outside the nucleus, E2 for APC/C
Ubc5	Functionally redundant with Ubc4
Ubc6	ERAD, K11-chain synthesis
Ubc7	ERAD
Ubc8	Regulation of gluconeogenesis
Ubc10/Pex4	Peroxisomal E2 import for peroxisome biogenesis
Ubc11	Unknown
Ubc13	DNA repair, dimerises with Mms2 for K63 chains
Mms2	DNA repair, dimerises with Ubc13 for K63 chains

Table 1.1 E2 enzymes identified in *S. cerevisiae* and their biological functions. Table adapted from (Finley *et al.*, 2012).

E3 ligase enzymes bind both the E2 and the substrate and convey substrate specificity to the modification. E3 enzymes fall into two main categories, those containing the really interesting new gene (RING) domain and those containing a HECT domain. HECT domain E3 ligases contain a cysteine residue at the active site which forms a thioester bond with ubiquitin prior to transferring the modifier to its substrate (Scheffner *et al.*, 1995). In contrast the RING domain E3s do not directly bind ubiquitin but facilitate transfer of

ubiquitin through conformational changes in the E2 stimulating the release of ubiquitin from the E2 (Ozkan *et al.*, 2005). Another group of E3 enzymes have been identified named the RING-in-between-RING (RBR) proteins, which appear to act as RING/HECT hybrids. RBR proteins bind to E2s with one RING domain and transfer ubiquitin to a conserved cysteine in the other RING domain leading to an E3~ubiquitin thioester. Two RBR proteins, Hel1 and Itt1 have been identified in *S. cerevisiae* (Finley *et al.*, 2012). Substrate recognition for RING domain E3 ligases is achieved either through a domain within the same polypeptide in the case of single subunit RING domain ligases or through a substrate recognition domain in multi-subunit RING ligases. Specific multi-subunit E3 enzymes such as cullin-RING-ligases (CRLs) and their substrate recognition will be discussed in greater depth later (see Section 1.2.4).

Ubiquitin can be removed from target substrates by deubiquitinating enzymes (DUBs) which catalyse the hydrolysis of the isopeptide bond between ubiquitin and the lysine residue on the target protein substrate. Over 80 DUBs have been identified in humans and these have been classified into five groups. Four of the DUB families are cysteine proteases which are dependent on an active site cysteine residue for their activity while the fifth family are metalloproteases. The catalytic domains of many of the cysteine protease DUBs contain a triad of amino acids which lower the pKa of the catalytic cysteine. This enables a nucleophilic attack on the isopeptide linkage between ubiquitin and the target lysine and subsequent deconjugation of the modifier (Komander *et al.*, 2009; Lee *et al.*, 2013). Twenty DUBs have been identified in *S. cerevisiae* which belong to 4 families. The majority of DUBs in *S. cerevisiae* belong to the Usp family of which there are 16 members. With the exception of 1 DUB all *S. cerevisiae* DUBs are thiol proteases and are involved in both removing ubiquitin from its substrates and also in activating ubiquitin precursors to ensure a pool of free ubiquitin. DUBs are functionally diverse in *S. cerevisiae* and vary in their subcellular localisation and substrate specificity. For example two *S. cerevisiae* DUBs, Ubp6 and Rpn11 are associated with the proteasome and are important for the removal of ubiquitin before proteins are degraded (Guterman and Glickman, 2004). Removal of ubiquitin in this way is important for maintaining a pool of free ubiquitin. Indeed, deletion of *UBP6* leads to increased sensitivity of *S. cerevisiae* cells to a range of drugs including translation inhibitors such as cycloheximide due to a decrease in the pool of free ubiquitin (Chernova *et al.*, 2003).

The action of Ubp6 leads to the release of unanchored free ubiquitin chains which are broken down through the actions of another DUB Ubp14 (Amerik AYu *et al.*, 1997).

1.1.3.2.2 Targets and functions of ubiquitination

The major role for ubiquitin is in directing proteins for degradation and thus the modification is important for a wide range of cellular processes beyond the scope of this introduction. The presence of seven lysine residues in ubiquitin itself (K6, K11, K27, K29, K33, K48, K63) allows ubiquitin to form several different types of chain on proteins which can lead to diverse effects. All ubiquitin linkages except K63 are thought to target proteins for degradation. K48 polyubiquitin chains are the classic signal for protein degradation and are recognised by receptors on the proteasome such as Rpn13 in *S. cerevisiae* leading to the subsequent degradation of the targeted protein (Husnjak *et al.*, 2008). K63 chains however have been linked to non-proteolytic signalling functions particularly as part of the immune response in mammals (Rieser *et al.*, 2013) and the response to oxidative stress in *S. cerevisiae* (Silva *et al.*, 2015).

1.1.3.3 SUMO

Small ubiquitin-like modifier (SUMO) is involved in a diverse range of processes including transcription, replication and DNA repair (Johnson, 2004). A single SUMO-encoding gene is present in *S. cerevisiae* (*SMT3*) and *S. pombe* (*pmt3*$^{+}$) while the human genome encodes four SUMO isoforms (SUMO-1-4). SUMO-2 and SUMO-3 share 97% identity and are described as SUMO-2/3 as antibodies cannot distinguish between the two isoforms (Saitoh and Hinchey, 2000). SUMO-1 and SUMO-2/3 are conjugated to different substrates and differ in their ability to form chains. While SUMO-1 and SUMO-2/3 expression is widely distributed, SUMO-4 has been shown to have tissue specific distribution with high levels found in the immune tissues, kidney and the pancreatic islet cells (Wang and She, 2008). Interestingly, SUMO-4 has been identified as a susceptibility gene for Type 1 diabetes mellitus. SUMO-2/3 and the SUMO modifiers of *S. cerevisiae* and *S. pombe* are able to form chains due to the presence of lysine residues in their N-terminal region (Golebiowski *et al.*, 2009). SUMO chains have been shown to form in response to heat shock and other stresses and act in signalling in the cellular defence to heat shock.

1.1.3.3.1 SUMO conjugation and deconjugation

In contrast to the ubiquitin conjugation pathway, SUMO is conjugated to target substrates using a single heterodimer E1 enzyme (Uba2/Aos1) and a single E2 enzyme (Ubc9). Interestingly, the E2 enzyme of the SUMO conjugation pathway is able to specifically recognise some target substrates and transfer SUMO without the aid of an E3 enzyme (Bernier-Villamor *et al.*, 2002). However, E3 enzymes increase the efficiency of the process. A number of E3 enzymes have been identified for the SUMO conjugation pathway. Siz1 and Siz2 identified in *S. cerevisiae* contain a SP-RING domain comparable to the RING finger domain found in ubiquitin E3 ligases. Deletion of both the *SIZ1* and *SIZ2* genes in *S. cerevisiae* leads to elimination of most but not all SUMO conjugates demonstrating the importance of these enzymes for SUMO conjugation (Johnson and Gupta, 2001). RanBP2 has also been shown to act as a SUMO E3 ligase in mammalian cells although its mechanism is different to either RING or HECT domain containing ligases (Pichler *et al.*, 2004). Mammalian RanBP2 has two repeated sequences designated IR1 and IR2 separated by a short spacer region (M). IR1 binds directly to Ubc9 and mutations in Ubc9 that prevent it binding to IR1 lead to a loss of the RanBP2 E3 SUMO ligase activity. Interestingly, SUMO-2/3 conjugation via RanBP2 requires a strong E2/E3 binding whereas SUMO-1 conjugation does not (Pichler *et al.*, 2004). The difference in the E2/E3 binding for different SUMO isoforms can be explained by the presence of a SUMO-1 binding site in the IR2 region of RanBP2 which binds only to SUMO-1, while the IR1 region of RanBP2 strongly interacts with Ubc9 (Tatham *et al.*, 2005). RanBP2 does not interact directly with substrates but is proposed to accelerate the transfer of SUMO from Ubc9 to the substrate (Pichler *et al.*, 2004; Tatham *et al.*, 2005).

SUMO-specific proteases (SENPs) are a group of cysteine proteases necessary both for SUMO activation and removal of SUMO from target substrates. Six SENPs have been identified in mammalian cells and two in *S. cerevisiae* (Ulp1 and Ulp2). Significantly, all SENPs in *S. cerevisiae* share a conserved C-terminal domain (Mukhopadhyay and Dasso, 2007). *ULP1* is an essential gene in *S. cerevisiae* and *ulp1* temperature sensitive mutants arrest in G2/M phase prior to anaphase at the non-permissive temperature (Li and Hochstrasser, 1999). In contrast, the *ULP2* gene is not essential for growth but *ulp2*Δ cells display increased sensitivity to temperature and a range of DNA damaging agents

and have defects in spindle/microtubule assembly. Ulp1 and Ulp2 have specific targets for SUMO deconjugation as demonstrated by the distinct patterns in the global high molecular weight SUMO conjugates detected in *ulp1* temperature sensitive and *ulp2*Δ strains.

1.1.3.3.2 Targets and functions of SUMOylation

Although the gene encoding SUMO (*SMT3*) is essential for viability in *S. cerevisiae* the equivalent genes (*pmt3*$^{+}$ and *SMT3*) are not essential in the distantly related *Schizosaccharomyces pombe* and the human fungal pathogen *Candida albicans* respectively (Tanaka *et al.*, 1999; Leach *et al.*, 2011). However loss of SUMO in *S. pombe* and *C. albicans* does lead to multiple phenotypes. For example, *S. pombe* cells lacking *pmt3*$^{+}$ display slow growth and increased sensitivity to a range of DNA damaging agents suggesting a role for SUMOylation in the DNA damage response (Tanaka *et al.*, 1999). Moreover, *pmt3*Δ cells display faulty chromosome segregation and elongated telomeres suggesting that SUMOylation plays a role in a wide range of nuclear events. Interestingly, similar phenotypes were also observed in *C. albicans* lacking *SMT3*. For example, *smt3*/*smt3* mutants display defects in nuclear segregation, slow growth and have elongated buds suggesting a role for SUMO in cell cycle regulation (Leach *et al.*, 2011). In contrast to *S. pombe* and *C. albicans*, *S. cerevisiae* cells lacking *SMT3* (or *UBC9* encoding the E2) arrest in G2/M phase of the cell cycle (Li and Hochstrasser, 1999). During anaphase the ubiquitin ligase APC/C is required for the degradation of cohesion between sister chromatids, allowing their separation and exit from mitosis (see Section 1.2.4.2). *ubc9*Δ and *smt3*Δ cells arrest with high levels of two APC/C target substrates Pds1 and Cdc5 suggesting that SUMOylation is required for a fully functioning APC/C in *S. cerevisiae*. Importantly, SUMO was shown not to be required for proteolysis in general as non-APC/C targeted proteins were efficiently degraded in the absence of *UBC9* or *SMT3* (Dieckhoff *et al.*, 2004). The involvement of SUMO for efficient APC/C activity demonstrates overlap between the ubiquitin and SUMO pathways with activity of an ubiquitin E3 ligase being dependent on the SUMO pathway.

SUMOylation has also been linked to the regulation of protein localisation. The small GTPase Ran regulates nuclear transport through conformational changes induced in the protein by GTP binding. RanGAP1 is the GTPase activating protein for Ran and is the

most abundant SUMO-1 conjugated protein in vertebrates (Mahajan *et al.*, 1997). SUMOylation of RanGAP1 on Lys 256 directs the protein from the cytoplasm to the nuclear pore complex (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). Interestingly, SUMOylation has been linked to the premature ageing disease Hutchinson-Gilford progeria syndrome (HGPS). HGPS is caused predominantly by a mutation in lamin A which causes the 'progerin' form of lamin A to remain tethered to the nuclear membrane where it disrupts the structure of the nuclear lamina (Kelley *et al.*, 2011). Cells from HGPS patients display a decrease in nuclear SUMO-2/3 conjugates and a disrupted Ran gradient. However, despite being a SUMO target RanGAP1 SUMOylation and localisation is not affected in these mutant cells. Interestingly, the SUMO pathway E2, Ubc9 is mis-localised to the cytoplasm in HGPS cells and directing Ubc9 into the nucleus restores the Ran gradient in these cells. Together, these observations suggest that correct localisation of Ubc9, SUMOylation and Ran-dependent transport play important roles in HGPS. SUMOylation has also been linked to protein localisation in *S. cerevisiae*. In particular, SUMOylation of the yeast importin Kap114 on Lys909 has been found to be required for its localisation and function as an importin (Rothenbusch *et al.*, 2012). Cells expressing Kap114 where Lys909 has been substituted to an arginine residue, which prevents SUMOylation, and cells lacking the SUMO conjugation machinery display nuclear accumulation of Kap114 and simultaneous cytoplasmic localisation of Kap114 cargo proteins. Moreover, SUMOylation of Kap114 has been demonstrated to be important for the efficient dissociation of Kap114 from its cargo.

1.1.3.4 Rub1

In *S. cerevisiae* Rub1 (Nedd8 in mammals) is the most similar of the Ubls to ubiquitin, sharing 76% sequence similarity. However, the targets of Rub1 conjugation are distinct from ubiquitin. Nedd8/*RUB1* is essential for viability in most model organisms, with the notable exception being *S. cerevisiae* where deletion of the *RUB1* gene leads to no discernible phenotypes (Lammer *et al.*, 1998). Interestingly, in humans deregulation of the Nedd8 pathway has been associated with disease phenotypes including cancer and an inhibitor of the Nedd8 pathway E1, is currently undergoing clinical trials as an anti-cancer therapeutic.

1.1.3.4.1 Rub1 conjugation and deconjugation

Similar to ubiquitin and other Ubls, Rub1 is initially produced as an inactive precursor in *S. cerevisiae* which is processed by Yuh1 (Linghu *et al.*, 2002). Although the ubiquitin C-terminal hydrolase UCH-L3 is able to process Nedd8 in mammals, deletion of UCH-L3 in mice does not cause a loss in viability suggesting the presence of other Nedd8 processing enzymes (Kurihara *et al.*, 2000). Indeed the Nedd8 processing enzyme NEDP1 has been identified that is conserved in *S. pombe* and mammals (although missing from *S. cerevisiae*) (Gan-Erdene *et al.*, 2003). The E1 and E2 enzymes of the Nedd8 conjugation pathway are highly conserved. The E1 is a heterodimer of Uba3 and Ula1 (APPB1 in humans) and a single E2 enzyme, Ubc12 has been identified. As described above, Nedd8 and ubiquitin share a high level of sequence similarity which has the potential to result in cross-talk between the conjugation pathways. However, the Nedd8 pathway employs a number of tools to prevent accidental ubiquitin charging. The residue at position 72 in the C-terminal tail region of ubiquitin and Nedd8 is the only difference between these proteins in this region and importantly this residue conveys specificity for the modifier. Position 72 is occupied by an arginine or an alanine in ubiquitin and Nedd8 respectively. Uba3 contains a conserved arginine residue at position 190 that clashes with Arg72 of ubiquitin but not with Ala72 of Nedd8 ensuring that Uba3 is charged only with Nedd8 (Souphron *et al.*, 2008). The interaction between the E1 and E2 enzymes of the Nedd8 pathway requires a unique N-terminal extension on Ubc12 that also prevents mischarging of Ubc12 with ubiquitin (Huang *et al.*, 2008b).

Several potential E3 enzymes have been identified for different Rub1/Nedd8 substrates. For example, in *S. cerevisiae*, Dcn1 has been identified as a potential E3 in the Neddylation of the cullin Cdc53 (Kurz *et al.*, 2005). Deletion of *DCN1* leads to accumulation of unmodified Cdc53 indicating a role for Dcn1 in the conjugation of Rub1 to Cdc53. Furthermore, Dcn1 directly interacts with both Cdc53 and Ubc12 to enhance Rub1 conjugation, possibly by positioning the charged E2 and the substrate in a favourable position (Kurz *et al.*, 2008).

Removal of Rub1/Nedd8 from cullins is catalysed by the conserved, multi-subunit COP9 signalosome (CSN). The Csn5 subunit is responsible for the catalytic activity of the CSN

due the metalloisopeptidase activity of its JAMM domain (Rabut and Peter, 2008). NEDP1 has also been demonstrated to act as a specific Nedd8 isopeptidase although unlike the CSN, NEDP1 is inefficient at deNeddylating cullins but can remove Nedd8 from other substrates. Deletion of the *nep1*$^{+}$ gene that encodes the NEDP1 homologue in *S. pombe* leads to an increase in Nedd8 conjugates, although the Neddylation status of the cullins remained the same as wild type suggesting Nep1 does not deNeddylate these proteins (Zhou and Watts, 2005).

1.1.3.4.2 Targets and functions of Neddylation

Until recently the only identified targets of Neddylation were cullins. Cullins act as scaffold proteins in the formation of the multi-subunit, ubiquitin E3, cullin-RING ligases (CRLs). The mammalian genome encodes 8 cullins while *S. cerevisiae* contains 3, Cul1/Cdc53, Cul3 and Cul4/Rtt101. The activity of CRLs is regulated by a number of mechanisms. In mammals, CAND1 (Lag2 in *S. cerevisiae*) binds to unmodified CRLs at both the N-terminus, where is competes with the Skp1 for binding, and at the C-terminus where it masks the Neddylation site. CAND1 was originally described as an inhibitor of CRL activity but more recently it has been demonstrated that CAND1 regulates the activity of CRLs by releasing Skp1 and the F-box proteins during inactivation allowing cells to alter their SCF complexes in response to a change in stimuli (Zemla *et al.*, 2013).

Neddylation of cullins activates CRLs by promoting the recruitment of the ubiquitin E2 to the complex (Kawakami *et al.*, 2001) and by inducing a conformational change which eliminates the CAND1 binding site and promotes ubiquitin ligase activity of the CRL (Duda *et al.*, 2008). The role of Neddylation in activation of CRLs is not simple and cycles of Neddylation and deNeddylation of cullins are important for the activity of CRLs. In *Caenorhabditis elegans* Cul3 is required for the degradation of MEI-1 at the mitosis to meiosis transition (Pintard *et al.*, 2003). Reducing the function of either the Nedd8 (NED-8) pathway or inactivation of the CSN pathway leads to a similar impairment of microtubule function likely caused by the presence of MEI-1 in both of these mutants, suggesting that cycles of Neddylation/deNeddylation of Cul3 are required for the ubiquitination and degradation of MEI-1. In *S. cerevisiae* the importance of Rub1 cycling is apparent as deletion of either *RUB1* or *CSN5* together with temperature sensitive

mutants of components of the SCF complex leads to exacerbation of the phenotypes associated with the individual temperature sensitive mutations (Lammer *et al.*, 1998; Cope *et al.*, 2002).

A number of tumour suppressor and oncoproteins have recently been identified as Neddylated substrates in mammalian cells. The tumour suppressor p53 is a transcription factor which upon activation is able to induce a pro-apoptotic, anti-proliferative programme and thus is considered as 'the guardian of the cell'. p53 is ubiquitinated through direct interaction with the oncoprotein Mdm2 leading to its degradation. Interestingly, both Mdm2 and p53 have been identified as substrates of Neddylation with Mdm2 acting as an E3 to promote the Neddylation of p53 leading to an inhibition of p53 transcriptional activity (Xirodimas *et al.*, 2004). The identification of these and other tumour suppressor and oncoproteins as Nedd8 substrates has linked Neddylation with cancer and, furthermore, deregulation of Nedd8 conjugation has been demonstrated in a number of cancers (Reviewed in (Watson *et al.*, 2011)).

As has been described above, ubiquitin/Ubls are involved in the regulation of many different cellular processes. Ubiquitin in particular, plays an important role in regulating a number of steps of the cell division cycle.

1.2 Regulation of the cell cycle

The eukaryotic cell division cycle is a highly organised, sequential process which must be tightly regulated to ensure correct segregation of genetic material. Mis-regulation of the cell cycle can lead to genetic instability and is the route of genetic diseases such as cancer. Studies in *S. cerevisiae* have been a valuable tool in providing insight into the mechanisms underlying cell cycle control and will be described below.

In eukaryotes, the cell division cycle is a process whereby DNA replication, mitosis and cytokinesis occur to produce two daughter cells from a single mother cell. In general, a single cell cycle is divided into four different stages: Gap phase 1 (G1), S-phase, Gap phase 2 (G2) and mitosis. A major regulatory stage of the cell cycle of *S. cerevisiae* occurs during G1 phase when cells must sense and interpret many signals including environmental and size cues and decide whether to commit to a cell cycle, mate with a cell of the opposite mating type or enter a stationary phase (G0) until conditions become favourable. In *S. cerevisiae* the decision of a cell to enter the cell cycle occurs at the end of G1 at Start (Forsburg and Nurse, 1991). Significantly, Start is the equivalent of the mammalian restriction point and is considered as the point where the cell commits to entering the cell cycle in these different organisms.

After commitment to the cell cycle at Start DNA replication, spindle pole body duplication and budding occurs as *S. cerevisiae* cells enter S-phase. Between S-phase and M-phase a second gap phase (G2) ensures that DNA replication has been fully completed before mitosis is initiated. During M-phase the chromosomes condense (prophase) and align on the metaphase plate (metaphase) through the attachment of spindle fibres to the spindle pole body at one end of the cell and to the kinetochore proteins located on the centromere of each chromosome. During anaphase the paired chromosomes are separated at the kinetochores and move to the opposite ends of the cell. Nuclear division occurs during telophase followed by cytokinesis where *S. cerevisiae* divides asymmetrically to produce two new daughter cells of different sizes.

The cell division cycle is highly regulated by a variety of mechanisms including transcriptional regulation, protein stability and protein modifications. A basic overview of these mechanisms in *S. cerevisiae* will be outlined below.

1.2.1 Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs), as their name suggests, are a group of conserved serine/threonine kinases that depend upon the binding of a cyclin for their activity. In *S. cerevisiae* five CDKs have been identified, Cdc28, Kin28, Ssn3, Ctk1 and Pho85 which together with their activating cyclins have roles in processes such as transcription (Kin28, Ssn3 and Ctk1) and regulation of phosphate and glycogen metabolism (Pho85). Pho85 and Cdc28 both play key roles in cell cycle regulation and are described in more detail below.

1.2.1.1 Pho85

The non-essential CDK Pho85 has been demonstrated to play a role in both phosphate sensing and cell cycle regulation. Pho85 is activated by 10 different cyclins which direct the kinase to different substrates. The best characterised substrate of Pho85 is the transcription factor Pho4 which activates the expression of genes involved in phosphate metabolism and vacuole formation. Recently a link between the role of Pho85 in phosphate metabolism and cell cycle regulation has been identified (Menoyo *et al.*, 2013). Pho80 is the main cyclin that activates Pho85 in its phosphate sensing role while Pho81 acts as a CDK inhibitor (Schneider *et al.*, 1994). Under conditions where phosphate is limited Pho85/Pho80 are inhibited by Pho81 leading to the dephosphorylation and nuclear localisation of Pho4 and the subsequent transcription of genes involved in phosphate metabolism (Springer *et al.*, 2003). It has also been proposed that Pho85 can regulate Cln3, a Cdc28 cyclin that acts upstream of Start, in response to changes in cellular phosphate. In replete phosphate conditions Pho85-Pho80 phosphorylates Cln3 stabilising the cyclin, potentially by blocking its degradation. However, when phosphate levels fall, a decrease in Pho85-directed Cln3 phosphorylation leads to Cln3 degradation and a G1 phase cell cycle arrest (Menoyo *et al.*, 2013).

1.2.1.2 Cdc28

Cdc28 is the major coordinator of cell cycle events in *S. cerevisiae*. *CDC28* is an essential gene in *S. cerevisiae* and so its function in regulating the cell division cycle at many different stages has been established using temperature sensitive *cdc28* mutants (Mendenhall and Hodge, 1998). Original *cdc28* temperature sensitive mutants arrested

in G1 phase when moved to the non-permissive temperature, suggesting that Cdc28 was required for cell cycle progression through Start (Hartwell, 1974). However, further studies showed that *cdc28* temperature sensitive mutants moved to the restrictive temperature shortly after Start arrested mainly in G2 phase demonstrating a role for Cdc28 later in the cell cycle (Reed and Wittenberg, 1990). Cdc28 is a stable protein and its expression does not alter during the cell cycle (Betting and Seufert, 1996). Cdc28 is therefore regulated to act at different stages of the cell cycle by multiple mechanisms including phosphorylation, association with stage specific cyclins and cyclin-dependent kinase inhibitors.

The activity of Cdc28 is regulated during the cell cycle through both activating and inhibitory phosphorylation events. For full activation Cdc28 must first be phosphorylated by the CDK-activating kinase, Cak1, on Thr169 before binding to a cyclin. In fact a large pool of phosphorylated Cdc28 is found in the cell throughout the cell cycle available for quick activation by association with the appropriate cyclin (Ross *et al.*, 2000). Cdc28 has been shown to be phosphorylated on Tyr19 after UV or HU treatment suggesting a role for this modification in the DNA damage response. Phosphorylation of Tyr19 has indeed been shown to inactivate Cdc28 leading to a subsequent delay in entry into S-phase.

1.2.2 *Cyclins*

Cdc28 is activated by the association of different cyclins during the cell cycle which directs its activity towards specific substrates at specific cell cycle stages (Figure 1.4). The differential expression, stability and the overlapping functions of some cyclins offers flexibility to cell cycle regulation. In *S. cerevisiae*, Cdc28 interacts with 3 G1 cyclins (Cln1-3) which are responsible for G1 to S phase progression, bud emergence, spindle pole body duplication and activation of the Cdc28-interacting B-type cyclins (Clb1-6) which then regulate further DNA replication and control entry into mitosis. Simultaneous deletion of the three *CLN* genes leads to G1 phase arrest however the presence of any one of the *CLN* genes is able to complement the arrest phenotype of the triple mutant demonstrating redundancy among the cyclins (Richardson *et al.*, 1989).

The specificity of an individual cyclin is achieved by several mechanisms including regulation of transcription, localisation and sensitivity to inhibitors. For example, in

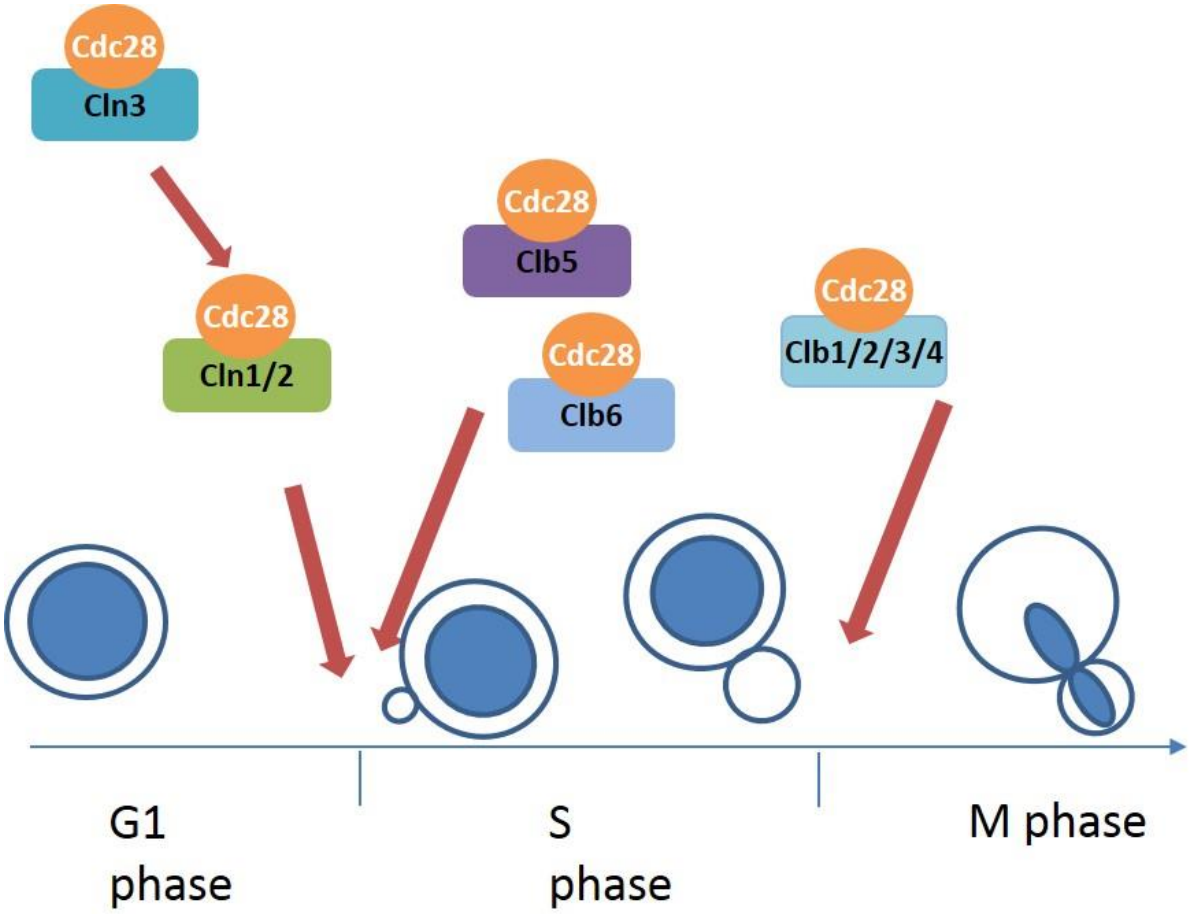


Figure 1.4 Cyclins in the budding yeast cell cycle. The G1 cyclins Cln1, 2 and 3 initiate DNA replication, budding and spindle pole body duplication and activate the expression of the B-type cyclins. The S-phase cyclins Clb5 and Clb6 further activate DNA replication. The M-phase cyclins Clb1, 2, 3 and 4 promote formation of the spindle and initiate mitosis. Figure adapted from Bloom, 2007.

S. cerevisiae, each cyclin gene is expressed at different times during the cell cycle except the *CLN3* gene, a key upstream activator of cell cycle initiation, which is expressed throughout the cell cycle but whose levels peak during late M-early G1 phase (Tyers *et al.*, 1993). Cln3-Cdc28 is responsible for initiating the transcription of the other G1 cyclin encoding genes *CLN1* and *CLN2* through the phosphorylation of the transcriptional repressor Whi5 and the subsequent activation of the SBF transcription factor (Costanzo *et al.*, 2004; de Bruin *et al.*, 2004). The expression of the B-type cyclins occurs sequentially in the cell cycle, *CLB5/6* expression occurs in late G1/S phase followed by *CLB3/4* and finally *CLB1/2*. Cln1, Cln2 and Clb5-Cdc28 complexes are also able to phosphorylate Whi5 providing a potential feedback loop regulating their own expression (Figure 1.7).

Cyclin expression is also controlled by ubiquitin-mediated degradation by the 26S proteasome. Ubiquitination of the G1 cyclins, Cln1 and Cln2 is mediated by the Skp1-Cullin-F-box complex (SCF) ubiquitin ligase SCF^{Grr1} (see Section 1.2.4.1.2). In contrast, the Clb6 B-type cyclin is targeted for ubiquitination by the ubiquitin ligase SCF^{Cdc4} (see Section 1.2.4.1.1), while the other B-type cyclins are targeted for degradation by the anaphase promoting complex (APC) ubiquitin ligase (see Section 1.2.4.2) (Bloom and Cross, 2007). Regulation of cyclins by ubiquitin-mediated proteasomal degradation will be discussed further in Section 1.2.4.

1.2.3 *Cyclin-dependent kinase inhibitors*

Cyclin-dependent kinase activity can also be directly inhibited by cyclin-dependent kinase inhibitors (CKIs). Two CKIs Sic1 and Far1 have been identified in *S. cerevisiae* which bind to and inhibit Cdc28-Clb and Cdc28-Cln kinases respectively.

1.2.3.1 *Sic1*

The CKI, Sic1, is able to bind tightly to Cdc28-Clb kinases but not Cdc28-Cln kinases blocking their activity until late G1/S phase. The inhibitory action of Sic1 is alleviated through its phosphorylation by Cdc28-Cln kinases which target it for degradation by the ubiquitin-proteasome system (see Section 1.2.4). Indeed, in the absence of *CLN1* and *CLN2*, DNA replication is delayed until cells reach a larger size, consistent with the

phosphorylation and degradation of Sic1 being vital for the appropriate timing of DNA replication (Schneider *et al.*, 1996; Verma *et al.*, 1997). Sic1 must be phosphorylated on six of the nine available phosphorylation sites before degradation is triggered (Nash *et al.*, 2001). Multi-site phosphorylation has been proposed to act as a mechanism to protect Sic1 from fluctuations in Cln levels providing a switch to trigger Sic1 degradation only at the appropriate time. Indeed a strain that expresses Sic1 containing a single optimal phosphorylation site is able to inhibit Cdc28-Clb complexes but undergoes DNA replication prematurely leading to genomic instability.

Sic1 must be degraded for Cdc28-Clb activation and DNA replication to occur. Early studies demonstrated that strains containing temperature sensitive mutations in components of the SCF^{Cdc4} ubiquitin ligase (Cdc34, Cdc4, Cdc53 and Skp1) display a G1 phase arrest phenotype together with high levels of Sic1 and low levels of Clb activity suggesting that the SCF^{Cdc4} complex was involved in the degradation of Sic1 (Schwob *et al.*, 1994). Indeed, the F-box protein, Cdc4 has been shown to bind to phosphorylated Sic1 leading to its degradation by the ubiquitin-proteasome pathway (Verma *et al.*, 1997) (Section 1.2.4.1.1).

Sic1 has also been shown to be phosphorylated *in vivo* by the CDK Pho85 and the protein is stabilised in *pho85*Δ cells (Nishizawa *et al.*, 1998). Pho85 phosphorylation of Sic1 is especially important for cell cycle re-entry in G1/S after DNA damage (Wysocki *et al.*, 2006). DNA damage caused for example, by ionising radiation leads to the activation of the effector kinase Rad53 (see section 1.2.5.3) and one downstream outcome of this is to prevent cyclin accumulation in G1 phase and to increase the stability of Sic1. Pho85 phosphorylation of Sic1 when Cdc28-Cln levels are decreased after DNA damage has been proposed to allow timely re-entry into the cell cycle by targeting Sic1 for degradation and activating Cdc28.

1.2.3.2 *Far1*

A second CKI, Far1, has been shown to inhibit the Cdc28-Cln kinases in *S. cerevisiae* particularly in response to mating pheromone. In response to a and α mating pheromones, *S. cerevisiae* cells of the opposite mating type undergo alterations in gene transcription and cell morphology and arrest their cell cycle in G1 phase to allow mating (Chang and Herskowitz, 1990). Far1 is necessary for the G1 phase arrest in response to

pheromone and *far1*Δ cells are unable to arrest in response to α-factor. Deletion of *CLN2* but not *CLN1* or *CLN3* in *far1*Δ cells restores the G1 phase arrest in response to α-factor and Far1 directly inhibits Cdc28-Cln1 and Cln2 (Peter and Herskowitz, 1994).

Far1 mRNA levels have been shown to fluctuate during the cell cycle, peaking in G1 phase. In response to α-factor Far1 protein levels increase by several fold (McKinney *et al.*, 1993). Similar to the regulation of Sic1, temperature sensitive mutations in components of the SCF^{Cdc4} complex are sensitive to the levels of Far1 suggesting a role for SCF^{Cdc4} in the degradation of Far1. Indeed, further studies demonstrated that Far1 is phosphorylated on Ser87 by Cdc28-Cln kinases triggering its degradation via SCF^{Cdc4} mediated ubiquitination (Henchoz *et al.*, 1997).

1.2.4 *Ubiquitin and cell cycle regulation*

As previously discussed (see Section 1.1), conjugation of ubiquitin to substrate proteins requires an enzymatic pathway containing E1, E2 and E3 enzymes. Furthermore, substrate specificity is achieved through different combinations of E2 and E3 enzymes. As described above key regulators of the cell cycle are degraded by the ubiquitin-proteasome system to ensure proper and timely transition through the cell cycle. Ubiquitination of these cell cycle regulators is directed by two different types of ubiquitin E3 ligases, SCF and APC, which target specific substrates to ensure cell cycle progression.

1.2.4.1 *SCF*

In *S. cerevisiae*, the conserved, multi-component SCF ubiquitin ligases consist of 4 subunits, Skp1, Cdc53 (Cul1 in mammals), a RING protein (Hrt1) and an F-box protein, which form a complex with the ubiquitin E2, Cdc34 to direct ubiquitin conjugation to specific target substrates. The importance of SCF complexes for the degradation of cell cycle regulators is demonstrated in temperature sensitive mutants of *cdc34*, *cdc4*, *cdc53* and *hrt1* in *S. cerevisiae*, which arrest at the non-permissive temperature with multiple, elongated buds and which are unable to undergo DNA replication due to their inability to degrade the CKI, Sic1 (Schwob *et al.*, 1994; Seol *et al.*, 1999). The stability of the cyclin Cln2 also increases in *cdc34* and *cdc53* mutants suggesting that an SCF complex is also necessary for Cln2 degradation (Willems *et al.*, 1996).

The crystal structure of the mammalian SCF complex has enabled the role of each of the SCF components in the complex to be studied (Zheng *et al.*, 2002). Cul1 is an elongated, scaffold protein which positions the other subunits and allows docking of the ubiquitin E2. Binding of Hrt1 to the C-terminus of Cul1 recruits ubiquitin while Skp1 binding to the N-terminus of Cul1 leads to F-box recruitment.

SCF complexes are activated by Rub1/Nedd8 modification of Cdc53/Cul1 which has been proposed to enhance the binding of Cdc34 to the SCF complex (Saha and Deshaies, 2008). Negative regulators of SCF complexes have also been identified. Cand1 in mammals and Lag2 in *S. cerevisiae* bind preferentially to unmodified Cul1/Cdc53 inhibiting the actions of the SCF complex (Siergiejuk *et al.*, 2009). In the case of Cand1, binding to Cul1 inhibits Skp1 association with the SCF complex thus preventing formation of a complete SCF. Lag2 binding to Cdc53 on the other hand appears to prevent the ubiquitination activity of the SCF complex either by binding to Cdc34 directly or by eliciting a conformational change that prevents Cdc34 accessing the E3 ligase (Liu *et al.*, 2009). These studies demonstrate the multiple levels of regulation that governs the activity of SCF complexes.

Substrate specificity of the SCF complex is governed by association with different F-box proteins of which 21 have been identified in *S. cerevisiae*. Below, 3 of the F-box proteins that have been linked to cell cycle regulation in *S. cerevisiae* will be discussed.

1.2.4.1.1 *SCF^{Cdc4}*

As described earlier, SCF^{Cdc4} is responsible for directing the ubiquitination and subsequent degradation of the CKIs Sic1 and Far1. Interestingly, SCF^{Cdc4} also directs the ubiquitination of two other important cell cycle regulators, the DNA replication protein Cdc6 and the Gcn4 transcription factor.

In *S. cerevisiae*, Cdc6 is essential for the formation of prereplicative complexes (pre-RCs) at replication origins during late G1 phase. To ensure that replication origins fire just once per cycle, pre-RCs are disassembled after origin firing and, importantly, this requires the degradation of Cdc6. Although strains expressing temperature sensitive mutations of *cdc34*, *cdc4* and *cdc53* are unable to degrade Cdc6 at the non-permissive temperature (Drury *et al.*, 1997), Cdc6 degradation is more complex than these observations imply (Drury *et al.*, 2000). Prior to G1 phase, Cdc6 is degraded in a SCF^{Cdc4}-

independent manner. However, in late G1/S phase Cdc6 is phosphorylated by Cdc28-Cln and rapidly degraded via the SCF^{Cdc4}-mediated ubiquitination pathway.

Gcn4 is a transcriptional activator whose translation and stability is increased in response to amino acid starvation and governs the transcription of amino acid biosynthetic genes (Hope and Struhl, 1985). Again, analyses of *cdc34*, *cdc4*, *cdc53* and *skp1* temperature sensitive mutants implicated SCF^{Cdc4} in the degradation of Gcn4 (Meimoun *et al.*, 2000). Prior to SCF^{Cdc4}-directed ubiquitination, Sic1, Far1 and Cdc6 first undergo phosphorylation by Cdc28. In contrast, Gcn4 phosphorylation is directed by Pho85, which phosphorylates Gcn4 on Thr165 prior to its ubiquitination by SCF^{Cdc4}.

1.2.4.1.2 *SCF^{Grr1}*

The observation that the G1 cyclins Cln1 and Cln2 were more stable in *grr1* mutants suggested that Grr1, a protein previously identified as being required for glucose repression, was also involved in targeting the G1 cyclins for degradation (Barral *et al.*, 1995). Grr1 was subsequently shown to be an F-box protein, able to form complexes with Skp1 and Cdc53 (Skowyra *et al.*, 1997) and to recognise substrates through 12 leucine rich repeats located within the protein (Flick and Johnston, 1991).

1.2.4.1.3 *SCF^{Met30}*

Met30 was originally identified for its role as a component in the methionine biosynthesis pathway in *S. cerevisiae* however, the observation that temperature sensitive *met30* mutants arrest predominantly in G1 phase at the non-permissive temperature suggested a further role for Met30 in cell cycle regulation (Patton *et al.*, 2000). Interestingly, deletion of *MET4*, a transcriptional activator, is able to overcome the requirement for Met30 in *S. cerevisiae* and importantly Met30 was shown to act as an F-box protein directing the ubiquitination of Met4. Met4 is required to induce genes involved in the synthesis of sulphur metabolites such as cysteine, methionine and S-adenosylmethionine and is also important for the cellular response to heavy metal stress induced by cadmium (Kaiser *et al.*, 2006). Unusually, ubiquitination of Met4 by SCF^{Met30} does not trigger its degradation but maintains a pool of inactive protein ready to respond to changes in the environment. Full activation of Met4 leads to cell cycle arrest at several positions and as such Met4 must be kept inactive to prevent cell cycle arrest (Patton *et al.*, 2000). For example, in response to cadmium or decreases in the levels of

sulphur containing compounds, rapid deubiquitination leads to activation of Met4 and cell cycle arrest (Kaiser *et al.*, 2000).

In addition to its essential role in Met4 inactivation, Met30 may also play a role in regulating the expression of the G1 cyclins, Cln1 and Cln2. In particular, *met30* temperature sensitive mutants are unable to initiate S-phase and have decreased expression of the G1 cyclins *CLN1* and *CLN2* (Patton *et al.*, 2000). However, the basis of the G1 phase arrest is still unknown as overexpression of *CLN1* and *CLN2* is insufficient to drive *met30* mutants into S-phase (Su *et al.*, 2005).

1.2.4.2 *APC/C*

The APC/C is a multi-subunit cullin RING ligase (CRL), ubiquitin E3 ligase. Substrate specificity for the APC/C in *S. cerevisiae* is achieved through association of one of two activator subunits, Cdc20 and Cdh1, which recognise substrates through C-terminal WD40 domains (Visintin *et al.*, 1997). During early mitosis, phosphorylation of several subunits of the APC/C by Cdc28-Clb leads to its association with Cdc20 (APC/C^{Cdc20}). A key target of APC/C^{Cdc20} is Pds1. Pds1 is an inhibitor of anaphase, and cells lacking Cdc20 arrest in metaphase, unable to pass through to anaphase due to their inability to degrade Pds1 . Cohesion between sister-chromatids depends on the cohesin complex consisting of 4 subunits, Scc1, Scc3, Smc1 and Smc3. At the metaphase to anaphase transition, when chromatids are aligned on the metaphase plate, ubiquitination of Pds1 by APC/C^{Cdc20} activates separase, a protease that promotes chromosome segregation, mediating the dissociation of the cohesin subunit Scc1 from the cohesin complex allowing separation of chromatids (Cohen-Fix *et al.*, 1996; Uhlmann *et al.*, 1999). APC/C^{Cdc20} is also responsible for the degradation of the B-type cyclins, Clb2 and Clb5 allowing cells to exit mitosis (Shirayama *et al.*, 1999; Wasch and Cross, 2002).

In contrast to Cdc20, Cdh1 is present throughout the cell cycle but is only bound to the APC/C during G1 phase. At the G1-S phase transition, Cdh1 is inactivated by phosphorylation directed by the S-phase CDKs, particularly Cdc28-Clb5. During late mitosis the decrease in Cdc28 activity due to APC/C^{Cdc20} and the activation of the Cdc14 phosphatase leads to the dephosphorylation and subsequent activation of Cdh1. Interestingly, although deletion of the *CDH1* gene has little effect on cell viability or cell cycle progression *cdh1*Δ cells have high levels of the cyclin Clb2 and the spindle

component Ase1 consistent with Cdh1 being necessary for mitotic exit (Visintin *et al.*, 1997).

1.2.5 *Cell division cycle checkpoints*

The cell cycle must be coordinated to ensure that each stage is complete before the next is started. For example, it is essential that DNA replication is complete before mitosis occurs to ensure that the mother and daughter cell each receive the correct compliment of genetic material. To ensure cell cycle fidelity there are a number of cell cycle checkpoints in place. A checkpoint is defined as a point where progression of the cell cycle arrests until certain minimum requirements have been met. Several cell cycle checkpoints have been identified in *S. cerevisiae* and these will be discussed below.

1.2.5.1 *Morphology checkpoint*

The morphology checkpoint operates between G2 and M phases and is activated by perturbations in bud morphology, the actin cytoskeleton or cell wall synthesis. The morphology checkpoint couples the nuclear cell cycle to bud formation and acts to prevent the cell from undergoing multiple DNA replication events in the absence of budding.

The Swe1 kinase in *S. cerevisiae* is the homologue of Wee1 in *S. pombe*. Swe1 accumulates in G1 and S phases and its degradation occurs at the end of G2 to ensure the cell moves from G2 to M phase. Exposure of cells to stresses such as heat or osmotic shock has been shown to cause actin depolarisation and delayed bud formation (Delley and Hall, 1999). Swe1 accumulates in response to the stresses which delay bud formation suggesting that Swe1 levels are involved in triggering the morphology checkpoint (Sia *et al.*, 1996).

Swe1 phosphorylation of Cdc28 on Tyr19 decreases Cdc28 kinase activity leading to a delay in mitosis (Booher *et al.*, 1993). Swe1 activity in turn is regulated by its localisation. At G2/M phases, Swe1 translocates from the nucleus to the bud neck where it is hyper-phosphorylated and subsequently undergoes degradation mediated by SCF^{Met30} (Kaiser *et al.*, 1998). Hyper-phosphorylation of Swe1 has been shown to be vital for entry into mitosis. After Swe1 is degraded, Tyr19 phosphorylation of Cdc28 is

removed by Mih1 phosphatase leading to active Cdc28-Clb2 and entry into mitosis (Booher *et al.*, 1993).

1.2.5.2 *Spindle Checkpoint*

The spindle checkpoint ensures correct chromosome segregation by delaying anaphase until all sister chromatids have achieved bipolar attachment. Checkpoint activation occurs in response to unattached kinetochores or kinetochores lacking in tension and leads to inhibition of the APC/C activator Cdc20. The majority of spindle checkpoint genes were identified in two genetic screens in *S. cerevisiae*, one that identified the mitotic arrest deficient (*MAD*) genes required for mitotic exit dependent on completion of spindle assembly (Li and Murray, 1991) and another that identified the budding uninhibited by benzimidazole (*BUB*) genes required for cell cycle arrest in response to loss of microtubule function (Hoyt *et al.*, 1991). The checkpoint genes identified in these screens: *MPS1*, *MAD1*, *MAD2*, *MAD3* (*BUBR1*), *BUB1* and *BUB3* are highly conserved and, although mutants display chromosome segregation defects, all but *MPS1* are non-essential in *S. cerevisiae* (Li and Murray, 1991).

During a normal cell cycle there is no delay before anaphase to complete biorientation which may explain the non-essential nature of many of the genes whose products are required for the spindle checkpoint. The most upstream signal for spindle checkpoint activation appears to be phosphorylation of the mitotic spindle protein, Spc105 by the kinase Mps1 leading to the recruitment of downstream checkpoint components to the kinetochore (London *et al.*, 2012). While Bub1 and Bub3 always localise to the kinetochore during mitosis, Mad1 and Mad2 only localise to unattached kinetochores. Mad1 is stably bound to kinetochores while Mad2 exists in two pools (De Antoni *et al.*, 2005). One pool of Mad2 is stably bound to Mad1 at the kinetochore and another pool of Mad2 rapidly cycles on and off the kinetochore. Binding of Mad2 to Mad1 induces a closed form of Mad2 which remains bound to Mad1 at the kinetochore. The second pool of soluble 'open' Mad2 is able to bind to the Mad1/Mad2 complex at the kinetochore where it is converted to the closed form. Closed Mad2 binding to Cdc20 as part of the mitotic checkpoint complex (Mad2, Mad3, Bub3, Cdc20) is a powerful inhibitor of the APC/C (Biggins, 2013). Silencing of the checkpoint is determined by PP1-

dependent dephosphorylation of the Mps1-mediated phosphorylation of Spc105 (London *et al.*, 2012).

1.2.5.3 *DNA damage checkpoint*

Faithful replication of DNA is crucial to maintain genomic integrity and cell viability therefore damage to DNA must be repaired. DNA damage must first be detected by damage sensors which are able to activate the checkpoint leading to a delay in cell cycle progression until the damage has been repaired. Many forms of DNA damage are processed to form single-stranded DNA (ssDNA) and it is the binding of the RPA protein to this ssDNA that often leads to checkpoint activation (Branzei and Foiani, 2006). In *S. cerevisiae*, checkpoint activation leads to the recruitment of the checkpoint clamp (Ddc1, Rad17 and Mec3) and Mec1 kinase to the site of the damage. Activated Mec1 then phosphorylates a large number of proteins and its activity is highly regulated. Two targets of Mec1 are the effector kinases Rad53 and Chk1. After initial activation, Rad53 and Chk1 undergo further phosphorylation stimulated by Rad9 leading to the phosphorylation of key downstream targets including those important for cell cycle arrest (Sweeney *et al.*, 2005).

1.2.5.4 *Start*

Start is the equivalent of the restriction point in mammalian cells where cells commit to cell division regardless of changes in upstream signals. Cells must reach a critical size before they commit to Start (Dungrawala *et al.*, 2012). Alongside external factors such as nutrient availability, a number of genes have been identified that play a role in cell size regulation. A screen of small size mutants found that regulators of Start and ribosome biogenesis were necessary for size control suggesting a link between cell growth and division (Jorgensen *et al.*, 2002). A major upstream regulator of Start is the cyclin Cln3. Cln3 is a low abundant and unstable protein whose expression unlike other cyclins does not vary much during the cell cycle (Tyers *et al.*, 1993). However, Cln3 is regulated at the post-transcriptional level. For example, during early G1 phase Cdc28-Cln3 complexes are sequestered at the ER by an association with the Hsp70 chaperone protein Ssa1 (Vergés *et al.*, 2007). In late G1 phase Cdc28-Cln3 binding to Ssa1 is displaced by Ydj1, a DNAJ-related co-chaperone allowing Cdc28-Cln3 to move to the nucleus and initiate Start. During G2/M phases Ssa1 undergoes phosphorylation on

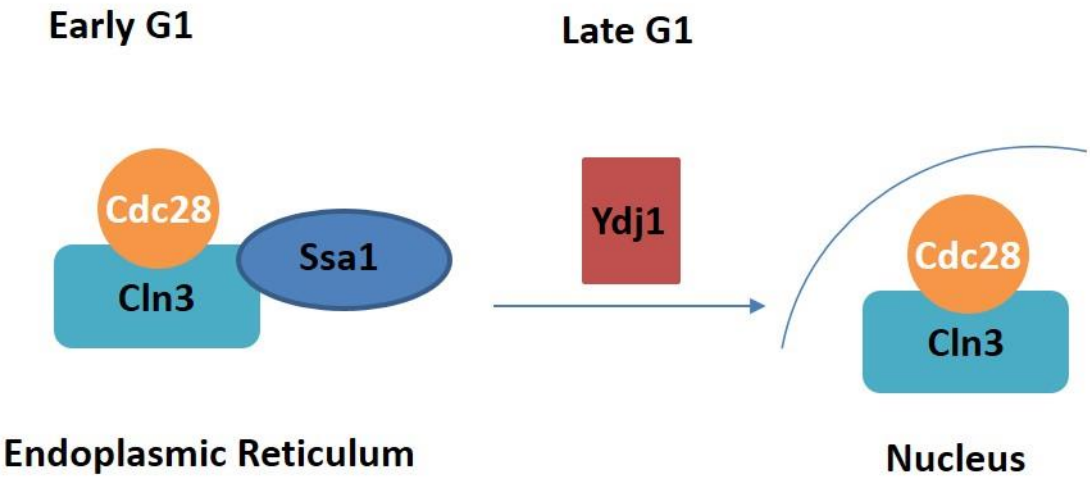


Figure 1.5 Cln3 regulation by Ssa1 and Ydj1 during the cell cycle. In early G1 phase Cdc28-Cln3 is sequestered at the ER through interactions between the J-like domain in Cln3 and the Hsp70 protein Ssa1. In late G1, Cln3 binding to Ssa1 is displaced by Ydj1 allowing Cln3 to translocate to the nucleus and initiate Start.

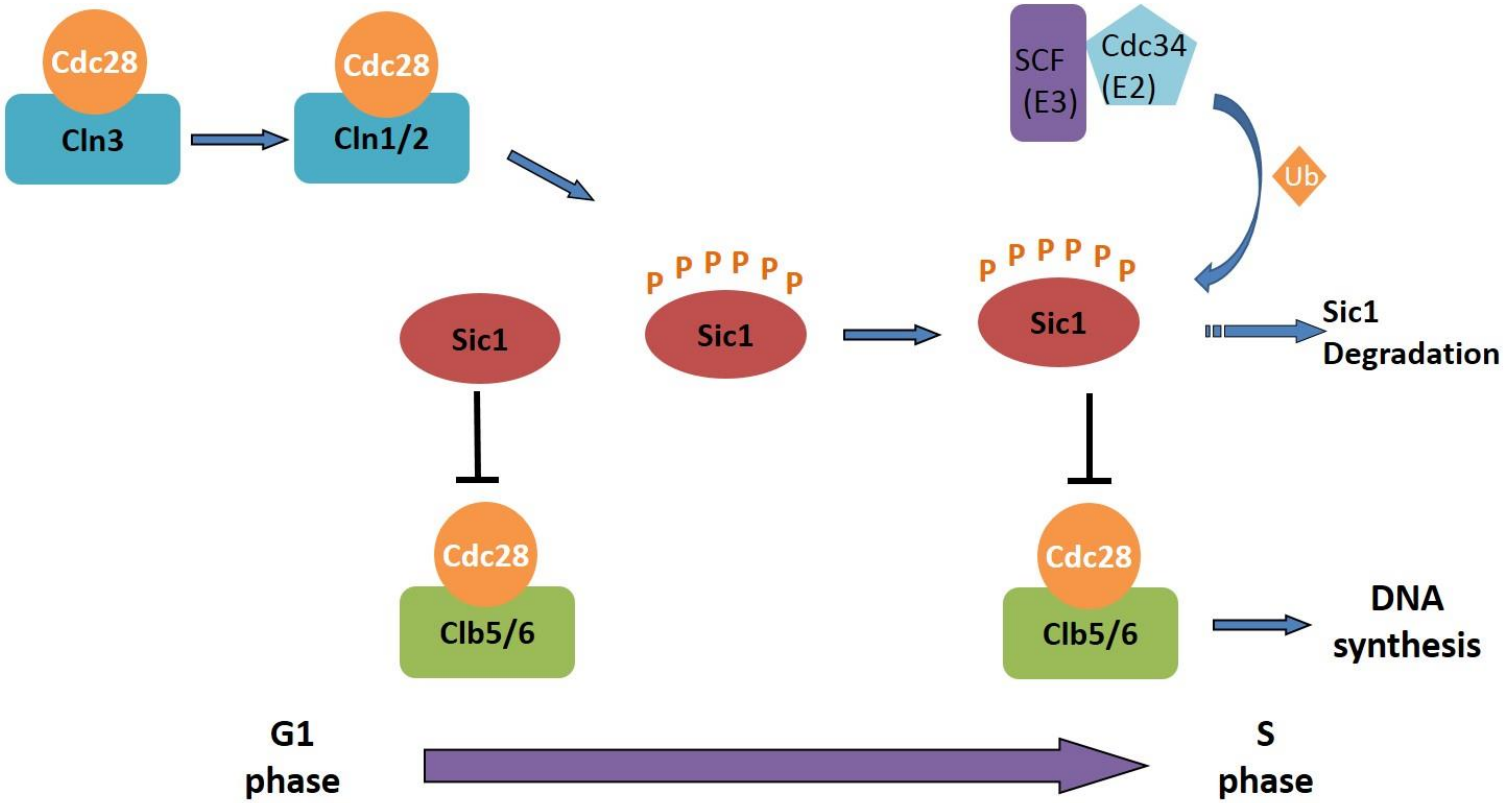
Thr36 by the CDKs Cdc28 or Pho85 disrupting the interaction between Ssa1 and Ydj1 and freeing Ssa1 to interact with Cln3 again (Truman *et al.*, 2012)(Figure 1.5).

As the upstream activator of Start, Cln3 has been shown to be sensitive to nutritional changes (Gallego *et al.*, 1997) and cellular growth rate (Tyers *et al.*, 1993). Both Cln3 and Ydj1 are rate limiting for cell cycle entry and over expression of either leads to the cells entering the cell cycle at a smaller size (Ferrezuelo *et al.*, 2012). Activated Cdc28-Cln3 enters the nucleus and activates the transcription factors SBF (consisting of Swi6-Swi4) and MBF (consisting of Swi6-Mbp1) through the inhibition of Whi5 as described in section 1.2.2. The activation of SBF and MBF leads to the transcription of nearly 200 cell cycle related genes encoding proteins involved in the three major processes that are initiated at Start: initiation of DNA replication, spindle pole body duplication and bud formation. The expression of the cyclins Cln1 and Cln2 is regulated by SBF and MBF. Cln1 and Cln2 themselves have been shown to act in a positive feedback loop to activate their own expression to ensure irreversible Start initiation (Skotheim *et al.*, 2008). A key target of Cdc28-Cln1/2 is Sic1 which inhibits Cdc28-Clb kinases (see Section 1.2.3.1). Phosphorylation of Sic1 is directed by Cdc28-Cln1/2 and leads to its degradation by the ubiquitin-proteasome system. Ubiquitination of Sic1 lifts its inhibitory action on Cdc28-Clb leading to the initiation of S-phase (Figure 1.6).

1.2.6 *Aspects of the cell cycle conserved between S. cerevisiae and mammalian cells*

Given the high level of conservation between *S. cerevisiae* and mammalian cells in many aspects of cell cycle regulation *S. cerevisiae* is a powerful genetic model for studying the mechanisms underlying this regulation.

In both yeast and human cells, the cell cycle is driven by the actions of CDKs. As discussed earlier, a single CDK, Cdc28 pairs with a number of cyclins during the cell cycle to determine proper cell cycle progression in *S. cerevisiae*. The mammalian cell cycle is regulated by more CDKs that, together with activating cyclins, control progression through each stage of the cell cycle demonstrating the increase in complexity of the cell cycle in higher eukaryotic cells. Progression through S phase in mammalian cells is promoted by the Cdk2-cyclinA complex whose activity is inhibited by the CKI $p27^{KIP1}$ (Figure 1.7). Interestingly, although Sic1 shares a low level of sequence homology with $p27^{KIP1}$ and another mammalian CKI $p21^{CIP1}$, the three CKIs have been shown to share



. **Figure 1.6 The G1 to S phase transition in S. cerevisiae is known as Start.** See text for details.

structural similarity in their inhibitory domain (Barberis *et al.*, 2005). Moreover, Sic1 is able to bind to and inhibit the activity of Cdk2-cyclinA *in vitro* while KIP1 over expression in a *sic1*Δ mutant was shown to rescue the cell cycle defects associated with loss of Sic1. Together these results demonstrate that Sic1 and $p27^{KIP1}$ can be considered as structural and functional homologues.

High levels of $p27^{KIP1}$ are found in quiescent cells which decrease after growth stimulation as the cells enter S-phase (Pagano *et al.*, 1995). Both Sic1 and $p27^{KIP1}$ undergo ubiquitin-mediated degradation following CDK-dependent phosphorylation. As in yeast, ubiquitination of $p27^{KIP1}$ is directed by an SCF ubiquitin ligase with the F-box protein Skp2 (Carrano *et al.*, 1999). Skp2 binds phosphorylated $p27^{KIP1}$ more predominantly in S-phase than G1 phase consistent with its identified role in allowing S-phase entry through $p27^{KIP1}$ degradation.

The human homologue of *CDC34* is able to complement a temperature sensitive *cdc34* strain at the non-permissive temperature (Plon *et al.*, 1993). Furthermore the ubiquitination of $p27^{KIP1}$ has been shown to be mediated through Cdc34 (Block *et al.*, 2005).

Both the cell cycle and the ubiquitin/Ubl conjugation pathways are regulated by and in response to ROS. How these ROS are produced and how the cell responds to these species are focused upon in the next section.

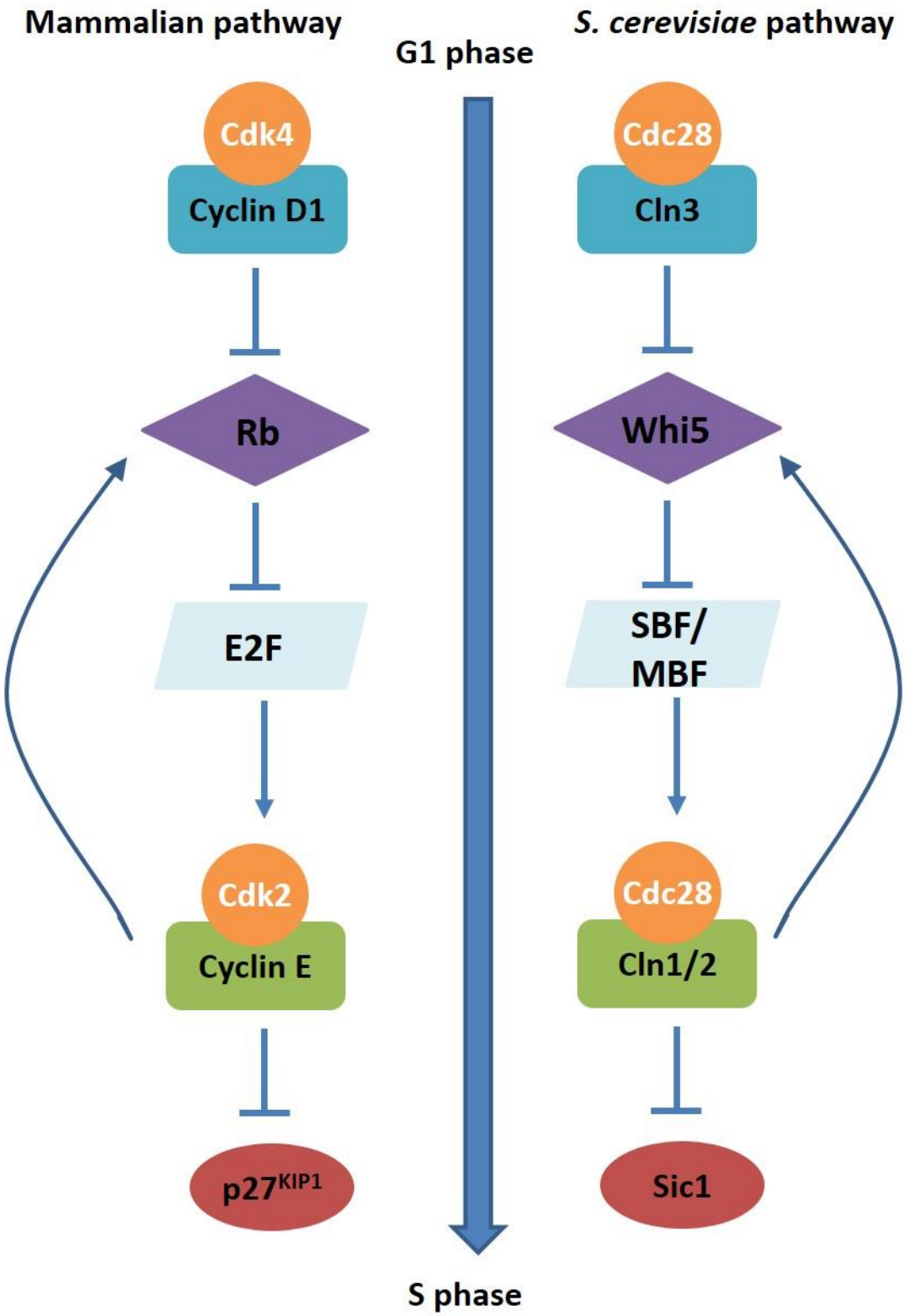


Figure 1.7. The transition from G1 to S phase in mammalian and *S. cerevisiae* shares functional homology. In mammalian cells phosphorylation of Rb by CyclinD-Cdk4 leads to the expression of E2F-regulated genes including CyclinE which in complex with Cdk2 drives further phosphorylation of Rb. In *S. cerevisiae*, Cdc28-Cln3 phosphorylates Whi5 leading to the expression of genes including *CLN1* and *CLN2* which are regulated by the SBF and MBF transcription factors. Adapted from Costanzo 2004

1.3 Oxidative Stress

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide anion ($O_2^{\cdot-}$) arise as a result of normal cellular metabolism and also from the exposure of cells to external stimuli such as heavy metals and UV radiation. Oxidative stress occurs if the levels of ROS exceed cellular antioxidant capabilities and can lead to damage of cellular macromolecules such as proteins, lipids and DNA. This damage has been linked to diseases such as cancer. To limit the detrimental effects of high levels of ROS the cell employs a number of defence mechanisms including antioxidant pathways and cell cycle delay. However, while the detrimental effects of ROS have long been established, ROS have also been shown to play key roles in cellular signalling. To use a potentially cytotoxic molecule in signalling the cell must tightly regulate its generation, transduction and removal. How the cell detects the level of ROS and mounts the appropriate downstream response is one of the main focuses of this study.

1.3.1 Types and sources of ROS

There are many types of ROS that can be either non-radical such as hydrogen peroxide (H_2O_2) or free radicals such as the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot}$). Free radicals are defined as 'any species capable of independent existence that contains one or more unpaired electron' and can thus act as powerful oxidants (Halliwell and Gutteridge, 2007).

While relatively stable, $O_2^{\cdot-}$ is the precursor of other types of ROS. Dismutation of $O_2^{\cdot-}$ either spontaneously, or through the action of superoxide dismutase enzymes (SODs), leads to the production of H_2O_2. H_2O_2 itself is only a weak oxidising agent but reactions between H_2O_2 and metal ions can lead to the formation of the highly reactive $OH^{\cdot}$, which accounts for most if not all of the damage to DNA in H_2O_2-treated cells (Spencer *et al.*, 1995).

Leakage of electrons from the mitochondrial electron transport chain produces the largest pool of intracellular ROS and it has been suggested that between 1% and 3% of oxygen reduced in the mitochondria may form $O_2^{\cdot-}$ although the true percentage may be less (Halliwell and Gutteridge, 2007). The transition metals that are central to the activity of many enzymes are also endogenous sources of ROS. The ability of transition metals to accept or donate electrons and their reaction with H_2O_2 can lead to the

formation of $OH^{\cdot}$. ROS can also arise from environmental sources such as UV light and xenobiotics.

1.3.1.1 The electron transport chain

Cellular energy in the form of ATP is produced predominantly in the mitochondria by the electron transport chain. Free energy from the oxidation of glucose and the citric acid cycle are stored as NADH and $FADH_2$. During respiration electrons are released from NADH and $FADH_2$ and transferred to O_2 via four complexes (complex I-IV) to form H_2O. Simultaneously protons are pumped against their concentration gradient from the inner membrane space to the outer membrane increasing the pH of the mitochondrial matrix. The energy harnessed through this proton motive force drives protons back through ATP synthase in the inner mitochondrial membrane leading to the formation of ATP from ADP and phosphate (Brand *et al.*, 2004).

Leakage of electrons from the mitochondrial electron transport chain is a major cause of cellular ROS. Complexes I and III have been identified as the major sites of superoxide generation in the mitochondria of higher eukaryotes. *S. cerevisiae* does not have a complex I but instead has three rotenone insensitive NADH dehydrogenases located at the mitochondrial inner membrane space (Herrero *et al.*, 2008). Two of the NADH dehydrogenases have their active sites facing the mitochondrial intermembrane space rather than the mitochondrial matrix and these 'external' NADH dehydrogenases account for approximately half of the mitochondrial ROS production in yeast (Fang and Beattie, 2003). Similar to higher eukaryotes, the other 50% of ROS produced by the electron transport chain in *S. cerevisiae* arises from the cytochrome *bc1* complex (complex III). Mitochondria are thought to consume more than 90% of oxygen used by the cell. Donation of electrons by the electron carriers of the electron transport chain to molecular oxygen can lead to the formation of $O_2^{\cdot -}$ which can be dismutated to H_2O_2. Subsequent reactions of H_2O_2 with transition metals can lead to the formation of highly reactive $OH^{\cdot}$.

1.3.1.2 Transition metals

Most transition metals exist with unpaired electrons and thus can be thought of as radicals. Transition metals act as co-factors for many proteins and are essential for a large number of critical cellular functions. Iron for example is required for iron sulphur

(Fe-S) clusters which are involved in electron transfer, catalysis and many regulatory processes (Lill, 2009). Many of the biological roles of transition metals arise from their ability to accept or donate electrons (Halliwell and Gutteridge, 2007). This ability can also have detrimental effects in the cell as oxidation of oxygen by transition metals can produce ROS. In the Fenton reaction, unpaired iron (Fe(II)) reacts with H_2O_2 to form the highly reactive $OH^{\cdot}$. Superoxide is also able to react with ferric iron (Fe(III)) in the Haber Weiss reaction producing Fe(II) in a redox cycling reaction (Figure 1.8). The interaction of transition metals with cellular components such as DNA, proteins and lipids can therefore lead to oxidative damage of these molecules under oxidising conditions.

$$O_2^{\cdot-} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$$ **Haber Weiss**

(i) $Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe2^+ + O_2$

Fenton reaction

(ii) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$

Figure 1.8 The Haber Weiss and Fenton reactions.

1.3.1.3 The immune system

ROS can also have beneficial roles in the producing organism for example, the first line of immune defence against pathogens is their engulfment by the phagocytic cells of the immune system which produce ROS to kill them. Upon phagocytosis the level of ROS produced in the phagosome increases due to the conversion of O_2 to $O_2^{\cdot-}$ by the NADPH oxidase (Nox) and its subsequent dismutation to H_2O_2. The phagocytic Nox consists of 2 integral membrane proteins and several cytosolic proteins which remain separate under resting conditions. Cell stimulation in response to a pathogen leads to assembly of the Nox enzyme and the formation of $O_2^{\cdot-}$ (Dupre-Crochet *et al.*, 2013). It is unclear how this 'respiratory burst' kills the pathogen but its importance is demonstrated in patients with

chronic granulomatous disease, where a mutation results in the absence or dysfunction of the proteins that form the phagocytic Nox and is characterised by recurrent bouts of infection.

1.3.1.4 UV and ionising radiation

Direct exposure of skin cells to UV radiation can cause damage to the skin. Although a fairly weak oxidising agent, UVA light is able to cause damage to DNA by stimulating the production of, for example, thymine dimers through indirect ROS production by photosensitisers (Marrot and Meunier, 2008). In contrast, ionising radiation are high in energy and are able to displace electrons from molecules. For example, ionising radiation passing through water can form $H^{\cdot}$ and $OH^{\cdot}$ (Halliwell and Gutteridge, 2007).

1.3.2 Intracellular damage by ROS

ROS are able to react with cellular macromolecules such as proteins, lipids and DNA. Consequences of oxidative damage to these cellular molecules will be discussed below.

1.3.2.1 Proteins

Oxidative damage to proteins can be either reversible or irreversible. For example, $OH^{\cdot}$ can cause cleavage of the protein backbone or oxidise the side chains of amino acids to aldehydes or ketones resulting in protein carbonyls (Moller *et al.*, 2011). Significantly, protein carbonylation is an irreversible protein oxidation event which cannot be reversed by the antioxidant systems. Protein carbonyl groups accumulate within cells and are a useful tool for measuring oxidative damage in cells (Costa *et al.*, 2002). A correlation between the level of oxidised proteins within the cell and ageing has been identified with older cells displaying higher levels of oxidised proteins compared to younger cells (Cabiscol *et al.*, 2014). Interestingly, specific groups of proteins such as heat shock proteins and those involved in amino acid metabolism have been shown to be particularly prone to carbonylation suggesting functions that may particularly be affected in ageing cells.

The thiol groups of methionine and cysteine are particularly susceptible to oxidation. Oxidation of cysteine residues can lead to the formation of reversible disulphide bridges or sulphenic acid groups (Figure 1.9). Sulphenic acid groups can be oxidised to sulphinic or oxidised further to irreversible sulphonic groups. Cysteine residues with a low pKa

are particularly susceptible to oxidation. Interestingly, few cysteine residues exist with a low pKa within the cell and thus these can be specifically targeted in redox cellular signalling pathways and will be discussed later (See Section 1.3.5).

1.3.2.2 Lipids

Lipids along with proteins make up a large proportion of cellular membranes. Initiation of lipid peroxidation occurs when a hydrogen atom is abstracted from a methylene (-CH_2-) group by several ROS species including $OH^{\cdot}$, resulting in a carbon radical (Halliwell and Gutteridge, 2007). Polyunsaturated fatty acids are particularly susceptible to hydrogen abstraction due to the presence of a carbon double bond which weakens the C-H on the carbon atom surrounding the double bond. The initiation reaction forms a lipid radical which is able to react with O_2 to form the peroxyl radical. Propagation occurs as the peroxyl radical itself is able to abstract hydrogen from an adjacent fatty acid side chain (Catala, 2010). Lipid peroxidation can have a variety of effects on the lipid bilayer that makes up cell and organelle membranes, for example, decreasing their fluidity and potentially damaging membrane proteins.

1.3.2.3 DNA

The frequency of oxidative DNA damage has been estimated at 10^4 lesions/cell/day in humans and thus is potentially a major source of DNA mutation (Halliwell and Gutteridge, 2007). ROS are able to attack DNA either by targeting the deoxyribose sugar or by modifying individual bases which can lead to several different products. For example, reaction of guanine with $OH^{\cdot}$ can lead to the formation of 8-hydroxyguanine (8-OHdG), an abundant base mutation in mammalian cells. 8-OHdG is able to base pair with either cytosine or adenine during DNA replication and, if left unrepaired, the GC→AT transversion becomes a permanent mutation in the DNA (Cheng *et al.*, 1992). 8-OHdG is repaired by the base excision repair pathway which uses DNA-glycosylases to remove the modified bases (Lu *et al.*, 2001).

1.3.3 Implications in disease and ageing

As described above, the oxidation of DNA, lipids and proteins has potential to damage these essential macromolecules and thus affect their function. As a consequence it is not surprising that oxidative stress has been linked with a range of disease pathologies

and ageing. The brain is particularly sensitive to oxidative damage due to its high requirement for oxygen together with the presence of high levels of fatty acids and iron. Indeed, high levels of protein carbonyls and 8-OHdG, two markers of oxidative damage to protein and DNA have been found in the brains of patients with Alzheimer's disease (Butterfield *et al.*, 2001).

The relationship between ROS and cancer appears complex. For example, one of the hallmarks of cancer is an oxidative switch whereby cancer cells survive with a much higher level of cellular ROS due to changes in cellular metabolism and aberrant antioxidant levels. Furthermore, although a number of solid tumours have been shown to have an increased level of 8-OHdG it is currently unknown whether ROS play a role in cancer initiation (Foksinski *et al.*, 2000; Sanchez *et al.*, 2006). Production of ROS is associated with the inflammatory response and increases in ROS are associated with a number of pathologies. For example, ROS regulate numerous stages in angiogenesis and dysregulation of ROS signalling pathways in cancer cells leads to an abnormal vascular pattern (Kim and Byzova, 2014). Thus, while ROS are implicated in cancer and in fact many diseases, it is important to establish whether ROS are the cause or a secondary consequence of the disease. This is particularly important as many disease pathologies are linked with inflammation which is associated with an increase in ROS.

ROS have been linked to longevity/the ageing process. For example, the oxygen theory of ageing states that ageing is a result of the deleterious effects of oxidative damage on cellular constituents (Harman, 1956). A large body of research at least partly supports this theory. For example, old *S. cerevisiae* mother cells display an increase in intracellular ROS even in the absence of extracellular sources (Laun *et al.*, 2001). Furthermore, the levels of protein carbonyls have been shown to increase exponentially in the last third of the lifespan in a number of different human cell types as well as in *C. elegans*, rat liver and house flies (Levine, 2002). Mutations in antioxidant pathways also suggest an involvement of ROS in ageing. For example, deletion of the mitochondrial superoxide dismutase (MnSOD) in *S. cerevisiae* leads to a loss of viability proposed to be due to the build-up of mitochondria-produced ROS inhibiting specific respiratory enzymes (Longo *et al.*, 1999). Consistent with the yeast studies overexpression of MnSOD in *Drosophila* has been shown to increase lifespan (Sun *et al.*, 2002). However, experiments exploring the effects of dietary antioxidants on the lifespan of a range of organisms have been

inconclusive. These data along with the increased prevalence of neurodegenerative diseases and cancer in the older population implicate ROS, at least in part, in the ageing process.

1.3.4 Defences against ROS

Cells have a number of antioxidant mechanisms in place to prevent ROS-induced cellular damage. These antioxidant pathways work together with other mechanisms such as repair pathways and cell cycle regulation to prevent cellular damage. Below, the antioxidant defences of *S. cerevisiae* and the regulatory mechanisms underlying their expression will be discussed.

1.3.4.1 Transcriptional regulation in the defence against ROS

Yap1 is an AP-1 like transcription factor that induces the expression of a number of antioxidant genes including *TRX1*, *TSA1* and *GSH1* encoding thioredoxin, thioredoxin peroxidase and γ-glutamylcysteine respectively. Yap1 binds specifically to Yap1 recognition elements (YREs) found in many gene promoters although some Yap1 targets do not contain YREs suggesting additional recognition sites (Lee *et al.*, 1999). In non-stressed conditions, Yap1 is localised to the cytoplasm. However, in response to oxidative stress Yap1 accumulates in the nucleus and this nuclear localisation is linked to changes in the redox status of two cysteine rich domains that prevent its association with the nuclear export factor Crm1 (Yan *et al.*, 1998; Delaunay *et al.*, 2000). Interestingly, while exposure to either H_2O_2 or the thiol oxidising agent diamide leads to Yap1 nuclear localisation the regulation of Yap1 in response to these two agents differs (Delaunay *et al.*, 2000; Kuge *et al.*, 2001). In response to diamide, Yap1 forms an intra-molecular disulphide bond between closely located cysteine residues in the C-terminal while H_2O_2 stimulates the formation of an intra-molecular disulphide bond between specific cysteine residues in the N and C-terminal regions of Yap1. Yap1 regulation in response to H_2O_2 also requires either Gpx3 or Ybp1 (Delaunay *et al.*, 2002; Veal *et al.*, 2003). Gpx3 promotes the activation of Yap1 through the formation of a transient inter-molecular disulphide bond between itself and Yap1 followed by a thiol exchange reaction leading to the intra-molecular Yap1 disulphide described above and recycling of Gpx3 (Delaunay *et al.*, 2002). Ybp1 has also been demonstrated to be important for the efficient nuclear accumulation of Yap1 in response to H_2O_2 (Veal *et al.*, 2003).

Significantly, a *ybp1*Δ*gpx3*Δ mutant is no more sensitive to H_2O_2 than either the *ybp1* or *gpx3* single mutant suggesting that Ybp1 and Gpx3 act through the same pathway to regulate Yap1.

Approximately half of the proteins induced by oxidative stress are dependent on Yap1 while the other half are dependent on both Yap1 and another transcription factor Skn7 (Mulford and Fassler, 2011). Unlike Yap1, Skn7 is predominantly localised to the nucleus in both stressed and unstressed conditions (Raitt *et al.*, 2000). In addition to the oxidative stress response Skn7 has also been implicated in the regulation of cell wall synthesis and the cell cycle (Herrero *et al.*, 2008).

The Msn2/4 transcription factors are associated with the general response to environmental stresses not just oxidative stress. The Msn2/4 transcription factors bind to and activate genes containing the stress response element in response to a range of environmental signals including H_2O_2, osmotic and heat stresses (Hasan *et al.*, 2002). Msn2/4 control the expression of 27 proteins upregulated in response to H_2O_2, 7 of these are also Yap1 targets while 1 is also a Yap1/Skn7 target.

Met4 is a bZip transcriptional activator that regulates the expression of genes involved in the synthesis of the sulphur containing amino acids cysteine and methionine. Met4 lacks a DNA binding domain and hence is localised to the promoters of its target genes in a complex with either Met28 and Cbf1 (Kuras *et al.*, 1997) or Met28 and Met31 or Met32 (Blaiseau *et al.*, 1997) depending on the Met4-regulated gene. In response to cadmium-induced ROS, Met4 induces the expression of *GSH1* which encodes γ-glutamylcysteine synthetase which catalyses the first, rate limiting step in the production of glutathione (see Section 1.3.4.3.1) (Dormer *et al.*, 2000). However, in response to oxidative stress induced by H_2O_2 and glutathione depletion *GSH1* expression is regulated by Yap1 instead of Met4 (Dormer *et al.*, 2002; Wheeler *et al.*, 2003). These studies suggest that the expression of *GSH1* is co-regulated by Yap1 and Met4 depending on the source of the ROS.

1.3.4.2 Enzymatic defences against ROS

A number of enzymatic antioxidant defence mechanisms exist in *S. cerevisiae* which directly detoxify ROS and these will be discussed below.

1.3.4.2.1 Superoxide dismutases

Superoxide dismutases (SODs) catalyse O_2^- to H_2O_2 and O_2 and their activity requires redox metal ions. *S. cerevisiae* expresses 2 SODs that differ in their reactive metal ion and their cellular localisation. The Cu/Zn-dependent Sod1 represents approximately 90% of the total SOD in the cell. The activity of Sod1 is dependent upon the chaperone Ccs1 which delivers copper to Sod1 and is also required for the formation of a disulphide bond, essential for Sod1 activity (Furukawa *et al.*, 2004). Sod1 is localised predominantly to the cytosol although a proportion of the enzyme also localises, together with Ccs1, to the mitochondrial intermembrane space where it offers protection against mitochondrial ROS-induced oxidative damage (Sturtz *et al.*, 2001). In contrast to Sod1, Sod2 is manganese-dependent and localises mainly to the mitochondrial intermembrane space.

Analyses of SOD mutants in *S. cerevisiae*, has offered clues to the function of each enzyme. In particular, *sod1* mutants display poor growth under respiratory conditions, a loss of viability in stationary phase, and increased sensitivity to external superoxide-generating oxidants such as paraquat and menadione (Longo *et al.*, 1996; Herrero *et al.*, 2008). *sod2* mutants are unable to grow under respiratory conditions and are hypersensitive to hyperoxia demonstrating that *SOD2* is essential for the defence against superoxide generated by the mitochondrial transport chain (van Loon *et al.*, 1986; Herrero *et al.*, 2008).

1.3.4.2.2 Catalases

S. cerevisiae contains 2 catalases, Cta1 and Ctt1, and both enzymes utilise the redox properties of a haem group to reduce H_2O_2 to H_2O and O_2. Cta1 is localised to the peroxisome where it is proposed to be involved in the detoxification of H_2O_2 produced during fatty acid oxidation in peroxisomes. In contrast, Ctt1 is cytoplasmic and its role remains unclear (Herrero *et al.*, 2008). Interestingly, in the absence of stress, cells deficient in either *ctt1*, *cta1* or both *ctt1* and *cta1* grow at the same rate as wild type cells suggesting that unlike SODs, catalase is not required for scavenging of endogenous ROS (Izawa *et al.*, 1996) . However, *cta1ctt1* double mutants display an increased sensitivity to H_2O_2 in stationary phase when compared to wild type or single catalase

mutant cells. These results suggest that catalase enzymes are necessary for stationary cells to tolerate oxidative stress but are dispensable under normal growth conditions.

1.3.4.2.3 Peroxidases

Peroxidase enzymes use electron donors to reduce H_2O_2 to H_2O using catalytic cysteine thiols. There are two classes of peroxidase enzymes named after their electron donor: glutathione peroxidases (Gpx) which use glutathione and thioredoxin peroxidases (peroxiredoxins (Prxs)) which utilise thioredoxin reductase.

Prxs were originally divided into two groups: 1-Cys and 2-Cys Prxs based on the number of cysteine residues used in catalysis. The 2-Cys Prxs have now been further subdivided into typical and a-typical Prxs. The typical 2-Cys Prxs have two cysteine residues at their active site, named the peroxidatic and resolving cysteine. The first step in the catalytic reduction of H_2O_2 by Prxs is the oxidation of the peroxidatic cysteine to a sulphenic acid (SOH). The oxidised peroxidatic cysteine of typical 2-Cys Prxs then forms a disulphide with the resolving cysteine of another Prx protecting the peroxidatic cysteine from further oxidation (Figure 1.9). The mechanism for atypical 2-Cys Prxs is the same as typical 2-Cys Prxs except that both the peroxidatic and its corresponding resolving cysteine residues are within the same protein and an intermolecular disulphide is formed during substrate reduction (Wood *et al.*, 2003). On the other hand, 1-Cys Prxs have only a peroxidatic cysteine and are therefore unable to form a disulphide. All Prxs require an electron donor to complete their catalytic cycle and restore enzyme activity. In the case of the *S. cerevisiae* mitochondrial 1-Cys Prx, Prx1, oxidised Prx1 is thought to be recycled first by glutathionylation which is then reduced by the thioredoxin reductase Trr2 (Greetham and Grant, 2009) or by the glutaredoxin, Grx2 (Pedrajas *et al.*, 2010). There are five Prxs in *S. cerevisiae*, Tsa1, Tsa2, Dot5, Ahp1 and Prx1 which differ in their target substrates and subcellular localisation (Park *et al.*, 2000). For example Tsa1 and Tsa2 are two highly homologous, cytoplasmic Prxs which are required for cellular resistance to exogenous H_2O_2 (Garrido and Grant, 2002; Wong *et al.*, 2002). Interestingly, *tsa1*Δ cells are more resistant to peroxide stress than *tsa2*Δ cells but a double *tsa1*Δ*tsa2*Δ is more sensitive to these stresses than either of the single mutants suggesting that the two Prxs work together in the cellular resistance to peroxide stress

(Wong *et al.*, 2002). Dot5 is a nuclear Prx and has been demonstrated to be particularly important for cellular viability during stationary phase (Cha *et al.*, 2003).

Glutathione peroxidases (Gpxs) are thought to be the major enzymatic contributors to the cellular defence against peroxide stress (Morano *et al.*, 2012). Gpx detoxify ROS by catalysing the reduction of H_2O_2 and organic hydroperoxides to water and the corresponding alcohol. Higher eukaryotes encode 2 main types of Gpx, classical Gpx (cGpx) which only use glutathione as an electron donor and phospholipid hydroperoxide Gpxs (PHGPx) which may use other reducing partners such as thioredoxin instead of glutathione (Avery *et al.*, 2004). In contrast to higher eukaryotes where cGpxs are the most ubiquitous Gpx, the *S. cerevisiae* genome encodes 3 Gpxs (Gpx1-3) all of which are PHGPxs (Avery and Avery, 2001). The Gpx enzymes of *S. cerevisiae* can be considered as atypical 2-Cys Prxs as they form intermolecular bonds during catalysis which are reduced by thioredoxin (Delaunay *et al.*, 2002; Ohdate *et al.*, 2010).

1.3.4.3 Non-enzymatic defences against ROS

A number of small molecules have been identified with non-enzymatic antioxidant properties in *S. cerevisiae*. For example, addition of ascorbate (vitamin C) to the media of *sod1* mutant cells has been demonstrated to increase both the mean and maximal lifespan of these normally short lived mutants (Krzepilko *et al.*, 2004). Furthermore, ascorbate has been shown to act as a reductant for oxidised 1-Cys Prxs (Monteiro *et al.*, 2007).

The glutaredoxin (Grx) and thioredoxin (Trx) pathways in *S. cerevisiae* utilise NADPH from the pentose phosphate pathway and either thioredoxin reductase or glutathione and glutathione reductase to reduce oxidised proteins. The Grx and Trx pathways together with glutathione make up the major non-enzymatic cellular antioxidant defence mechanisms in *S. cerevisiae* and will be discussed below.

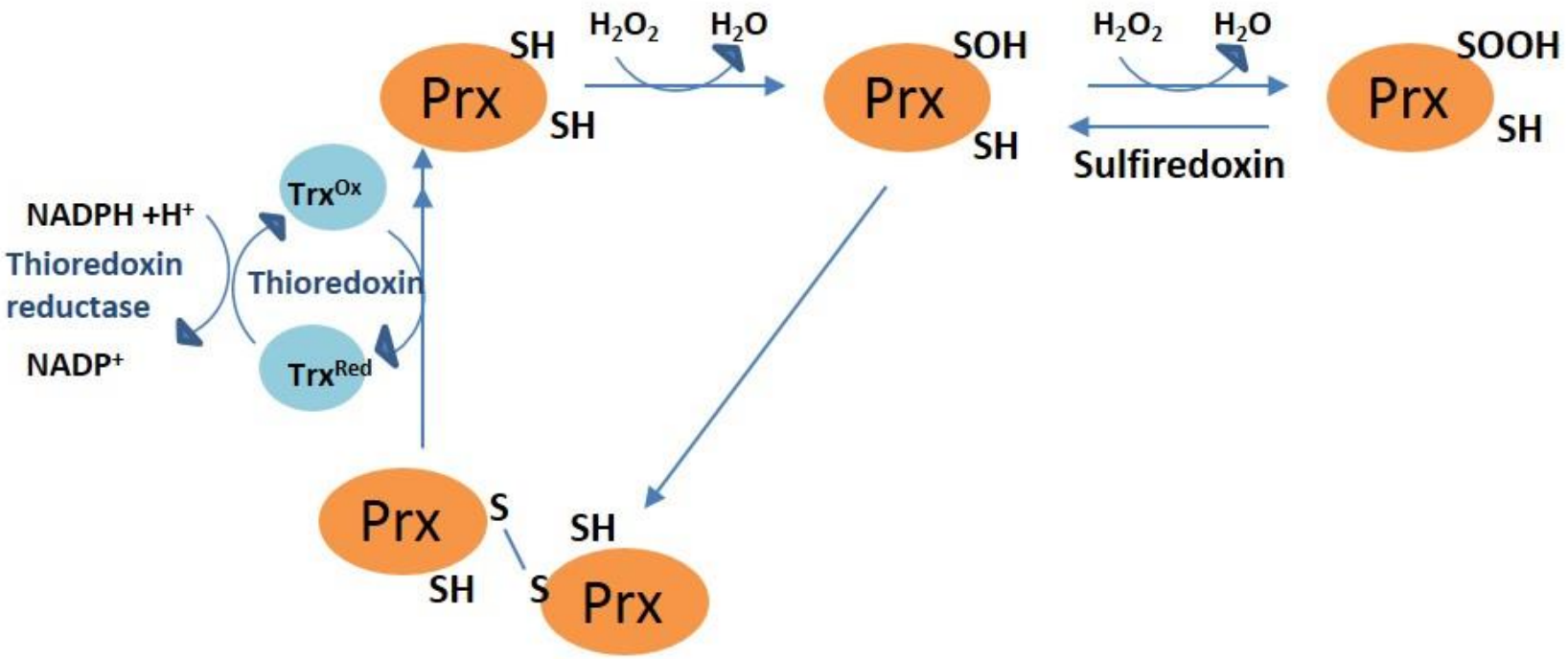


Figure 1.9 Redox cycling catalysed by typical 2-Cys Prxs. See text for details.

1.3.4.3.1 Glutathione

Glutathione is a highly abundant, low molecular weight peptide present in millimolar concentrations in the cytosol. Glutathione is synthesised in two ATP-dependent steps (Figure 1.10). In the first, rate-limiting step, γ-glutamyl-L-cysteine is formed from glutamic acid and cysteine catalysed by γ-glutamylcysteine synthetase encoded by *GSH1* in *S. cerevisiae*. In the second step, glutathione synthetase encoded by *GSH2*, catalyses the ligation of glycine with γ-glutamyl-L-cysteine forming glutathione.

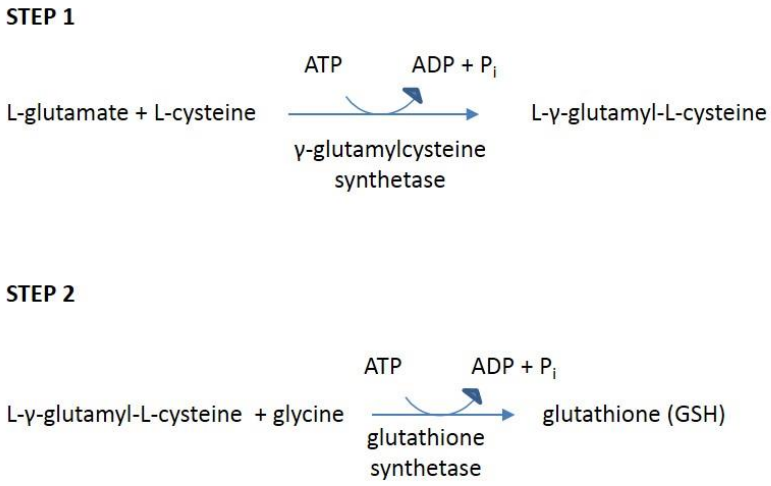


Figure 1.10 Glutathione is synthesised in a two-step process catalysed by γ-glutamylcysteine synthetase and glutathione synthetase. See text for details.

In unstressed *S. cerevisiae* cells glutathione predominantly exists in its reduced form (GSH). However, upon exposure to H_2O_2, the levels of GSH are reduced and an increase in oxidised glutathione (GSSG) and protein-bound glutathione are observed (Grant *et al.*, 1998). These results demonstrate the different modes through which glutathione functions as an antioxidant. Oxidation of the active site cysteine of glutathione enables glutathione to act as a ROS scavenger and GSSG can be reduced through a NADPH-dependent reaction catalysed by glutathione reductase to regenerate GSH. Binding of glutathione to oxidised sulphydryls in proteins (protein S-thiolation) is a protective mechanism to prevent further, irreversible oxidation. S-thiolation is a reversible process and reduction of these protective disulphides can occur directly via GSH or via glutaredoxins and thioredoxins (Grant, 2001).

Glutathione is essential in *S. cerevisiae* and cells lacking *GSH1* are unable to grow in the absence of exogenous glutathione and arrest their cell cycle after approximately seven generations (Spector *et al.*, 2001). Interestingly, the glutathione auxotrophy of the *gsh1*Δ mutant cannot be rescued by thiol reducing agents such as DTT nor by growth under anaerobic conditions suggesting that the essential nature of glutathione is not through its role as an antioxidant.

1.3.4.3.2 Glutaredoxins

Glutaredoxins (Grxs) are small, oxidoreductase proteins responsible for the reduction of GSH-protein mixed disulphides and protein disulphides. Grxs can be classified into two different groups: monothiol and dithiol depending on the number of active site cysteine residues they contain. *S. cerevisiae* expresses 2 dithiol Grxs, Grx1 and Grx2 which differ in their sensitivity to different oxidising agents (Luikenhuis *et al.*, 1998). Grx1 can protect cells from the superoxide anion while Grx2 is required for resistance to H_2O_2. There are three monothiol GRXs expressed in *S. cerevisiae* which play a role in iron homeostasis (Herrero *et al.*, 2008). Grx3 and Grx4 are localised to the nucleus where they are redundantly involved in the regulation of the Aft1 transcription factor (Pujol-Carrion *et al.*, 2006). Aft1 responds to iron depletion by moving from the cytoplasm to the nucleus where it regulates a group of genes involved in iron uptake. However, in a *grx3grx4* double mutant Aft1 is nuclear leading to accumulation of cellular iron. As previously described (see Section 1.3.1.2) iron is able to cause oxidative stress through the Fenton reaction and hence *grx3grx4* cells are highly sensitive to H_2O_2 and *t*-BOOH presumably due to activation of the Fenton reaction in these cells. Together, these results support an oxidative stress role for Grx3 and Grx4 by preventing accumulation of Aft1 in the nucleus thus preventing iron accumulation and subsequent oxidative stress due to activation of the Fenton reaction. In contrast to Grx3 and Grx4, the third monothiol Grx, Grx5 localises to the mitochondrial matrix (Herrero *et al.*, 2008). Deletion of *GRX5* leads to increased sensitivity to H_2O_2 and menadione, an increase in oxidative protein damage and an inability to grow when glycerol is the only carbon source (Rodriguez-Manzaneque *et al.*, 1999). Furthermore, Grx5 is involved in the biogenesis of Fe-S clusters and its absence causes inactivation of Fe-S cluster containing proteins (Rodriguez-Manzaneque *et al.*, 2002).

1.3.4.3.3 Thioredoxins

Thioredoxins (Trxs) are small, cysteine-containing oxidoreductase proteins (Morano *et al.*, 2012). The two conserved cysteine residues of Trxs participate in the reduction of enzymes that form a disulphide bond as part of their catalytic cycle. Oxidised Trxs are reduced back to their active form by Trx reductase.

S. cerevisiae has two cytosolic (Trx1, Trx2) and one mitochondrial (Trx3) Trxs. The cytosolic Trxs, particularly Trx2, have been shown to be important for the cellular response to externally added oxidants. Although the expression of *TRX2* increases in response to a range of oxidative stress agents, *trx2* mutants have an increased sensitivity only to H_2O_2 and *t*-BOOH but not to diamide (Kuge and Jones, 1994; Garrido and Grant, 2002). Trxs have also been shown to be important in maintaining viability in stationary phase cells. The presence of either *TRX1* or *TRX2* maintains viability of *S. cerevisiae* cells in stationary phase but loss of both results in a rapid loss of viability (Garrido and Grant, 2002). The essential role of Trxs in stationary phase is by acting as reductants for Prxs.

1.3.5 ROS in signalling

While the detrimental effects of ROS have long been established ROS, especially H_2O_2 have been shown to be important regulators of eukaryotic cell signalling (Figure 1.11). To act successfully as a signalling molecule H_2O_2 must have certain characteristics (Hancock, 2009):

1. It must be able to be produced rapidly and close to the site of action.
2. It must be detected and the appropriate response triggered.
3. It must be removed rapidly to attenuate the signal.

While H_2O_2 can arise from a number of endogenous sources, the formation of a localised pool of H_2O_2 for signalling is produced by a family of NADPH oxidases (Nox). Similar to those found in phagocytic cells (see Section 1.3.1.3) the non-immune Nox family produce $O_2^{\cdot -}$ by transferring electrons from NADPH to O_2. Dismutation of $O_2^{\cdot -}$ produces H_2O_2 which is longer lasting than $O_2^{\cdot -}$ and able to diffuse through cell membranes (Lassegue and Griendling, 2010).

H_2O_2 elicits different responses in cells depending on its concentration and the cell type. For example, the concentration of H_2O_2 that causes mammalian cell apoptosis can vary

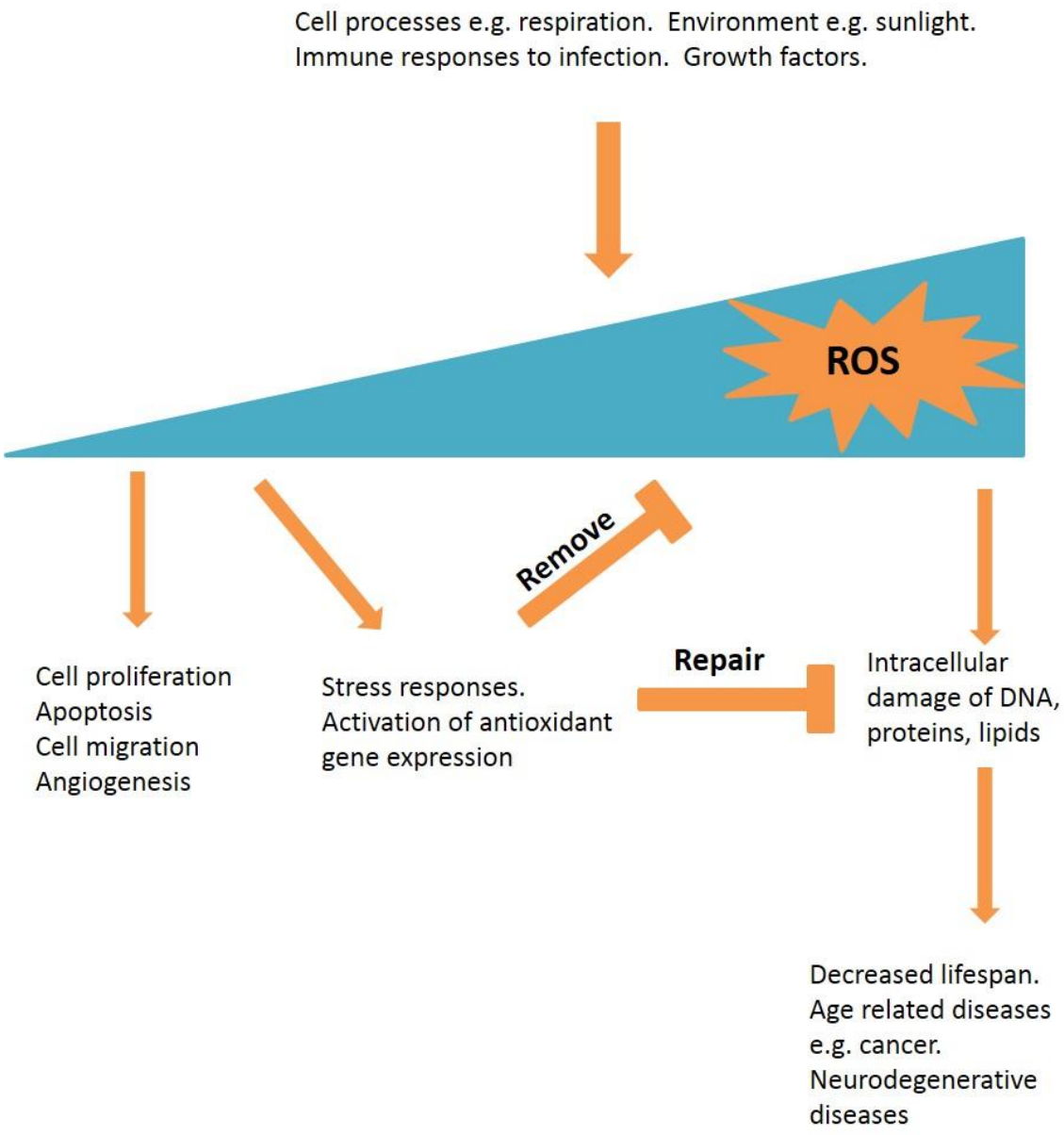


Figure 1.11 The concentration-dependent effects of ROS. ROS can be both harmful and beneficial to a cell. At high concentrations, ROS are able to damage cellular components such as DNA, protein and lipids which has been linked to decreased lifespan and to age-related diseases. However, at lower concentrations, ROS have a beneficial role in cellular signalling. It is therefore important for the cell to establish the level of stress that it is being exposed to and to induce the appropriate response.

20-fold depending on the cell type (Chen *et al.*, 2005). To elicit the correct response, H_2O_2 must first be sensed by H_2O_2-sensitive sensor proteins. As discussed earlier (see Section 1.3.2.1) cysteine residues with a low pKa are particularly sensitive to oxidation. The existence of a small number of deprotonated cysteine containing proteins thus allows specific targeting of these proteins, triggering defined affects within cells. Some examples of these redox-sensitive proteins are discussed below.

A number of mechanisms exist to attenuate the signal. As previously discussed, cells contain numerous antioxidant pathways that are able to eliminate H_2O_2 thus removing the signalling molecule itself. A major consequence of H_2O_2 signalling is the oxidation of cysteine residues and these must be reduced in order to attenuate the signal once the appropriate response has occurred. Reduction of these oxidised cysteine thiols is carried out by the Trxs and Grxs as described previously (Hancock, 2009).

A diverse range of pathways have been shown to involve ROS sensing and signalling to elicit numerous effects and several notable examples will be discussed below.

1.3.5.1 Protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) are a large family of proteins with important roles in signal transduction in response to many stimuli. PTPs are defined by a cysteine-containing signature motif at their catalytic site. Interestingly, the catalytic cysteine of this motif exists with a low pKa due to the surrounding chemical environment, which both enhances the role of the cysteine as a nucleophile in catalysis and also increases its susceptibility to oxidation (Tonks, 2005).

Oxidation of the active site cysteine inhibits the nucleophilic activity of the PTP and thus inhibits its activity. However, for the oxidation of PTPs to act in signalling, further, irreversible oxidation must be prevented. In the case of the PTP PTP1B, following oxidation a cyclic sulphonamide is formed, inducing a structural change in the active site and preventing further oxidation (Salmeen *et al.*, 2003; van Montfort *et al.*, 2003). Alterations in the active site have been demonstrated to both protect the catalytic cysteine from irreversible oxidation and also to expose the oxidised cysteine to reducing agents which enables reduction of the enzyme back to its active form. However, other PTPs, for example the cell cycle regulator Cdc25, contain a second cysteine residue (Buhrman *et al.*, 2005). In response to oxidation, Cdc25 forms an intra-molecular

disulphide bond between the catalytic cysteine and this second cysteine which protects the catalytic cysteine residue from further, irreversible oxidation.

1.3.5.2 OxyR

The OxyR transcriptional regulator of *Salmonella typhimurium* and *Escherichia coli* activates the expression of specific genes in response to H_2O_2 (Marinho *et al.*, 2014). Interestingly, OxyR itself undergoes direct oxidation by H_2O_2 and thus acts as both a H_2O_2 sensor and signal transducer. OxyR is able to bind to DNA in both its oxidised and reduced form but only activates transcription in its oxidised form (Storz *et al.*, 1990). Oxidation of OxyR alters the contact between the transcriptional regulator and DNA leading to the activation of OxyR target genes including several that encode antioxidants.

1.3.5.3 Ubiquitin and Ubls

The common and widespread use of catalytic cysteine residues in ubiquitin/Ubl conjugation and deconjugation pathways provides a potential route by which these protein modifiers could be redox-regulated (Figure 1.12). Indeed the global levels of SUMO (Saitoh and Hinchey, 2000; Zhou *et al.*, 2004), Nedd8 , ubiquitin (Bossis and Melchior, 2006) and Urm1 (Van der Veen *et al.*, 2011) modification increase in response to oxidative stress conditions suggesting a role for these modifiers in stress responses. However, at lower concentrations of ROS a signalling role for the mammalian SUMO and Nedd8 and the *S. cerevisiae* ubiquitin pathways have been identified. In the case of the mammalian SUMO pathway, while high concentrations of H_2O_2 (100 mM) increased the global levels of SUMO conjugation, at lower concentrations (1 mM) SUMO conjugation is inhibited (Bossis and Melchior, 2006). At low and high levels of H_2O_2 the E1 and E2 enzymes of the SUMO pathway form an inhibitory disulphide that prevents SUMO conjugation. However, SUMO conjugates accumulate at high but not lower levels of H_2O_2 because the deconjugation machinery is also inhibited at high levels of H_2O_2. Chemotherapeutic drugs used in the treatment of acute myeloid leukaemia (AML) have been shown to induce ROS production which may be part of their mechanism for inducing cell death (Bossis *et al.*, 2014). Treatment of AML cells in culture with these chemotherapeutic drugs stimulates the formation of the SUMO E1-E2 disulphide. Interestingly, AML cells which are chemoresistant are unable to

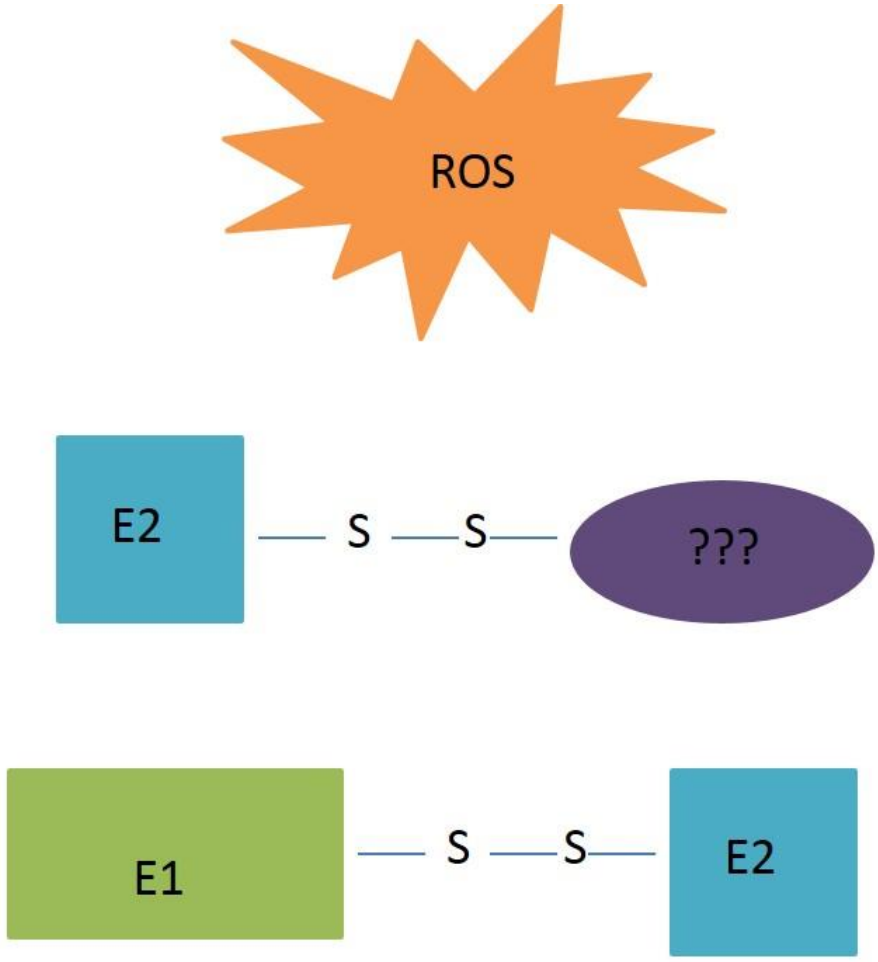


Figure 1.12 The known mechanisms of redox regulation of the ubiquitin and Ubl conjugation pathways in mammalian cells. The E1 and the E2 of the mammalian SUMO pathway form a disulphide in response to H_2O_2 (Bossis and Melchior, 2006) while the E2 of the mammalian Nedd8 pathway forms a complex with an unidentified partner in response to bacterial-induced H_2O_2 production in the gut epithelia (Kumar et al, 2007).

form the E1-E2 disulphide and display reduced levels of ROS. Together, these results suggest that the formation of the E1-E2 disulphide and the consequent SUMO deSUMOylation is important for drug-induced apoptosis in AML cells.

Redox-regulation of the Nedd8 pathway has also been demonstrated to affect specific targets in mammalian cells. Upon exposure to bacteria, the epithelia in the gut rapidly generate ROS. The Nedd8 pathway E2, Ubc12 has been shown to form a high molecular weight (HMW) complex in these cells suggesting that, similar to the SUMO pathway, the Nedd8 pathway is also redox-regulated in mammalian cells (Kumar *et al.*, 2007). However, in contrast to the SUMO pathway, Ubc12 does not complex with the E1 of the Nedd8 conjugation pathway. Analysis of Cul1, a component of the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase, revealed that Neddylation of Cul1 is abolished upon oxidation of Ubc12. This loss of Cul1 Neddylation has been proposed to inactivate $SCF^{\beta\text{-}TrCP}$ and in cells treated with concentrations of H_2O_2 that result in oxidation of Ubc12 the immunoregulatory NFκB pathway was attenuated by inhibition of IκB-α a normal target for $SCF^{\beta\text{-}TrCP}$.

1.3.6 ROS and regulation of the cell cycle

Evidence from both mammalian cells and yeast has suggested that ROS can have a dual role in cell cycle regulation. In response to high levels of ROS, cells arrest their cell cycle to prevent and repair oxidative damage. However, in mammalian cells low levels of ROS can trigger cell proliferation. As ROS can have opposing effects on cell proliferation, the redox state of the cell must be tightly regulated. In fact one of the hallmarks of cancer is redox imbalance due to the increased metabolism and the aberrant levels of antioxidants in cancer cells (Glasauer and Chandel, 2014). Below, redox control of cell proliferation in both mammalian cells and yeast will be discussed.

1.3.6.1 ROS in normal cell cycling

In response to oxidative stress, cells arrest their cell cycle to prevent and repair oxidative damage. In multicellular organisms, ROS can be produced in response to a range of factors including growth factors and cytokines which can lead to the stimulation of cellular proliferation (Chiu and Dawes, 2012). For example, a transient increase in H_2O_2 has been detected in a rat vascular smooth muscle cell line after stimulation with platelet derived growth factor or a human cancer cell line in response to epidermal growth factor (Sundaresan *et al.*, 1995; Bae *et al.*, 1997). In both mammalian cells and *S.*

cerevisiae the existence of a redox cycle alongside the cell division cycle has become apparent. For example, experiments in mouse embryonic fibroblasts demonstrated an increase in pro-oxidants from early G1 phase (Menon *et al.*, 2003). Importantly, treatment of cells with the antioxidant N-acetyl cysteine prevented the cells entering G1 phase suggesting that this increase in oxidation is required for transition from G1 to S phase of the cell cycle. The existence of an oxidative shift has also been observed in a human cell line (Goswami *et al.*, 2000). Using a pro-oxidant fluorescent probe, HeLa cells were shown to have maximal ROS in late S, G2 and M phases compared to lower levels in G1 phase. In *S. cerevisiae*, the cell division cycle is synchronised to a metabolic cycle where the cell division cycle is initiated late in the oxidative phase and DNA synthesis and mitosis occurs in the reductive phase (Tu *et al.*, 2005). This study found that very few cells replicate their DNA or divide during the oxidative phase of the metabolic cycle suggesting the synchrony of the cell division and metabolic cycles may act as a mechanism to protect DNA from oxidative damage.

1.3.6.2 Oxidative stress and the cell cycle in S. cerevisiae

As described above, the normal cell cycle of both mammalian cells and *S. cerevisiae* is tightly linked to the cellular redox status. However, high levels of ROS have the potential to damage DNA. The cell must therefore be able to sense the level of ROS and employ mechanisms to both detoxify the ROS and to trigger checkpoints that lead to cell cycle arrest.

A number of antioxidant mechanisms have been linked to cell cycle regulation in *S. cerevisiae*. For example, *sod1*Δ cells show an extended G1 phase when grown in air (Lee *et al.*, 1996). Interestingly, switching *sod1*Δ cells from growth in N_2 to growth in 100 % O_2 led to a permanent G1 phase arrest and *sod1*Δ cells lost viability after 3 hours of growth under these conditions. Deletion of *GSH1* which encodes the first, rate-limiting step in glutathione synthesis (Figure 1.10) has also been demonstrated to lead to G1 phase arrest when cells are grown in the absence of glutathione for approximately 7 generations however the mechanisms behind this arrest remain unknown (Spector *et al.*, 2001).

In *S. cerevisiae*, different oxidising agents have been shown to arrest the cell cycle at different points and by different mechanisms. For example, menadione and paraquat,

which both generate superoxide, arrest the cell cycle in G1 phase in a manner that is independent of the DNA damage checkpoint protein Rad9, while H_2O_2 leads to a Rad9-dependent cell cycle arrest in G2 phase (Nunes and Siede, 1996; Flattery-O'Brien and Dawes, 1998). Work from our lab has also shown that H_2O_2 and diamide induce a G1 phase arrest in synchronised cells (Doris *et al.*, 2012). Linoleic acid hydroperoxide (LoaOOH) is a toxic metabolite of lipid peroxidation which causes a Rad9-independent G1 phase cell cycle arrest (Chiu and Dawes, 2012). The mechanism behind the LoaOOH-dependent G1 phase arrest has now been elucidated by the observation that Swi6, a component of the MBF and SBF transcription factors is directly oxidised upon exposure to LoaOOH (Chiu *et al.*, 2011). Furthermore, oxidation of Swi6 prevents the expression of G1 cyclins leading to G1 phase arrest.

1.4 Aims and Objectives

It is vital for cells to detect and respond to oxidative stress to prevent damage to cellular macromolecules such as DNA but also to allow the cell to repair damage to prevent it being passed on to future generations. However, how the cell detects oxidative stress and responds appropriately remains largely unclear. Modification of proteins by ubiquitin and Ubl modifiers affects a diverse range of processes including cell division. Moreover, the use of catalytic cysteine residues in their conjugation pathways opens up the possibility that these pathways may be regulated by ROS. Thus in this thesis we will use *S. cerevisiae* as a model to study potential regulators of the cell cycle in this model organism in response to oxidative stress.

To achieve this aim the specific objectives for this project are:

1. To investigate the relative sensitivities of ubiquitin/Ubl conjugation pathways to oxidation.
2. To investigate the potential roles of identified G1/S phase regulators in oxidative stress-dependent cell cycle control.
3. To identify the pathways involved in the G1 phase arrest in response to glutathione depletion.

Chapter 2: Materials and Methods

2.1 Yeast strains

The *S. cerevisiae* strains used in this study are derived from W303-1a (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) or BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*).

S. pombe strains in this study are derived from CHP428 (h^+ *ade6-M216 his7-366 leu1-32 ura4-D18*).

All strains used in this study are listed in Tables 2.1 and 2.2.

2.2 Yeast techniques

2.2.1 Growth conditions

S. cerevisiae strains were grown in either rich YPD media (1% w/v Bacto-yeast extract, 2 % w/v Bacto-peptone, 2% w/v glucose, -/+ 2 % w/v agar) or minimal SD media (0.67% w/v Bacto-yeast nitrogen base without amino acids, 2% w/v glucose, -/+ 2% w/v agar). For selective growth, SD was supplemented with adenine sulphate (20 mg/ml), L-histidine hydrochloride (10 mg/l), L-leucine (20 mg/l), L-tryptophan (10 mg/l), uracil (8 mg/l) and L-methionine (10 mg/l) (all supplied by Sigma), as required. For glutathione depletion experiments cells were grown in SD media supplemented with 1 mM L-glutathione reduced (Sigma) prior to glutathione removal. Unless otherwise stated, strains were grown at 30°C.

S. pombe strains used in this study were grown in rich YE5S media (0.5 % w/v yeast extract, 3 % w/v glucose and 225 mg/L adenine, histidine, leucine, uracil and lysine hydrochloride) or when selection was required in minimal media (EMM) (3 g/L potassium hydrogen phallate, 2.2 g/L Na_2HPO_4, 5 g/L NH_4Cl, 2% w/v glucose, 20 ml/L salts (52.5 g/L $MgCl_2.6H_2O$, 0.735 g/L $CaCl_2.2H_2O$, 50 g/L KCl and 2 g/L $NaSO_4$), 1 ml/L vitamins (1 g/L pantothenic acid, 10 g/L nicotinic acid, 10 g/L inositol and 10 mg/L biotin), 0.1 ml/L minerals (5 g/L boric acid, 4 g/L $MnSO_4$, 4 g/L $ZnSO_4.7H_2O$, 2 g/L $FeCl_2.6H_2O$, 0.4 g/L molybdic acid, 1 g/L KI, 0.4 g/L $CuSO_4.5H_2O$ and 10 g/L citric acid) plus the required amino acid supplements as outlined previously (Moreno *et al.*, 1991).

Strain	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Gift from David Lydall
ELR5 *	*MATa UBA1-TAP*	Ghemmaghami et al (2003)
ELR6 *	*MATa UBA2-TAP*	Ghemmaghami et al (2003)
ELR7 *	*MATa UBA3-TAP*	Ghemmaghami et al (2003)
ELR8 *	*MATa UBA4-TAP*	Ghemmaghami et al (2003)
ELR10 *	*MATa UBC1-TAP*	Ghemmaghami et al (2003)
ELR11 *	*MATa UBC2-TAP*	Ghemmaghami et al (2003)
ELR12 *	*MATa CDC34-TAP*	Ghemmaghami et al (2003)
ELR13 *	*MATa UBC4-TAP*	Ghemmaghami et al (2003)
ELR14 *	*MATa UBC5-TAP*	Ghemmaghami et al (2003)
ELR15 *	*MATa UBC6-TAP*	Ghemmaghami et al (2003)
ELR16 *	*MATa UBC7-TAP*	Ghemmaghami et al (2003)
ELR17 *	*MATa UBC8-TAP*	Ghemmaghami et al (2003)
ELR18 *	*MATa UBC10-TAP*	Ghemmaghami et al (2003)
ELR19 *	*MATa UBC13-TAP*	Ghemmaghami et al (2003)
ELR20 *	*MATa MMS2-TAP*	Ghemmaghami et al (2003)

ELR21 *	*MATα UBC9-TAP*	Ghemmaghami et al (2003)
CLC9 *	*his3-DAmP*	Gift from David Lydall
CLC27 *	*cdc34-DAmP*	Gift from David Lydall
CLC30 *	*uba1-DAmP*	Gift from David Lydall
KD66 (W303-1a)	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	Thomas and Rothstein (1989)
KD65 (W303-1a)	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	Thomas and Rothstein (1989)
ELR49 #	*MATα rub1::LEU2*	This study
ELR52 #	*MATα far1::HIS3*	This study
ELR58 #	*MATα gsh1::LEU2 far1::HIS3*	This study
ELR60 #	*MATα gsh1::LEU2 rub1::LEU2*	This study
ELR61 #	*MATα csn5::HIS3*	This study
ELR71 #	*MATα gsh1::LEU2 YDJ1-3HA::kanMX6*	This study
ELR72 #	*MATα gsh1::LEU2 csn5::HIS3*	This study
ELR75 #	*MATα FAR1-13Myc::TRP1*	This study
ELR76 #	*MATα gsh1::LEU2 far1::HIS3 sic1::URA3*	This study
ELR77 #	*MATα gsh1::LEU2 FAR1-13Myc::TRP1*	This study

ELR102 #	*MATa gsh1::LEU2 MET4-18Myc::TRP1*	This study
ELR120 #	*MATa rub1::LEU2 Sic1-13Myc::kanMX6*	This study
KD19 #	*MATa sic1::URA3*	Lab stock
KD79 #	*MATa CDC34-13Myc::kanMX6*	Lab stock
KD117 #	*MATa met4::TRP1*	Lab stock
KD118 #	*MATα gsh1::LEU2 met4::TRP1*	Lab stock
KD125 #	*MATa gsh1::LEU2 sic1::URA3*	Lab stock
KD183 #	*MATa gsh1::LEU2 SIC1 T173A-13Myc::kanMX6 CDC34-13Myc::kanMX6*	Lab stock
KD192 #	*MATa gsh1::LEU2 hog1::LEU2 SIC1-13Myc::kanMX6*	Lab stock
KD225 #	*MATa YDJ1-3HA::kanMX6*	Lab stock
KD231 #	*MATa YDJ1-3HA::kanMX6-PGAL1*	Lab stock
KD233 #	*MATa gsh1::LEU2 YDJ1-3HA::kanMX6-PGAL1*	Lab stock
KD257 #	*MATa CDC34-13Myc::kanMX6 SIC1-13Myc::kanMX6*	Lab stock
KD294 #	*MATa gsh1::LEU2 CDC34-13Myc::kanMX6 SIC1-13Myc::kanMX6*	Lab stock

KD484#	*MATα MET4-18Myc::TRP1*	Lab stock
KD502 #	*MATα gsh1::LEU2*	Lab stock

Table 2.1. *S. cerevisiae* strains used in this study. Strains marked with an * are derived from the BY4741 background while those marked with # are derived from W303-1a.

Strain	Genotype	Source
CHP428	*h⁺ ade6-M216 his7-366 leu1-32 ura4-D18*	Lab stock
ER1	*h⁺ ade6-M216 his7-366 leu1-32 ura4-D18 ubc9-3PK::ura4⁺*	This study

Table 2.2. *S. pombe* strains used in this study.

2.2.2 Yeast transformations

2.2.2.1 S. cerevisiae transformations

DNA was introduced into *S. cerevisiae* cells using a protocol based on the high efficiency lithium acetate method (Schiestl and Gietz, 1989). Briefly, 50 ml of mid-log phase growing (~4 x 10^6 cells/ml) were pelleted (3000 x g, 2minutes), washed in sterile water and resuspended in 1 ml LiAc/TE solution (0.1 M LiAc, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH8]). 200 µl of cells were added to 50 µg of boiled salmon sperm DNA (Ambion) and 0.1-10 µg DNA. 1 ml of LiAc/TE/PEG solution (0.1 M LiAc, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH8], 40% w/v PEG 2000) was added to the transformation mix which was incubated with agitation at 30°C for 30 minutes, followed by a heat shock at 42°C for 15 minutes. Cells were pelleted (7000 x g, 30 seconds), resuspended in sterile water and plated onto appropriate selective media. Selection plates were incubated at 30°C for at least 2 days or until colonies appeared.

2.2.2.2 S. pombe transformations

DNA was introduced into *S. pombe* using the lithium acetate method previously described (Moreno *et al.*, 1991). 100 ml of mid-log phase growing cells were pelleted (3000 x g, 3 minutes), washed in 1 ml sterile water and resuspended in 1 ml LiAc/TE (0.1 M LiAc [pH 7.5], 10 mM Tris-HCl [pH 7.5], 1 mM EDTA). 100 µl of cells and 20 µg sheared salmon sperm DNA (Ambion) were added to the transforming DNA (~ 1 µg), mixed gently and incubated at room temperature for 10 minutes. 260 µl of LiAc/TE/PEG (0.1 M LiAc [pH 7.5], 1 mM EDTA and 50% v/v PEG 4000) were added following incubation and gently mixed before incubation at 30˚C for 30-60 minutes. 43 µl of DMSO were added and the cells incubated at 42˚C for 5 minutes. Cells were pelleted, washed in 1 ml sterile water and then resuspended in 150 µl sterile water. Cells were plated on EMM agar plates with the required supplements and incubated at 30˚C until colonies were visible.

2.2.3 Strain constructions

2.2.3.1 Gene tagging S. cerevisiae

Expression of Far1 tagged at the C-terminus with 13 Myc epitopes from the normal chromosomal locus was achieved by integration of a PCR-amplified cassette created using the primers Far1TagF (Primer 1) and Far1TagR (Primer 2) (Table 2.3) with the

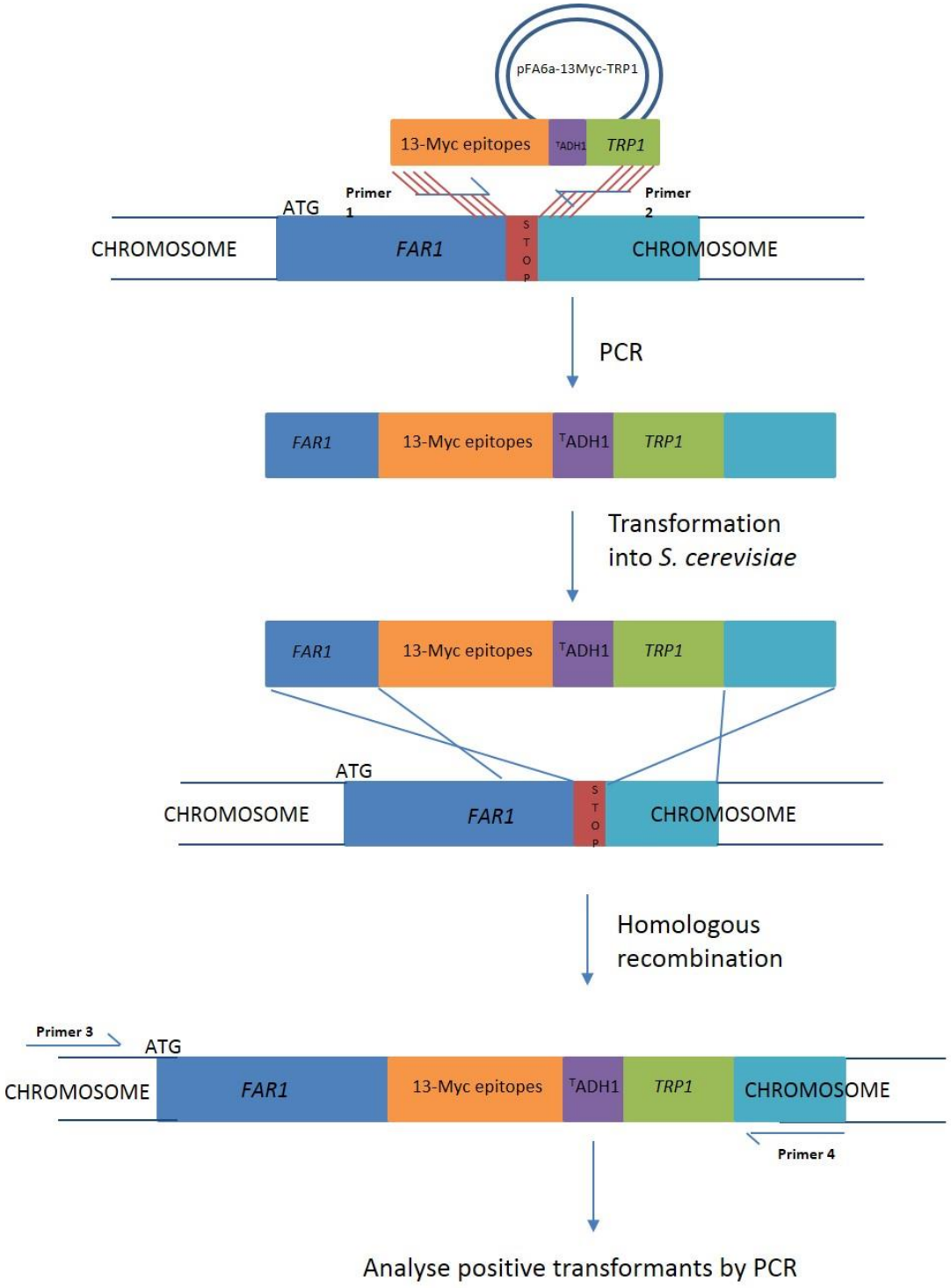

pFA6a-13Myc-TRP1
13-Myc epitopes
TADH1
TRP1
ATG
Primer 1
Primer 2
CHROMOSOME
FAR1
STOP
CHROMOSOME
PCR
FAR1
13-Myc epitopes
TADH1
TRP1
Transformation into S. cerevisiae
FAR1
13-Myc epitopes
TADH1
TRP1
ATG
CHROMOSOME
FAR1
STOP
CHROMOSOME
Homologous recombination
Primer 3
ATG
CHROMOSOME
FAR1
13-Myc epitopes
TADH1
TRP1
CHROMOSOME
Primer 4
Analyse positive transformants by PCR

Figure 2.1. Schematic diagram of the method to construct Far1-13Myc from its normal chromosomal locus. Primer 1 contains 40 nucleotides homologous to the sequence located immediately upstream of the *FAR1* Stop codon and 20 nucleotides homologous to the 5'end of the 13-Myc sequence. Primer 2 contains 40 nucleotides homologous to the sequence immediately downstream of the *FAR1* stop codon and 20 nucleotides homologous to the 3' sequence of *TRP1*. PCR with primer 1 and primer 2 and the plasmid pFA6a-13Myc-TRP1 as a template generated a PCR fragment that was transformed into wild type *S. cerevisiae*. Homologous recombination leads to the replacement of the stop codon of *FAR1* with the sequences encoding 13-Myc epitopes fused in frame with the *FAR1* gene followed by the sequence encoding the *TRP1* gene, allowing selection of positive transformants by growth on media lacking tryptophan. Successful epitope tagging was confirmed by PCR using primers 3 and 4.

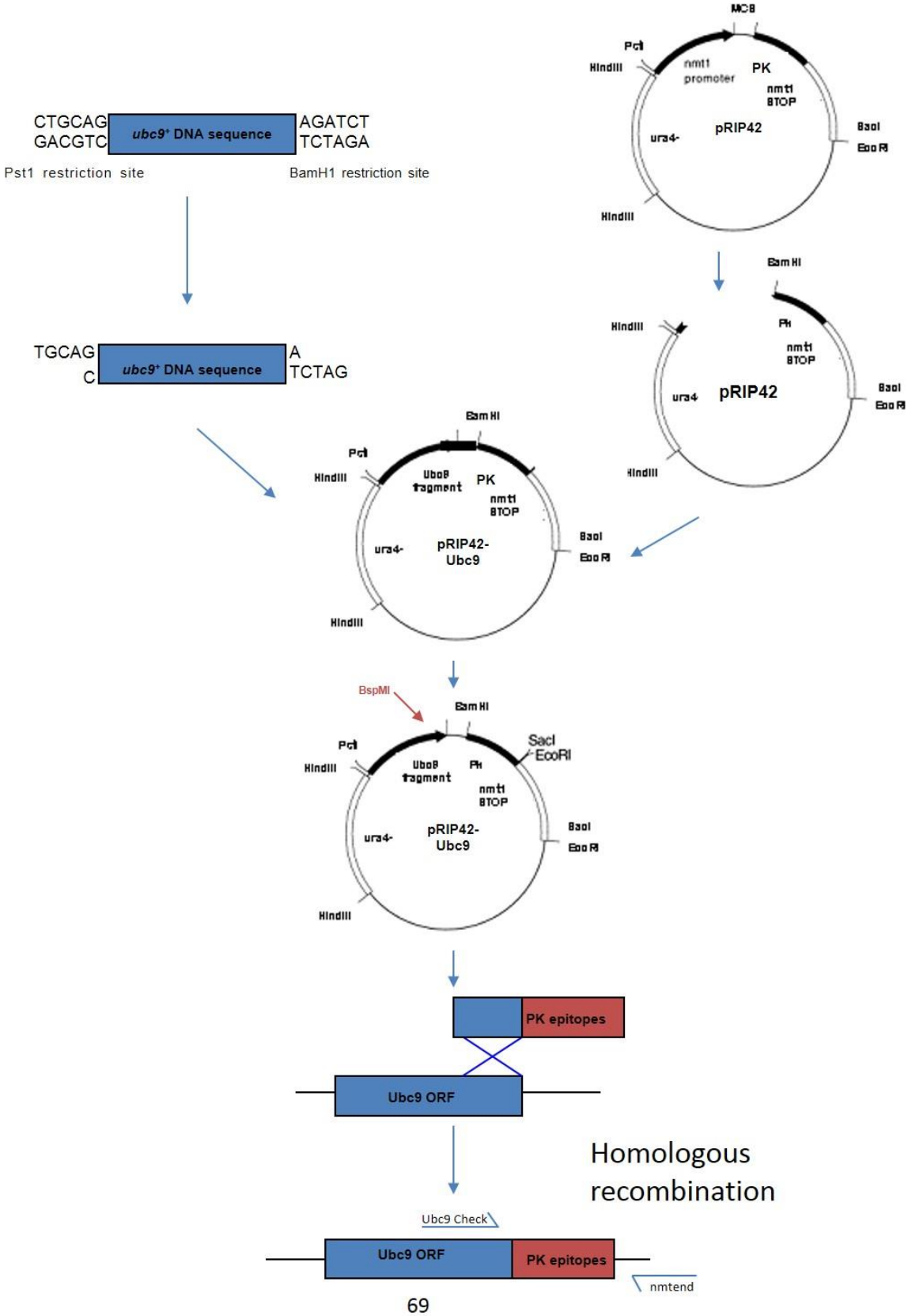
plasmid pFA6a-13Myc-TRP1 as a template as described by (Longtine *et al.*, 1998) using the strategy shown in Figure 2.1. Positive transformants were selected by their ability to grow on media lacking tryptophan and integration of the epitope-tag at the correct chromosomal locus was confirmed by PCR using the primers Far1TagChkF and Far1TagChkR (Primers 3 and 4 respectively) which flank the region.

2.2.3.2 Gene tagging S. pombe

Expression of Ubc9 tagged at the C-terminus with 3 PK epitopes, from the normal chromosomal locus was achieved using the strategy shown in Figure 2.2. Briefly, a PCR fragment encoding the C-terminus of *ubc9*$^{+}$ with restriction sites for BamH1 and Pst1 was amplified from CHP428 using the primers Ubc9 Forward and Ubc9 Reverse (Table 2.3). The vector pRIP42-Ubc9 was constructed by digestion of the *ubc9*$^{+}$ PCR fragment with BamH1 and Pst1 and ligation of the fragment with BamH1/Pst1 digested pRIP42. To promote homologous recombination into the yeast genome, pRIP42-Ubc9 was linearised using the restriction enzyme BspM1 prior to transformation. Positive transformants were obtained and correct integration confirmed using Ubc9 Check and Nmtend oligonucleotide primers (Table 2.3 and Figure 2.2)

2.2.3.3 Gene deletion

Genes were substituted with a selectable marker in the genome of *S. cerevisiae* using the one-step homologous recombination method previously described by (Rothstein, 1991) and shown in Figure 2.3. The specific gene was replaced in W303-1a using the appropriate oligonucleotide primers 1 and 2 (Table 2.3) and either YDp-L, YDp-H or YDp-U plasmid as a template (Berben *et al.*, 1991). Primer 1 contains 90 nucleotides homologous to the region located directly upstream of the ATG of the target gene and 20 nucleotides homologous to the region directly upstream to the 5' end of the selectable marker gene in the YDp plasmid. Primer 2 contains 90 nucleotides homologous to the DNA sequence located immediately downstream of the stop codon of the target gene and 20 nucleotides homologous to the 3' end of the selectable marker gene. PCR fragments were then introduced into the relevant strain and successful transformants were selected by growth on media lacking either leucine, histidine or uracil respectively.


CTGCAG
GACGTC
ubc9+ DNA sequence
AGATCT
TCTAGA
Pst1 restriction site
BamH1 restriction site
TGCAG
C
ubc9+ DNA sequence
A
TCTAG
MCB
PstI
HindIII
nmt1 promoter
PK
nmt1 BTOP
pRIP42
ura4-
BamHI
BaoI
EcoRI
pRIP42
ura4
Ubc9 fragment
pRIP42-Ubc9
BspMI
SacI
EcoRI
PK epitopes
Ubc9 ORF
Homologous recombination
Ubc9 Check
nmtend

69

Figure 2.2. Schematic diagram of the strategy for tagging Ubc9 of *S. pombe* with 3 PK epitopes at the normal chromosomal locus. Primers were designed to amplify the last 200 bp of the *ubc9*$^{+}$ encoding sequence up to but not including the Stop codon. pRIP42-Ubc9 was formed by cloning the amplified *ubc9*$^{+}$ fragment into the BamH1 and Pst1 restriction sites of the vector pRIP42. pRIP42-Ubc9 was linearised by digestion with BspM1 and transformed into wild type *S. pombe*. Positive transformants were confirmed by PCR using the Ubc9Check and nmtend primers.

Correct gene replacement was confirmed by PCR using the respective primer 3 and 4 check oligonucleotide primers designed to flank the specific gene locus.

2.2.4 Mating, meiosis induction and tetrad dissection

S. cerevisiae strains of opposite mating types were crossed on YPD agar and the resulting diploids were sporulated on sporulation media (1% w/v potassium acetate, 0.1 % w/v yeast extract, 0.05 % w/v glucose) by incubation at 30°C for 2-3 days. Cells were then resuspended in 50 µl 5% v/v glusulase solution (PerkinElmer) and incubated at 30°C for 20-30 minutes. The cell suspension was gently mixed with 300 µl YPD and 20 µl were pipetted linearly onto a YPD plate. Spores were separated using a tetrad dissector (Singer Instruments) and plates were incubated at 30°C for 2-3 days.

2.2.5 Stress sensitivity and viability assays

For stress sensitivity assays 10-fold serial dilutions of mid-log phase growing *S. cerevisiae* cells were spotted, using a 48-pronged replica plater instrument (Sigma) onto YPD plates containing the indicated oxidising agents.

For viability assays 10-fold serial dilutions of mid-log phase growing *S. cerevisiae* cells or cells grown for the indicated period of time in media lacking GSH (SD –GSH) were spotted onto SD media supplemented with 1 mM GSH (SD +GSH).

Plates were incubated for 2-3 days at 30°C unless otherwise stated.

2.2.6 Cell cycle synchronisation

S. cerevisiae cells were grown in either YPD or SD +GSH until mid-log phase and blocked in late G1 phase by the addition of 15 µg/ml α-factor (CRUK) for 1 hour followed by a second addition of 5 µg/ml α-factor for a further hour. To release the cells from the cell cycle arrest the culture was filtered using the vacuum filtration rapid Filtermax system (TPP) and washed with four times the initial culture volume of pre-warmed YPD/SD+GSH. At each time point the percentage of budded cells were counted and cells were collected for DNA content analysis (1 ml) and protein extraction (2 ml) respectively, as indicated.

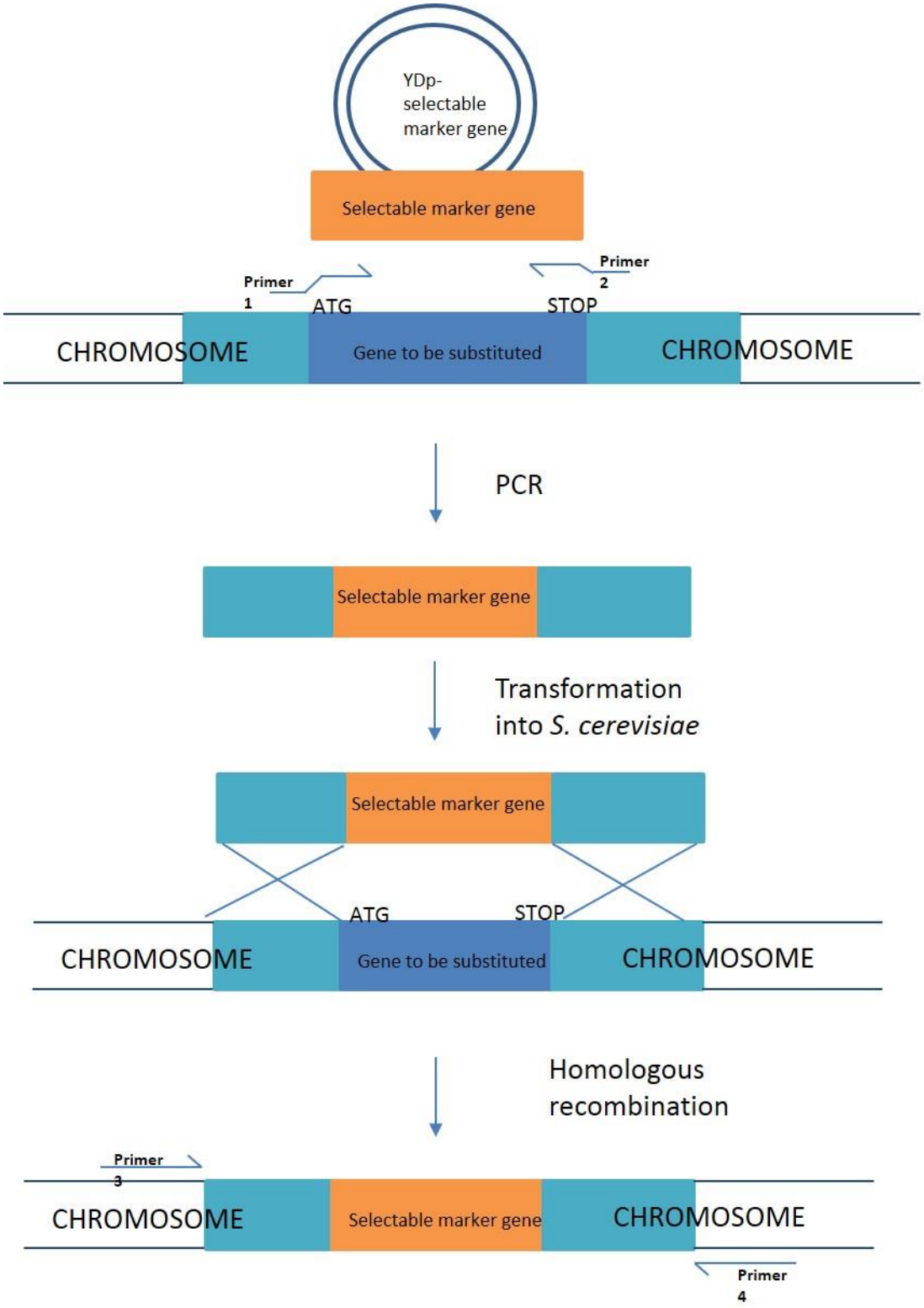

YDp-
selectable
marker gene
Selectable marker gene
Primer
1
Primer
2
ATG
STOP
CHROMOSOME
Gene to be substituted
CHROMOSOME
PCR
Selectable marker gene
Transformation
into S. cerevisiae
Selectable marker gene
ATG
STOP
CHROMOSOME
Gene to be substituted
CHROMOSOME
Homologous
recombination
Primer
3
CHROMOSOME
Selectable marker gene
CHROMOSOME
Primer
4

Figure 2.3. Schematic diagram of the one-step homologous recombination method used to delete specific genes in *S. cerevisiae*. Primer 1 contains 90 nucleotides homologous to the sequence located immediately upstream of the ATG codon of the gene to be substituted and 20 nucleotides homologous to the 5' end of the selectable marker gene. Primer 2 contains 90 nucleotides homologous to the sequence located immediately downstream of the stop codon of the gene to be substituted and 20 nucleotides homologous to the 3' end of the selectable marker gene. PCR using primer 1 and primer 2 and the plasmid YDp containing the selectable marker gene as a template generates a PCR fragment that is transformed into wild type *S. cerevisiae*. Homologous recombination results in substitution of the gene to be deleted with a selectable marker gene. Successful deletion was verified by PCR using primers 3 and 4, designed to bind upstream and downstream of the region that has been deleted.

2.2.7 S. cerevisiae genomic DNA extraction

DNA was extracted from cells using a protocol based on that previously described by (Hoffman and Winston, 1987). 5 ml of overnight *S. cerevisiae* culture were pelleted by centrifugation (3000 x g, 2 minutes), washed in 1 ml of sterile water and resuspended in 200 µl STET solution (2% v/v Triton X-100, 1% w/v SDS, 100 mM NaCl, 10 mM Tris-HCl [pH8]). 200 µl of phenol/chloroform/isoamyl alcohol (25:24:1, pH8) were added and the cells lysed with glass beads using a Mini Beadbeater (Biospec Products). 200 µl of TE solution (10 mM Tris-HCl [pH7.4], 1 mM EDTA [pH8] were added prior to centrifugation (13000 x g, 5 minutes). The upper aqueous phase was added to a fresh Eppendorf tube and 1 ml 100% ethanol added to precipitate the DNA (13000 x g, 2 minutes). The DNA pellet was resuspended in TE solution containing 75 µg/ml RNase (Sigma) and incubated at 37°C for 5 minutes. 10µl of 4 M ammonium sulphate and 1 ml 100% ethanol were added and the DNA precipitated (13000 x g, 2 minutes). The DNA pellet was finally resuspended in 50 µl TE and stored at -20°C.

2.2.8 S. pombe genomic DNA extraction

10 ml of overnight *S. pombe* culture were pelleted by centrifugation (2000 x g, 3 minutes), washed in 1 ml of sterile water and resuspended in 200 µl chromosomal breakage buffer (10 mM Tris HCl [pH8], 1 mM EDTA, 100 mM NaCl, 1% w/v SDS, 2% v/v Triton X-100). 200 µl of phenol/chloroform/isoamyl alcohol (25:24:1, pH8) were added and the cells lysed with glass beads using a Mini Beadbeater (Biospec Products). 500 µl of chromosomal breakage buffer were added prior to centrifugation (9000 x g, 5 minutes). The aqueous phase was transferred to a fresh Eppendorf and 50 µl of 3M sodium acetate and 1 ml 100% ethanol were added and the DNA precipitated at -20°C for 30 minutes. DNA was pelleted by centrifugation (13000 x g, 15 minutes), washed in 400 µl 70% ethanol and pelleted by centrifugation (13000 x g, 5 minutes). The DNA pellet was air dried before being resuspended in 100µl nH_2O and stored at -20°C.

2.2.9 RNA extraction

50 ml of mid-log phase growing, or glutathione depleted, *S. cerevisiae* cells were pelleted by centrifugation (3000 x g, 2 minutes), washed in 1 ml sterile water, pelleted again (3000 x g, 2 minutes) and the pellet snap frozen in liquid nitrogen. Pellets were thawed on ice and resuspended in 750 µl TES solution (10 mM Tris-HCl [pH7.5], 10 mM

EDTA [pH8], 0.5 % w/v SDS) with 750 µl acidic phenol: chloroform 5:1 (Sigma). Samples were incubated at 65˚C for 1 hour with a 10 second vortex step every 10 minutes. Samples were incubated on ice for 1 minute, vortexed for 20 seconds and pelleted by centrifugation for 15 minutes (1300 x g, 4˚C). The aqueous phase was then transferred to a heavy phase lock tube (Eppendorf) containing 700 µl acidic phenol 5:1 (Sigma), mixed by inversion and centrifuged for 5 minutes (13000 x g, 4˚C). 700 µl of the aqueous layer were then transferred to a light phase lock tube (Eppendorf) containing 700 µl of phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma), mixed by inversion and centrifuged for a further 5 minutes (13000 x g, 4˚C). 500 µl of the aqueous layer were then transferred to tubes containing 1.5 ml 100% ethanol and 50 µl of 3M NaAc [pH 5.2]. Following incubation -80˚C for 30 minutes, the precipitated RNA was pelleted by centrifugation (10 minutes, 13000 x g), the supernatant removed and 500 µl 70% ethanol added. Following centrifugation (1 minute, 13000 x g), the supernatant was removed and the pellet air-dried for 5 minutes at room temperature. The pellet was dissolved in 100 µl of RNase free water and stored at -80˚C.

2.2.10 S. cerevisiae protein extraction

50 ml of mid-log phase growing *S. cerevisiae* cells were pelleted by centrifugation (3000 x g, 2 minutes) and the pellet snap frozen in liquid nitrogen. The pellet was thawed at room temperature and then resuspended in 150 µl ice-cold protein lysis solution (20 mM HEPES [pH 7.3], 350 mM NaCl, 10% v/v glycerol, 0.1% v/v Tween-20), containing protease inhibitors (0.097 trypsin inhibitor units/ml aprotinin, 2 µg/ml leupeptin, 2µg/ml pepstatin A, 105 µg/ml PMSF) and phosphatase inhibitors (1 mM NaF, 2 mM Na_3VO_4), and then added to 1 ml of ice-cold glass beads (0.5 mm, BioSpec Products). Cells were lysed using a Mini Beadbeater (Biospec Products) and the protein clarified by centrifugation (13000 x g, 10 minutes, 4°C). Protein concentrations were estimated by Bradford assay (Pierce).

For experiments to investigate protein oxidation the trichloroacetic acid (TCA) protein extraction method was used. Mid-log phase growing cells were collected into an equal volume of 20% w/v TCA, pelleted by centrifugation (3000 x g, 2 minutes) and the pellet snap frozen in liquid nitrogen. Pellets were thawed on ice, resuspended in 200 µl ice cold 10% w/v TCA and cells lysed with ice-cold glass beads using a Mini Beadbeater

(Biospec Products). The insoluble pellet was washed three times in 200 µl acetone, solubilised in 200 µl of resuspension buffer (100 mM Tris-HCl [pH8], 1% w/v SDS, 1 mM EDTA) and the protein concentration estimated using the BCA protein assay kit (Thermo Scientific).

2.2.11 S. pombe protein extraction

100 ml of mid-log phase growing *S. pombe* cells were pelleted by centrifugation (2000 x g, 3 minutes), washed in 1 ml of sterile water and the pellet snap frozen in liquid nitrogen. The pellet was thawed at room temperature and resuspended in 200 µl of ice-cold lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.5 % w/v Nonidet P-40, 10 mM Imidazole) containing protease and phosphatase inhibitors as described in 2.2.10 before being added to 1 ml of ice-cold glass beads (0.5 mm, BioSpec Products). Cells were lysed using a Mini Beadbeater (BioSpec Products) before addition of a further 300 µl of lysis buffer. Protein was clarified by centrifugation (13000 x g, 10 minutes, 4°C) and protein concentrations estimated by Bradford assay (Pierce).

2.2.12 DNA content analysis

Approximately 5 x 10^6 cells were pelleted by centrifugation (2 minutes, 3000 x g), washed in 1 ml of sterile water, pelleted again (2 minutes, 3000 x g) and fixed in 1 ml of 70% ethanol overnight at 4˚C. Fixed cells were sonicated for 5 seconds to break up clumps and then pelleted by centrifugation (1 minute, 13000 x g). Cells were washed in 800 µl 50 mM sodium citrate [pH 7.2], pelleted again, resuspended in 500 µl RNase A solution (50 mM sodium citrate [pH 7.2], 0.25 mg/ml RnaseA (Thermo Scientific) and incubated at 37˚C overnight. 50 µl of proteinase K (20 mg/ml) (Roche) were added and the cells were incubated for 1-2 hours at 50˚C. Cell were sonicated again for 5 seconds to break up clumps and incubated with 500 µl of Sytox Green solution (50 mM sodium citrate [pH 7.2], 4 µM Sytox Green (LifeTechnologies)) for 1 hour in the dark at room temperature. A BD FACSCanto™ II (BD Biosciences) flow cytometer was used to measure DNA content and FACSDiva™ (BD Biosciences) software used in data analysis.

2.3 Molecular Biology and Bacterial Techniques

2.3.1 PCR

PCR reactions in this study were carried out using either the Expand™ High Fidelity PCR system (Roche) or Phusion® High-Fidelity DNA Polymerase (NEB). The DNA sequence of oligonucleotide primers used in this study are listed in Table 2.3.

2.3.1.1 Expand™ High-Fidelity PCR system

Each PCR reaction contained: 0.5 µl Expand™ High Fidelity Enzyme (3.5 units/µl), 1 µl template DNA, 10 µl 10 x High Fidelity Buffer, 85.5 µl water, 1 µl Forward/ Reverse Primer (100 pmol/µl), 1 µl dNTPs (10 mM). PCR was performed using the following parameters in a T3000 Thermocycler (Biometra):

Step 1 94˚C, 2 minutes

Step 2 94˚C, 20 seconds
Step 3 54˚C, 30 seconds
Step 4 72˚C, 1 minute/Kb
} x 35 cycles

Step 5 72˚C, 10 minutes

2.3.1.2 Phusion® High-Fidelity DNA Polymerase system

Each PCR reaction contained: 0.5 µl Phusion® High-Fidelity DNA polymerase (2000 units/ml), 1 µl template DNA, 0.5 µl Forward/reverse primer (100 pmol/µl), 1.5 µl $MgCl_2$ (1.5 mM), 10 µl 5 x Phusion GC buffer, 1 µl DMSO (2 % w/v), 0.5 µl dNTPs (10 mM), 34.5 µl water. PCR was performed using the following parameters in a T3000 Thermocycler (Biometra):

Step1 94˚C, 2 minutes

Step2 94˚C, 30 seconds
Step 3 54˚C, 1 minutes
Step 4 72˚C, 1 minute/kb
} x 35 cycles

Step 5 72˚C, 10 minutes

Primer name	Sequence 5’ → 3’
Ubc9 Forward	AACTGCAGAACCAATGCATTGGCCTTCTGGCACCGTTTGTCTC
Ubc9 Reverse	CGGGATCCCGTTGGAGCATTTTCACGAGCC
Ubc9 Check	CAGGTAGGTTTACTCCACCG
Nmtend	GCAGCTTGAATGGGCTTCC
Gsh1KO ChkF	GGCAACACCAGCTTCTCC
Gsh1KO ChkR	CCCAAATACAACAAGAACGG
Far1KOF	TCTATTTACTTTTATATTTCTTGACCATCCTTTACACAAAGTCTATAGATCCACTGGA AAGCTTCGTGGGCGTAAGAAGGCAATCTATTAGAATTCCCGGGGTCCGG
Far1KOR	TCAGTCATTGCGTAGTATAGACGTGGAGAAACGAAAAAAAAAAAAGGAAAAGCA AAAGCCTCGAAATACGGGCCTCGATTCCCGAACTAGCTTGGCTGCAGGTCGACGG
Far1KOChkF	TCACAGTATATAATATATTCA
Far1KOChkR	GATTTAATTATCGCCAATAGGT
Far1TagF	GGTAAAGCAGCAAAGAATTCATCAGACCCTGGAAGTTCCCAACCTCCGGATCCCC GGGTTAATTAA
Far1TagR	GAAAAGCAAAAGCCTCGAAATACGGGCCTCGATTCCCGAACTAGAATTCGAGCTC GTTTAAAC
Far1TagChkF	ACTGGAAAGCTTCGTGGGCG

Far1TagChkR	GATTTAATTATCGCCAATAGGT
Rub1KOF	AGCTGAAAAACCAAAATTCTGTTATTCAAATGAAGTATTCCGACAGAGGAATAAA TAAAGGAAGGTAATTAACTTCCTTACAGCCGTAACCGGAATTCCCGGGGATCCGG
Rub1KOR	AGCCTTCCAAAGTCCAAGTGAACTTATGTTCGTTTTGTCTTCTTTTCTAATGAACAC CTTCGATAAAATTCCATAAATGACGGAAAATGGTTGCTTGGCTGCAGGTCGACGG
Rub1KOChkF	ATTCCGATGGCGTCTTCGGGAAG
Rub1KOChkR	ACGGATAATTTGATAGATAC
Csn5koF	ACGCAGGAAGCGCTCTTATTAAAGGGTTTTTCAAATAAACTAAAAATGAATAACA ACGATCGAAAATCAATAAAAACTGCAGGAGGCAGCGAATTCCCGGGGATCCGG
Csn5KOR	GATATACTTATAGAGATTCAAGAAGGAATAACTATTAAAAGTACGTTGCAGATAA ACATAAGCTATTGCAGCGTCATTGGAGCAACGTTAGGAATGCTTGGCTGCAGGTC GACGG
Csn5KOChkF	GAAACTTTAGGAAAGGCGCG
Csn5KOChkR	TATCAAATTGTCACCCGG
F2 Chk	AACCCGGGGATCCGTCGACC
Uba1TAP	TTCATCGAAACTGCTGATCG
Uba2TAP	TCCACAGGATATTTCGCTCC
Uba3TAP	TAGATGGTGGAACAGAGGGG
Uba4TAP	ATTCAGGTTTCCCACAGCAG

Ubc1TAP	TAAAAGGCACATTTTTGGGC
Ubc2TAP	CCCCACCGGGTGTATCTGC
Cdc34TAP	AACGTTTACAGGGATGGCAG
Ubc4TAP	AGCCGGTCCAGTCGGCGATGATC
Ubc5TAP	CCGAAGAGATCCTCCTGCTTC
Ubc6TAP	AACCACCGGCTATCAGAATG
Ubc7TAP	CCACCTGGTATAGTGGCTGGTCC
Ubc8TAP	TACGAAAATGGGGTTTGGAG
Ubc10TAP	ACGTCTGATACATGTATGTCG
Ubc13TAP	TTGTCCAGTTTGTAGCAGCG
Mms2TAP	CAACGTAGAAGAAAGCAGCG
Ubc9TAP	TACACAATTTCATCCAGCGG

Table 2.3. The DNA sequence of the oligonucleotide primers used in this study.

Primer name	Sequence 5’ ⟶ 3’
ARN2F	TGACCTACGCAATGAACTCG
ARN2R	CAGCAGCATACCTCTGGACA
Cdc34F	CACTTCTGAATCGGCGTACA
Cdc34R	TGGTTGTTTCCATCGTCGTA
Ydj1F	CTGGTGGTGAATTTGCATTG

Ydj1R	GGACAATTCTTGGAGGCAAA
Cdc53F	CATTATGTCGTGGCGAATTG
Cdc53R	GTTAAGCTCGTGCCCTCTTG
Act1F	GCCTTCTACGTTTCCATCCA
Act1R	GGCCAAATCGATTCTCAAAA

Table 2.4. The DNA sequence of the oligonucleotide primers used for qRT-PCR in this study.

2.3.2 qRT-PCR RNA clean up and DNase digestion

RNA was extracted as described in 2.2.9 and purified using an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. 3 µg of purified RNA were subjected to DNase digestion to remove gDNA contamination using a Precision DNase kit (Primer Design) according to the manufacturer's protocol.

2.3.3 qRT-PCR conditions

qRT-PCRs in this study were performed using a Precision One-Step qRT-PCR mastermix with SYBR Green (Primer Design) using the following volumes: 10 µl qRT-PCR mastermix with SYBR Green, 2 µl RNA (typically 3 µg), 7 µl water, 1 µl primer mix (5 mMol). The DNA sequence of the oligonucleotide primers used for qRT-PCR in this study are listed in Table 2.4.

SYBR Green detection was recorded using a lightcycler (Corbett 6000) with the following settings:

Step 1 (Reverse Transcription)	10 minutes at 55˚C
Step 2 (Enzyme activation – Hotstart)	8 minutes at 95˚C
Step 3 (Denaturation)	10 seconds at 95˚C
Step 4 (Data Collection)	60 seconds at 60˚C.

Steps 3-4 were repeated 50 times, and at the finish a melt curve was generated to help confirm primer specificity.

2.3.4 Escherichia coli transformation and plasmid isolation

Plasmids were propagated by introducing them into *E. coli* SURE competent cells (*e14*$^{-}$ (*mcrA*$^{-}$) Δ(*mcrCB*$^{-}$*hsdSMR*$^{-}$*mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5* (*Kan*^{r}) *uvrC*[*F'proAB lac*^{Q}*ZΔm15 Tn10* (*Tet*^{r}) (Stratagene) using the standard calcium chloride method (Maniatis *et al.*, 1982). *E. coli* SURE cells transformed with plasmids containing the ampicillin resistance gene were grown in LB media (2 % w/v Bacto tryptone, 1 % w/v Bacto yeast extract, 1 % w/v NaCl [pH7.2]) containing 0.1 mg/ml ampicillin (Sigma). A GenElute Plasmid Miniprep kit (Sigma) was used to isolate plasmid DNA, according to manufacturer's instructions.

2.3.5 Restriction endonuclease digestion and DNA ligation

PCR products and plasmids were digested using the relevant restriction enzymes and buffers according to the manufacturer's instructions (Fermentas). Digested plasmids and PCR fragments were purified (see Section 2.3.6). DNA was ligated using DNA ligase (Fermentas) according to manufacturer's instructions.

2.3.6 Agarose gel electrophoresis and DNA purification

PCR products and plasmids were separated by electrophoresis using 1 % w/v agarose gels using TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH8]) and containing 5 µg/ml ethidium bromide, visualised using a transilluminator (Ultra-Violet Products). Where required, DNA was excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen), according to manufacturer's instructions.

2.3.7 Western Blotting

In this study, proteins were separated on either 10% w/v or 13% w/v SDS polyacrylamide gels. The gel recipes were based on the SDS (denaturing)-discontinuous buffer system of Laemmli (1970). 5 µl of PageRuler Prestained Protein Ladder (Thermo Scientific) were included to allow estimation of molecular weight. Separated proteins were transferred onto Protran® nitrocellulose membrane (GE Healthcare Life Sciences) using a Bio-Rad mini transfer apparatus, according to the manufacturer's instructions. The membrane was blocked in 10% w/v BSA for 30 minutes and incubated with the appropriate primary antibody (Table 2.5) diluted in 5% w/v BSA overnight at 4˚C with agitation. The membrane was then washed with TBST solution (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% v/v Tween®20) (3 x 5 minutes) prior to incubation for 1 hour at room temperature with the relevant secondary antibody (either anti-mouse HRP, anti-rabbit HRP or anti-goat HRP) diluted 1:2000 in 5% w/v BSA. The membrane was washed in TBST (3 x 10 minutes) and protein visualised using either the ECL detection system (Amersham Pharmacia Biotech) or ECL plus (Thermo Scientific).

To re-probe with a different antibody, nitrocellulose membranes were stripped by incubating at 50˚C for 30 minutes in stripping solution (2% w/v SDS, 62.5 mM Tris-HCl [pH 6.7], 100 mM β-mercaptoethanol). The membrane was then washed in TBST (3 x 10

minutes) and blocked in 10% w/v BSA before being incubated with the relevant primary and secondary antibodies as described above.

Antibody name	Working dilution in 5% w/v BSA	Supplier
Rad53 (yC-19)	1:1000	Santa Cruz Biotechnology
Cdc53 (yN-18)	1:1000	Santa Cruz Biotechnology
CBP	1:1000	Source Bioscience LifeSciences
HA	1:1000	Thermo Scientific
Myc 9E10	1:1000	SigmaAldrich
Anti-V5 tag (PK)	1:1000	Bio-Rad
Anti-Hog1 (y218)	1:1000	Santa Cruz Biotechnology
Anti-Donkey anti-goat IgG HRP	1:2000	Santa Cruz Biotechnology
Anti-Mouse IgG (whole molecule)	1:2000	SigmaAldrich
Anti-Rabbit IgG (whole molecule)	1:2000	SigmaAldrich

Table 2.5. Antibodies used in this study.

Chapter 3: Analysis of the relative sensitivities of yeast Ub/Ubl conjugation pathways to ROS

3.1 Background

The ability to detect environmental stress and initiate an appropriate response is key to cellular survival. Several signalling pathways have been identified in eukaryotes that are able to respond to ROS via reversible oxidation of cysteine residues in signalling proteins for example the protein tyrosine phosphatases (PTPs) which are inactivated by oxidation of their active site cysteine. The use of catalytic cysteine residues in their conjugation pathways raises the possibility that ubiquitin and Ubl conjugation may also be regulated by oxidation.

Global patterns of SUMO (Saitoh and Hinchey, 2000), Nedd8 and Urm1 (Van der Veen *et al.*, 2011) conjugation have been shown to increase in response to H_2O_2 in both yeast and mammalian cells suggesting a conserved role for a number of Ubl pathways in the cellular response to oxidative stress. However, in contrast to the above observations, lower concentrations of H_2O_2 have been shown to inhibit the mammalian SUMO conjugation pathway through the formation of a disulphide bond between the E1, Uba2 and the E2, Ubc9 blocking downstream SUMOylation (Bossis and Melchior, 2006). A similar mechanism has also been demonstrated for the mammalian Nedd8 (Rub1 in *S. cerevisiae*) conjugation pathway, whereby the E2, Ubc12 has been shown to form a disulphide with an unidentified partner after exposure to H_2O_2 produced by commensal bacteria in the gut, leading to inhibition of Nedd8 conjugation to the downstream substrate Cul-1 (Kumar *et al.*, 2007). These studies demonstrate a mechanism by which two Ubls may be involved in stress signalling.

Previous work from our lab had shown that in response to specific oxidising agents e.g. diamide and menadione cells arrest their cell cycle in G1 phase (O'Callaghan, 2004; Doris, 2008). Interestingly, the observed cell cycle arrest coincided with an increase in the stability of the CKI Sic1. As previously described (Section 1.2.4), Sic1 is ubiquitinated by a pathway involving the E1 Uba1 and the E2 Cdc34. It was predicted that the formation of an inhibitory disulphide bond between these enzymes as is seen in the mammalian SUMO pathway (Bossis and Melchior, 2006) could lead to an increase in the stability of Sic1. A high molecular weight (HMW) disulphide complex was indeed seen to

form between the catalytic cysteine residues of Uba1 and Cdc34 after treatment with specific oxidising agents. Importantly, the formation of the Uba1-Cdc34 complex occurred at the same time as the observed increase in Sic1 stability (Doris, 2008).

While Cdc34 was shown to be sensitive to a number of oxidising agents (Doris, 2008), *S. cerevisiae* has a number of ubiquitin and Ubl pathway enzymes and their relative sensitivities to diamide have yet to be explored. In this chapter we will investigate the sensitivity of a number of ubiquitin and Ubl pathway enzymes to the oxidising agents diamide and H_2O_2.

3.2 Cdc34 forms a HMW complex in response to oxidising agents independent of strain background or epitope tag

Previous work in our lab had shown that myc epitope-tagged Cdc34 forms a HMW complex with the ubiquitin pathway E1 Uba1 in response to both H_2O_2 and diamide in cells from the W303 background. However, the potential effects of the tag, strain background or the general specificity of this E1/E2 sensitivity to oxidation were not fully explored. To begin to address these questions, cells expressing tandem affinity purification (TAP) epitope-tagged Cdc34 from the normal chromosomal locus were obtained in the BY strain background (Ghaemmaghami *et al.*, 2003). This strain background has previously been shown to display both different sensitivities to oxidative stress compared with W303 and to utilise different signalling mechanisms to detect and respond to oxidative stress (Veal *et al.*, 2003). W303 cells expressing Cdc34-13Myc and BY cells expressing Cdc34-TAP were treated with 5 mM diamide and proteins analysed by western blotting (Figure 3.1). Significantly, in cells from both strain backgrounds, epitope-tagged Cdc34 was found to form a HMW complex after 30 minutes treatment with 5 mM diamide consistent with oxidation of Cdc34 (Figure 3.1). Thus, these data strongly suggest that the Cdc34-Uba1 disulphide complex is induced by oxidative stress in both a strain background and epitope tag independent manner.

3.3 E1s of the ubiquitin, SUMO, Rub1 and Urm1 conjugation pathways show differing sensitivities to oxidation

Having established that TAP-tagged Cdc34 forms a HMW complex in response to oxidative stress we next took advantage of the availability of many TAP-tagged E1/E2s in Ub/Ubl pathways in the BY strain background (Ghaemmaghami *et al.*, 2003) to

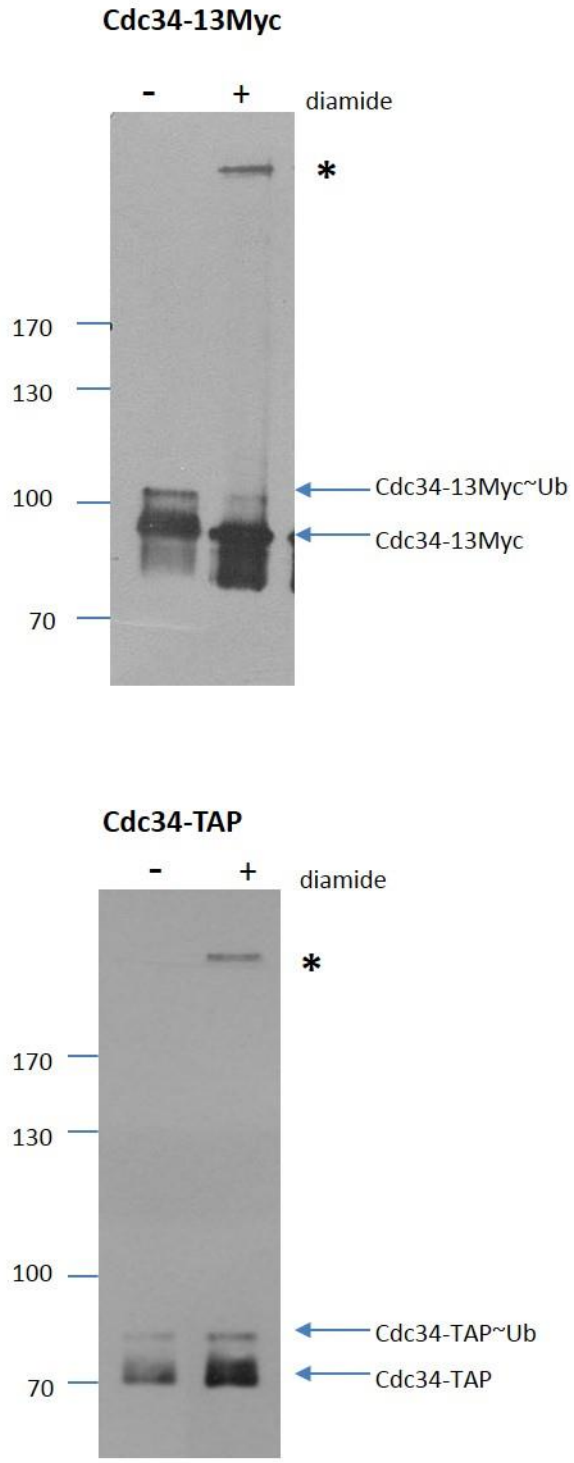
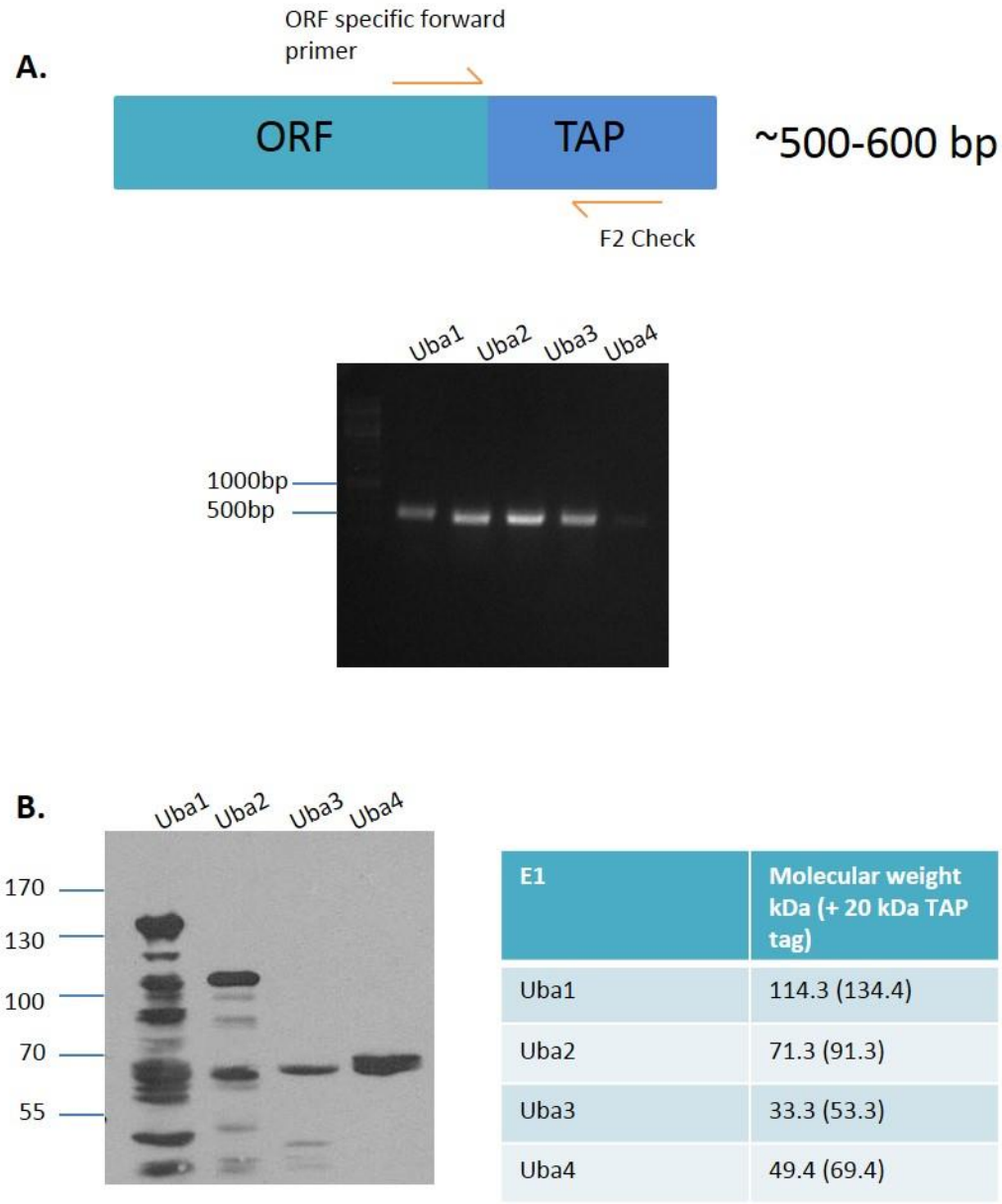


Figure 3.1 Cdc34 forms a high molecular weight complex after treatment with diamide in cells from different strain backgrounds. Cells expressing Cdc34-13Myc (KD79) or Cdc34-TAP (ELR12) from their normal chromosomal locus were treated with 5 mM diamide for 30 minutes. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting with anti-Myc (Cdc34-13Myc) or anti-CBP antibodies (Cdc34-TAP). * denotes the Cdc34 containing HMW complex.

explore the relative sensitivities of the different E1/E2 enzymes to oxidation. *S. cerevisiae* contains a number of E1/E2 enzymes in at least 10 Ubl conjugation pathways. Our data demonstrate that Uba1 in the ubiquitin pathway is sensitive to oxidation (Doris, 2008). Interestingly work in other systems has shown that the E1 of the mammalian SUMO pathway also displays an increased sensitivity to oxidation (Bossis and Melchior, 2006) however this has not been explored in *S. cerevisiae*. There is very little known about the Urm1 Ubl pathway but it has been linked with oxidative stress responses in both yeast and humans (Van der Veen *et al.*, 2011). Analysis of the available Ubl TAP-tagged genome library showed that strains potentially expressing the TAP-tagged E1s Uba1 (ubiquitin), Uba2 (SUMO), Uba3 (Rub1) and Uba4 (Urm1) were present. To confirm the identity of these strains, a PCR strategy was performed to demonstrate the localisation of the TAP-tagged cassette at the 3'end of each gene. PCR was performed using an E1-specific forward primer and a common reverse primer (F2 check) located in the TAP tag cassette (Figure 3.2 A). If correctly TAP-tagged, PCR for each gene product should amplify a product of approximately 500-600 bp. Significantly, this PCR strategy confirmed that the *UBA1*, *UBA2*, *UBA3* and *UBA4* genes have the TAP-tag cassette located at their 3'ends (Figure 3.2 A). Next, to ensure that each TAP-tagged protein was expressed, protein was extracted from Uba1-TAP, Uba2-TAP, Uba3-TAP and Uba4-TAP cells and analysed by western blotting. Specific bands corresponding to the approximate predicated size of TAP epitope-tagged Uba1, Uba2 and Uba4 were detected suggesting that these E1s were correctly TAP-tagged and expressed (Figure 3.2 B). The Uba3-TAP ran at a larger size than was expected however due to the lack of non-specific bands in this lane it was predicted that this band was Uba3-TAP (Figure 3.2 B).

Having established that Uba1-TAP, Uba2-TAP, Uba3-TAP and Uba4-TAP could be detected the relative sensitivities of these proteins to 5 mM diamide were explored. As with Cdc34-TAP (Figure 3.1), Uba1-TAP was shown to form a HMW complex following treatment with 5 mM diamide independent of strain background or epitope tag (Figure 3.3). Furthermore, the relative abundance of this HMW complex matched that of the HA epitope-tagged Uba1 expressed in the W303 strain background (Doris, 2008).

Previous studies in mammalian cells had demonstrated that Uba2 forms a HMW complex in response to H_2O_2 (Bossis and Melchior, 2006). Interestingly, in contrast to Uba1, no Uba2-TAP containing complex was detected in cells treated with 5 mM



E1	Molecular weight kDa (+ 20 kDa TAP tag)
Uba1	114.3 (134.4)
Uba2	71.3 (91.3)
Uba3	33.3 (53.3)
Uba4	49.4 (69.4)

Figure 3.2 Uba1, Uba2, Uba3 and Uba4 are all successfully TAP tagged at their normal chromosomal locus. [A] PCR was performed using genomic DNA from Uba1-TAP (ELR5), Uba2-TAP (ELR6), Uba3-TAP (ELR7) or Uba4-TAP (ELR8) as a template, the F2 check reverse primer and a strain specific forward primer as shown. PCR products were separated using gel electrophoresis on a 1% agarose gel and visualised under UV light. [B] Protein was extracted from mid-log phase growing Uba1-TAP, Uba2-TAP, Uba3-TAP and Uba4-TAP strains. Cell lysates were prepared under reducing conditions, separated by SDS-PAGE and analysed by western blotting with anti-CBP antibodies.

diamide (Figure 3.3). Furthermore, no changes in Uba4 were detected in response to diamide. Unexpectedly, Uba3-TAP was found to form a HMW complex in response to diamide (Figure 3.3). Previous work in mammalian cells had shown that the E2 but not the E1 of the Neddylation pathway forms a HMW complex in response to ROS (Kumar *et al.*, 2007). Hence, this is the first identification of such a HMW complex involving the Rub1 pathway E1 in eukaryotic cells. The identities of the components of this complex are unclear and require further investigation.

3.4 Oxidation of the SUMO pathway E1 and E2 enzymes is not conserved in either *S. cerevisiae* or *S. pombe*

Previous studies in mammalian cells demonstrated that the SUMO pathway E1/E2 enzymes specifically form a disulphide complex in response to H_2O_2. The initial study presented here of the equivalent E1 enzyme in the SUMO pathway in *S. cerevisiae* did not reveal any detectable sensitivity to oxidation by diamide. However, diamide and H_2O_2 are different oxidising agents with different effects and responses in cells (Flattery-O'Brien and Dawes, 1998; O'Callaghan, 2004) and hence it could be possible that the Uba2 and Ubc9 enzymes of the *S. cerevisiae* SUMO pathway could be more sensitive to H_2O_2 than diamide. To test this possibility, cells expressing Uba2-TAP were treated with a range of H_2O_2 concentrations and protein extracts analysed by western blot. Consistent with the results obtained using diamide, no HMW Uba2-TAP complex was detected when cells were treated with H_2O_2 (Figure 3.4 A). Hence, in contrast to mammalian cells Uba2 is not sensitive to the oxidising agents H_2O_2 or diamide.

It was possible that the E2 enzyme Ubc9 in *S. cerevisiae* is more sensitive to oxidation by diamide and/or H_2O_2 than Uba2, perhaps forming a different HMW complex than that observed in mammalian cells. To explore this possibility a strain expressing TAP-tagged Ubc9 from the normal chromosomal locus was obtained from the large scale TAP-tagged library (Ghaemmaghami *et al.*, 2003). Correct integration of the TAP-tagged cassette at the 3'end of the *UBC9* gene was confirmed by PCR and protein expression were analysed as previously described. Consistent with the analysis of Uba2-TAP, Ubc9-TAP did not form any detectable complexes when cells were treated with H_2O_2 (Figure 3.4 B). Differences observed in the ratio of Ubc9 and the Ubc9~SUMO thioester (Figure 3.4 B) were not repeated.

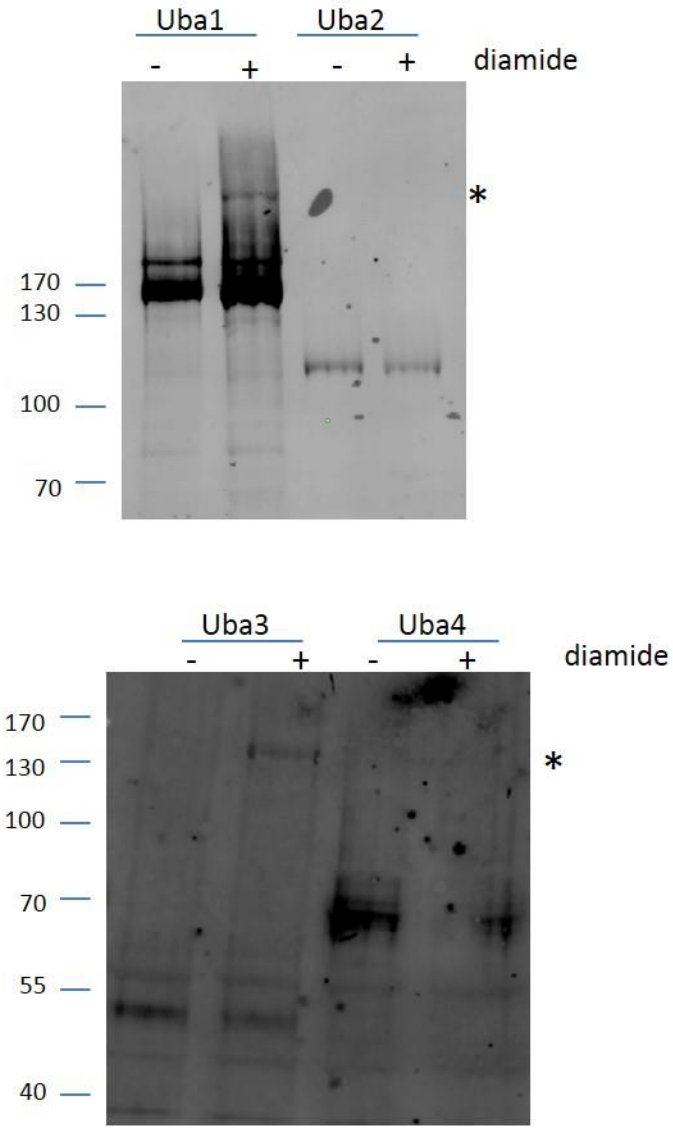


Figure 3.3 Uba1, Uba2, Uba3 and Uba4 show different sensitivities to diamide. Mid-log phase growing cells expressing either Uba1-TAP (ELR5), Uba2-TAP (ELR6), Uba3-TAP (ELR7) or Uba4-TAP (ELR8) from their normal chromosomal locus were treated with 5 mM diamide for 30 minutes. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and visualised by western blotting using anti-CBP antibodies. * denotes HMW complex.

As described above, analysis of Uba2 and Ubc9 suggested that these enzymes are much less sensitive to oxidation by oxidising agents in *S. cerevisiae* than in mammalian cells. In addition neither Uba2 nor Ubc9 formed a disulphide complex at levels of H_2O_2 shown to oxidise Uba2 and Ubc9 in mammalian cells (Figure 3.4 A and B, (Bossis and Melchior, 2006)). It was possible that this lack of sensitivity was specific to *S. cerevisiae* and not generally to eukaryotic cells. To investigate this specificity, the potential oxidation of the SUMO conjugation enzymes was examined in the evolutionarily distantly related *S. pombe*. Interestingly, in contrast to *S. cerevisiae*, the gene encoding the SUMO modifier is not essential in *S. pombe* (Tanaka *et al.*, 1999) suggesting that the SUMO pathways of the two yeasts could be regulated differently. Hence to test the oxidation of Ubc9 in *S. pombe*, a strain expressing Ubc9 tagged at the C-terminus with 3PK epitopes from the normal chromosomal locus was treated with concentrations of diamide that lead to the formation of the Cdc34 containing HMW complex and with concentrations of H_2O_2 which stimulate the formation of the Uba2~Ubc9 complex in mammalian cells (Bossis and Melchior, 2006). Significantly, there was no evidence that a HMW complex containing Ubc9 forms after exposure to these levels of ROS in *S. pombe* (Figure 3.4 C).

Taken together these results suggest that in contrast to mammalian cells, Uba2 and Ubc9 do not form disulphide complexes following H_2O_2 or diamide treatment in these widely divergent eukaryotic cells. Thus, the increased sensitivity to oxidation of Uba2 and Ubc9 may be confined to mammalian/higher eukaryotic cells.

3.5 Of the Ubiquitin pathway enzymes, Cdc34 is more sensitive to oxidation by diamide

As previously described, work in our lab had shown that the ubiquitin E2, Cdc34 was sensitive to oxidation. However, the previous study had only investigated Cdc34 and *S. cerevisiae* has at least twelve ubiquitin pathway E2 enzymes that help achieve substrate specificity. Here we extended the initial study to investigate the relative sensitivities of the ubiquitin pathway E2 enzymes.

Twelve known E2 enzymes were identified of which eleven were available in the large scale TAP epitope-tagged library (Ghaemmaghami *et al.*, 2003). To confirm the position of the TAP-tagging cassette at the 3'end of each gene, PCR was performed using a common reverse

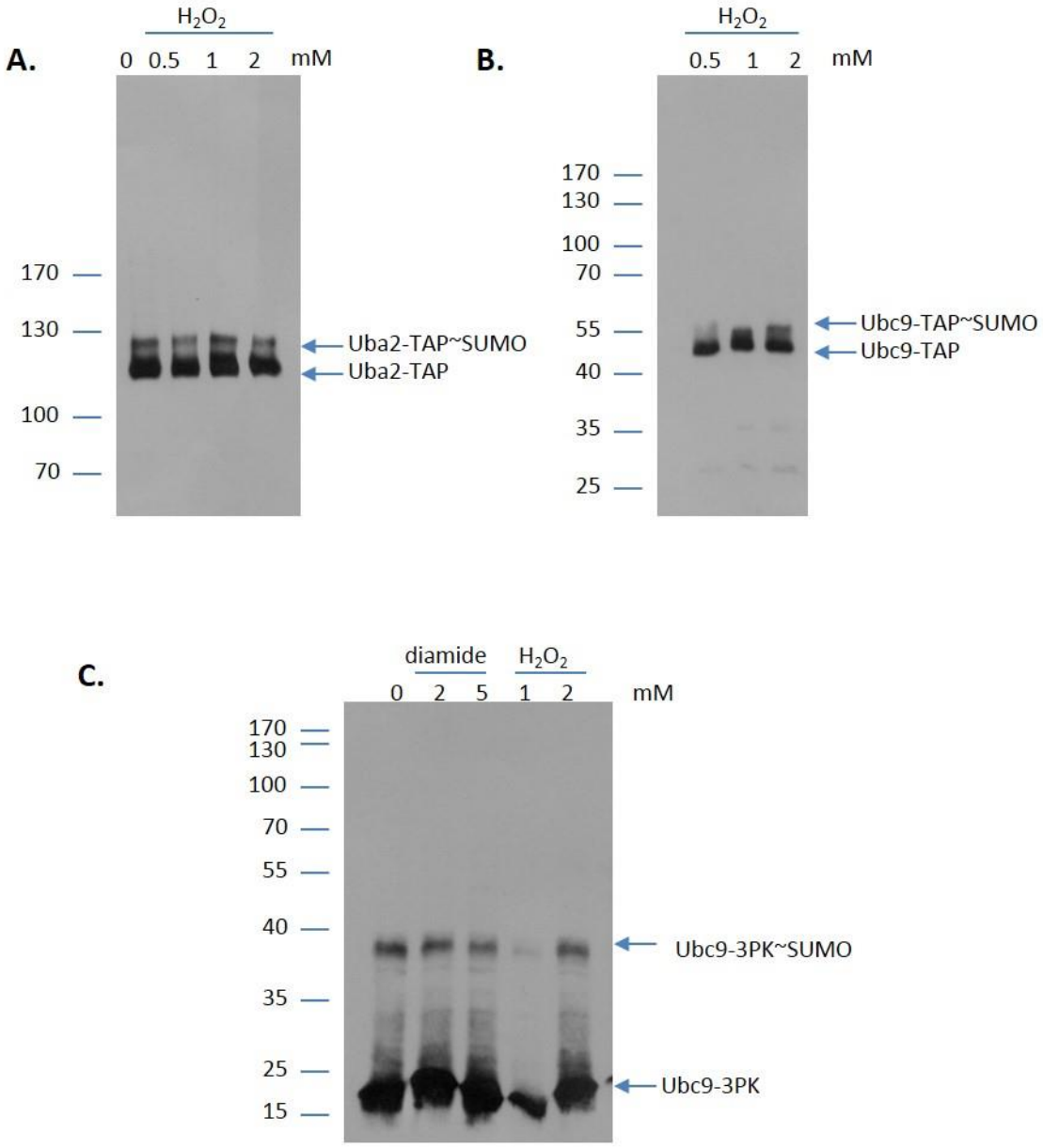
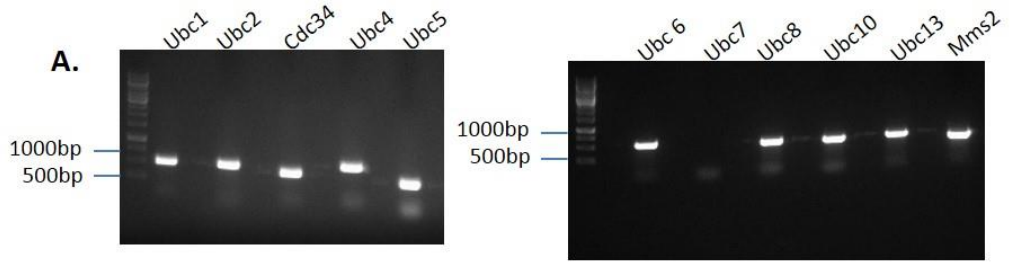
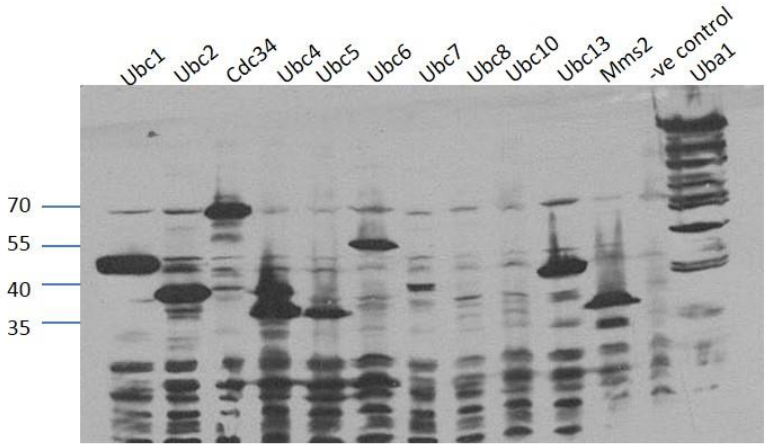


Figure 3.4 The E1 and E2 enzymes of the SUMO pathway do not form HMW complexes following oxidative stress in either *S. cerevisiae* or *S. pombe*. [A] and [B] Mid-log phase growing *S. cerevisiae* cells expressing either Uba2-TAP (ELR6) [A] or Ubc9-TAP (ELR21) [B] from their normal chromosomal locus were treated with the indicated concentration of H_2O_2 for 20 minutes. [C] Mid-log phase growing *S. pombe* cells expressing Ubc9-3PK (ER1) from the normal chromosomal locus were treated with the indicated concentration of either H_2O_2 or diamide for 20 or 30 minutes respectively. [A-C] Cell lysates were processed under non-reducing conditions, separated by SDS-PAGE. And analysed by western blotting using anti-CBP [A] and [B] or anti-PK [C] antibodies.



B.



E2	Molecular weight kDa (+ 20kDa TAP tag)
Ubc1	24.2 (44.2)
Ubc2	19.7 (39.7)
Cdc34	34.1 (54.1)
Ubc4	16.5 (36.5)
Ubc5	16.3 (36.3)
Ubc6	28.4 (48.4)
Ubc7	18.5 (38.5)
Ubc8	24.6 (44.6)
Ubc10	21.1 (41.1)
Ubc11	Not in library
Ubc13	17.5 (37.5)
Mms2	15.5 (35.5)

Figure 3.5 A number of ubiquitin pathway TAP epitope-tagged E2 enzymes expressed from their normal chromosomal locus can be detected by western blot analysis. [A] PCR was performed using genomic DNA extracted from the indicated TAP-tagged strains (ELR10-ELR20) with the F2 check reverse primer and a gene-specific forward primer (see Figure 3.2). PCR products were separated on a 1% agarose gel and visualised under UV light. [B] Protein was extracted from mid-log phase growing cells expressing the indicated TAP-tagged E2 enzyme. Cell lysates were prepared under reducing conditions, separated by SDS-PAGE and analysed using western blotting with anti-CBP antibodies.

primer (F2 check) and a specific forward primer as previously described (Figure 3.2 [A]). PCR analysis of all the E2 enzyme encoding genes except *UBC7* amplified a band product of approximately 500-600 bp as predicted (Figure 3.5 A). Next, protein extracts were obtained from each TAP epitope-tagged E2 expressing strain and western blot analyses performed to confirm visualisation of the TAP epitope-tagged protein (Figure 3.5 B). Of the eleven E2 enzymes, the more abundant proteins, Ubc1, Ubc2, Cdc34, Ubc4, Ubc6 and Ubc13 were detected by western blotting (Figure 3.5 B). Having identified five further E2 enzymes that could be detected by western blot we next investigated their sensitivity to diamide in comparison to Cdc34.

Cells expressing each of the identified TAP-tagged E2 enzymes were treated with 5 mM diamide for 30 minutes and protein extracts examined on non-reducing gels (Figure 3.6). Excitingly, Cdc34 alone was seen to form a HMW complex after treatment with 5 mM diamide (Figure 3.6). Thus taken together with the analysis of other Ubl pathways in *S. cerevisiae* these data indicate that Cdc34 is specifically sensitive to specific oxidising agents.

3.6 Strains with a decrease in *CDC34* or *UBA1* transcript levels show resistance to diamide

Previous data from our lab indicated that oxidation of Uba1/Cdc34 is specifically linked to regulation of the cell cycle and Sic1 (Doris, 2008). However we had not been able to provide evidence that the formation of the Uba1-Cdc34 HMW complex, the increased stability of Sic1 and the subsequent cell cycle delay that was observed after diamide treatment were directly linked. Previous attempts to make strains which expressed Cdc34/Uba1 that were more or less sensitive to oxidation had proven difficult due to the essential nature of both genes. However, a commercially available 'Decreased Abundance by mRNA Perturbation' (DAmP) strain collection has been constructed whereby the 3'UTR of many essential genes has been disrupted by the integration of a kanamycin cassette leading to a subsequent destabilisation of the transcript (Breslow *et al.*, 2008). This reduction in transcript levels provides a useful tool for studying essential genes and thus whether Cdc34-Uba1 HMW complex formation acts as protective mechanism for the cell in response to ROS.

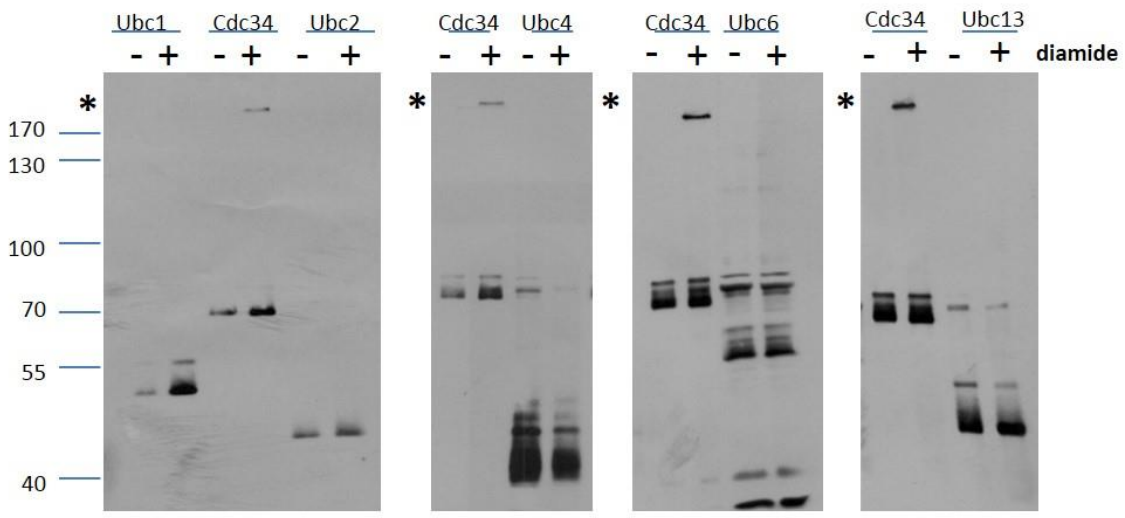


Figure 3.6 Cdc34 is specifically sensitive to oxidation by diamide. Mid-log phase growing cells expressing the indicated TAP epitope-tagged E2 enzymes were treated with 5 mM diamide for 30 minutes. Cell lysates were collected under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-CBP antibodies. * denotes Cdc34-containing HMW complex.

It is possible that insertion of the kanamycin cassette does not lower the levels of specific transcripts. Therefore, to test whether *CDC34* or *UBA1* transcript levels were reduced in the relevant DAmP strains qRT-PCR was performed. Importantly, the transcript levels for both *CDC34* and *UBA1* were found to be reduced (Figure 3.7 A). Next the sensitivity of these strains to diamide was examined. Interestingly, despite a decrease in the levels of *CDC34* and *UBA1* expression each strain grew well in the absence of stress in comparison with a control strain where the 3'UTR of the *HIS3* gene has been disrupted by the DAmP cassette (HIS3 DAmP) (Figure 3.7 B). Excitingly, both the *CDC34* and *UBA1* DAmP strains showed an increased resistance to diamide compared to the *HIS3* DAmP strain. These results demonstrate that weakening of the Uba1/Cdc34 pathway is necessary for cellular survival in response to diamide.

3.7 Discussion

While the detrimental effects of high levels of ROS have long been recognised, the importance of lower levels of ROS, such as H_2O_2 in cellular signalling has now become apparent. Significantly, a number of redox-sensitive proteins have now been identified, which are regulated through reversible oxidation of their catalytic cysteine residue. Significantly, the ubiquitin and Ubl family of protein modifiers use catalytic cysteine residues in their conjugation pathways and studies in mammalian cells and *S. cerevisiae* have shown that the conjugation pathways of the Nedd8, SUMO and ubiquitin pathways are sensitive to oxidising agents. Here, studies of the SUMO conjugation pathway in two evolutionary divergent yeast, *S. cerevisiae* and *S. pombe* have shown no evidence that the E1-E2 disulphide observed in mammalian cells in response to H_2O_2 is conserved. However, we show that the E1 of the Rub1 pathway does form a HMW complex in response to diamide, although the other components of this complex are currently unknown. In this chapter we have extended previous studies from our lab that showed that Uba1 and Cdc34 form a HMW complex in response to diamide to demonstrate that this Uba1-Cdc34 complex is formed regardless of strain background or epitope-tag. Importantly, here we have demonstrated that Cdc34 is specifically more sensitive to diamide than the other ubiquitin pathway E2 enzymes tested. While previous studies had shown that the formation of the Uba1-Cdc34 HMW complex coincided with an increase in the stability of the CKI Sic1 and cell cycle delay, we had been unable to

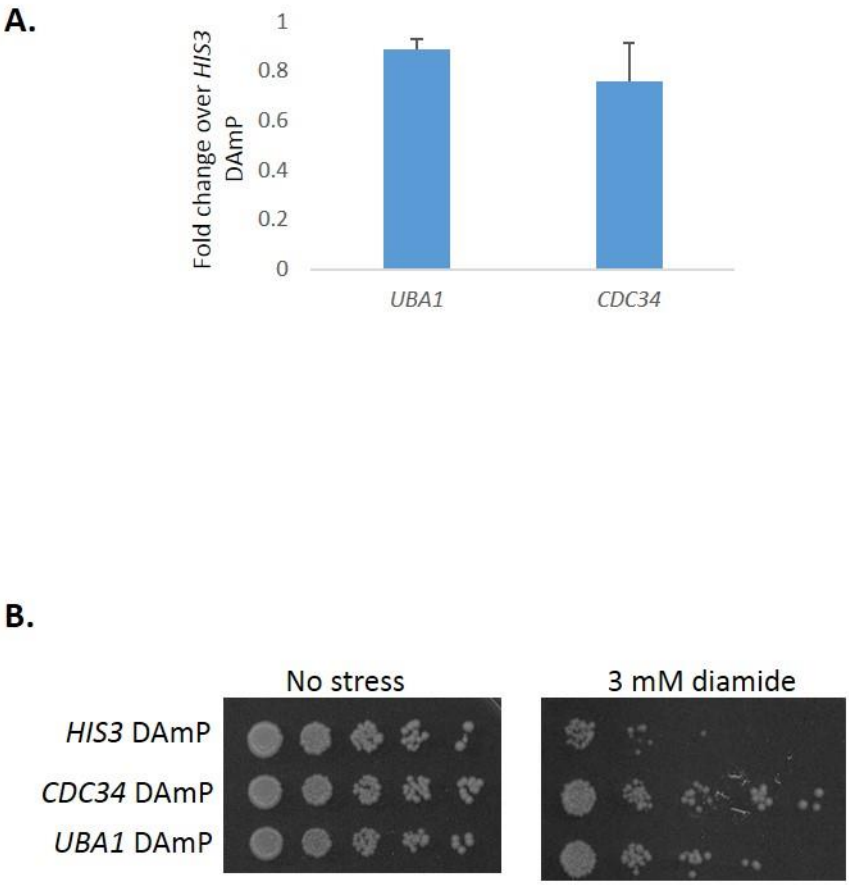


Figure 3.7. Reduction of *CDC34* or *UBA1* transcript levels increases resistance to diamide. [A] RNA was extracted from mid-log phase growing *HIS3* (CLC9), *CDC34* (CLC27) and *UBA1* (CLC30) DAmP strains and subjected to qRT-PCR to detect *CDC34* and *UBA1* transcripts. qRT-PCR was normalised to *ALG9* and fold changes over *HIS3* is shown. Fold changes are the mean of 3 biological repeats and error bars represent SE [B] 10-fold serial dilutions of the indicated mid-log phase growing DAmP strains were spotted onto YPD plates containing 3 mM diamide. Plates were incubated at 30°C for 2-3 days.

directly link these events. Here we have shown that cells that have a decreased expression of *CDC34* or *UBA1* are resistant to diamide providing direct evidence between the formation of the Uba1-Cdc34 disulphide and cell viability in response to diamide.

3.7.1 Redox regulation of the SUMO pathway

While there is evidence for the regulation of the mammalian SUMO pathway E1 and E2 enzymes by H_2O_2, the conservation of this sensitivity to oxidation has not been explored in the *S. pombe* or *S. cerevisiae* model systems. Significantly, we find that that the E1-E2 disulphide that forms in response to low levels of H_2O_2 in mammalian cells could not be detected in either *S. cerevisiae* or *S. pombe* treated with either diamide or H_2O_2 (Figure 3.4).

In mammalian cells, treatment with high concentrations of H_2O_2 (100 mM) leads to an increase in the global levels of SUMO conjugation while at lower concentrations of H_2O_2 (1 mM) SUMO conjugation is inhibited by the formation of an E1-E2 disulphide (Bossis and Melchior, 2006). The regulation of SUMO conjugation at different H_2O_2 concentrations appears to be due to the specific sensitivities of the conjugating and deconjugating enzymes. Indeed, the SENP cysteine proteases that remove SUMO from its targets are also sensitive to oxidation. Exposure of SENPs to H_2O_2 can lead to the oxidation of their catalytic cysteine residue to the irreversible Cys-SO_2 or Cys-SO_3 forms and a loss of enzyme activity (Xu *et al.*, 2008). The activity of the mammalian SENP1 has been shown to be abolished in response to H_2O_2. Reversible oxidation of the SENP1 active site cysteine residue leads to the formation of a protective disulphide with another protease and a subsequent loss of activity. Interestingly, another mammalian SENP, SENP2 does not form a disulphide under the same conditions providing a mechanism whereby specific SUMO targets could be affected in response to H_2O_2 . The human SENP3 has also been shown to be regulated under conditions of mild oxidative stress. Usually kept at a low basal level by ubiquitin-mediated degradation, in response to mild oxidative stress SENP3 is oxidised at two cysteine residues and binds to HSP90 which prevents its ubiquitination and subsequent degradation (Yan *et al.*, 2010).

In *S. cerevisiae*, as with mammalian cells the global level of SUMO conjugation increases after exposure to high levels of oxidative stress (Zhou *et al.*, 2004). Interestingly, in

contrast to mammalian cells, treatment of *S. cerevisiae* with 1 mM H_2O_2 leads to an increase in global SUMO conjugates. One of the *S. cerevisiae* SENP enzymes, Ulp1 has been shown to be particularly sensitive to oxidation even at low concentrations of H_2O_2, leading to the formation of a dimer (Xu *et al.*, 2008). Taken together, these studies alongside our data suggest that in response to H_2O_2 and diamide the SUMO pathway in *S. cerevisiae* may be regulated through the deconjugating machinery rather than the conjugating enzymes.

3.7.2 Redox regulation of the Rub1/Nedd8 pathway

The E2 of the mammalian Nedd8 pathway conjugation pathway, Ubc12 has been shown to form a HMW complex in response to H_2O_2 leading to the inhibition of Nedd8 conjugation to Cul-1 (Kumar *et al.*, 2007). Here, we have shown that the Rub1 pathway E1 (Uba3) of *S. cerevisiae* forms a HMW complex in response to diamide treatment (Figure 3.3). Further work is required to establish the components of the HMW complex in *S. cerevisiae* however the size of the complex suggests that it may not be a simple E1-E2 complex. It will however be interesting to investigate the components of this complex as this may provide clues to the regulation of the Rub1 pathway in response to oxidative stress.

The best established targets for Nedd8 modification are the cullins, a group of proteins which act as a scaffold for the formation of the multi-subunit, cullin-RING E3 ubiquitin ligases such as the SCF complexes. Addition of Nedd8 to cullins increases their ubiquitin E3 activity possibly by facilitating contact between the E2 and the modifier (Saha and Deshaies, 2008). The activation of CRLs by Nedd8 modification demonstrates cross-talk between the two pathways and thus a mechanism whereby oxidation events that affect the Nedd8/Rub1 pathway can also affect ubiquitination of certain substrates. This has been shown to be the case for the lung Epithelial Sodium Channel (ENaC) whose expression at the surface of epithelial cells increases in response to ROS. Increased ENaC under oxidative conditions is believed to arise as a result of inhibition of the Nedd8 conjugation pathway by oxidation of Ubc12 and subsequent inactivation of the CRL-E3 ubiquitin ligase responsible for the ubiquitin-mediated degradation of ENaC (Downs *et al.*, 2013). Furthermore, cross talk between the Nedd8 and ubiquitin pathways under oxidative stress conditions has been demonstrated. Similar to SUMOylation, the levels

of Nedd8 conjugation increase upon exposure to oxidative stress. Interestingly, however, the conjugation of Nedd8 is directed through the ubiquitin E1 enzyme and not the Nedd8 E1 leading to mixed ubiquitin and Nedd8 chains on substrates . The purpose behind this response to oxidative stress is currently unknown but cross-talk between the pathways in response to stress has the potential to expand the substrates targeted by these two pathways.

Nedd8/Rub1 is removed from its substrates by a highly conserved complex called the COP9 signalosome (CSN). Unlike the SUMO pathways isopeptidases, which use catalytic cysteine residues to hydrolyse the isopeptide bond between SUMO and its target substrate, the CSN is a metalloprotease (Cope *et al.*, 2002) and has currently not been shown to be redox-regulated.

3.7.3 Redox regulation of the ubiquitin pathway

We had previously established that Uba1 and Cdc34 are oxidised, forming a HMW complex in response to specific oxidising agents. However, the specificity of the oxidation of Cdc34 had not previously been explored. Excitingly, in this chapter we have shown that Cdc34 displays increased sensitivity to diamide relative to many other ubiquitin pathway E2 enzymes examined (Figure 3.6). Importantly, we show that the Uba1-Cdc34 HMW complex forms independent of strain background or epitope tag.

The BY and W303 strain backgrounds have different sensitivities to oxidising agents. Cells from the W303 background carry a mutation in the *YBP1* gene which abolishes its function. Ybp1 regulates the cellular response to oxidative stress by forming a stress-induced complex with the transcription factor Yap1 stimulating Yap1 oxidation, localisation to the nucleus and thus its activity as a transcription factor (Veal *et al.*, 2003). Interestingly cells lacking *YBP1* show an increased sensitivity to H_2O_2 and *t*BOOH but not to diamide. Cells from the BY strain background differ from W303 in their Yap1 dependent gene expression specifically they rely on Gpx3 while W303 cells are dependent on Tsa1 but not Gpx3 for Yap-dependent gene expression (Okazaki *et al.*, 2005). Although W303 and BY strains show differences in their stress responses, the Cdc34 HMW complex was formed independent of strain background in this study (Figure 3.1).

While we had previously shown that the formation of the Uba1-Cdc34 complex coincides with an increase in Sic1 stability and cell cycle delay in response to diamide we had been unable to show direct evidence to link these events. The formation of the Uba1-Cdc34 complex weakens the Cdc34 pathway and thus we proposed that the Cdc34 pathway must be weakened for diamide-induced cell cycle arrest and to maintain viability in response to this oxidising agent. Previous attempts to make Cdc34 mutants that were more or less sensitive to oxidation have been hampered by the essential nature of this gene however, here we have been able to utilise a commercially available DAmP strain collection to artificially weaken the Cdc34 pathway. Importantly, we have shown that cells with a decreased expression of either *CDC34* or *UBA1* show an increased resistance to diamide (Figure 3.7). These results suggest weakening of the Cdc34 pathway is essential for cell cycle arrest and to maintain viability after treatment with diamide. To further these studies it would be interesting to monitor how the ratio of overall Cdc34 to Cdc34 in the HMW complex differs between wild type cells and the *CDC34* DAmP strain.

The specific sensitivity of Cdc34 over the other ubiquitin pathway E2s to diamide is advantageous as it allows the targeting of specific Cdc34 targets such as Sic1. The basis of the increased Cdc34 sensitivity is unknown. Proteins which contain cysteine residues with a low pKa are particularly sensitive to oxidation. Interestingly, the active site cysteine residue of three ubiquitin E2s, mammalian UbcH10 and Ubc2 and Ubc13 from *S. cerevisiae* have been shown to exist with a high pKa (Tolbert *et al.*, 2005). Sequence alignment has shown that the residues that increase the pKa of the catalytic cysteine residue of these E2s are not conserved, however, acidic and basic residues are found in all three sequences that could modulate the pKa of the active site. Interestingly, the pKa of the active site must be lowered for the E2 to accept and transfer ubiquitin and it is thought that the E1 or ubiquitin itself must be able to lower the pKa of the active site cysteine of the E2 to allow ubiquitin to move through the pathway. The high pKa of the active site cysteine of E2 enzymes may act as a protective mechanism against unwanted reactions such as oxidation. It would thus be interesting to investigate the kinetics of the active site cysteine of Cdc34 to see whether it exists with a lower pKa than other E2s, underlying its increased susceptibility to oxidation.

As only a small proportion of Cdc34 is involved in the Uba1-Cdc34 complex it could be that only Cdc34 in a specific localisation is oxidised. In the case of the mammalian SUMO pathway, the E1-E2 disulphide that forms in activated macrophages after the oxidative burst was shown to involve only a fraction of both enzymes. Importantly, the E1-E2 disulphide was shown to form specifically in the cytoplasm although the majority of Uba2 and Ubc9 was nuclear (Bossis and Melchior, 2006). Although Cdc34 shares a similar cellular localisation to other ubiquitin E2s it could be possible that a small proportion of specifically localised Cdc34 is oxidised.

Similar to the SENP enzymes, several mammalian deubiquitinase enzymes (DUBs) have been shown to be redox regulated. Importantly, oxidative inactivation of the DUB USP1 was shown to trigger the DNA damage tolerance response after oxidative DNA damage (Cotto-Rios *et al.*, 2012; Lee *et al.*, 2013). Preliminary work from our lab has also suggested that specific DUBs in *S. cerevisiae* may show sensitivity to diamide (data not shown) offering another mechanism by which the ubiquitin pathways may be redox-regulated.

In this chapter we have established that Cdc34 is specifically more sensitive to oxidation by diamide than other ubiquitin pathway E2s that were investigated. These findings were published as part of a paper that identified Cdc34 as a stress-sensing mechanism to respond to oxidative stress (Doris *et al.*, 2012). Having established that Cdc34 oxidation was important in cell cycle regulation the next objective was to investigate other pathways that may be involved in regulation of the cell cycle in response to diamide.

Chapter 4: Regulation of the cell cycle in response to oxidative stress

4.1 Background

In *S. cerevisiae* exposure to different oxidising agents has been shown to lead to arrest at specific points in the cell cycle dependent on the oxidant. For example a G1 phase arrest occurs in response to menadione, H_2O_2 (Flattery-O'Brien and Dawes, 1998) and diamide (Doris *et al.*, 2012) while a G2/M phase arrest occurs in response to menadione (O'Callaghan, 2004), arsenite and H_2O_2. However, despite these studies the mechanisms underlying the various cell cycle responses are largely unknown. Recent work from our lab revealed that the ubiquitin pathway E2, Cdc34 is specifically sensitive to oxidation by diamide and H_2O_2, forming an inhibitory disulphide bond and leading to an increase in the stability of Sic1 and a subsequent G1 phase arrest (Doris *et al.*, 2012)(Figure 4.1). Only a proportion of Cdc34 and Uba1 is oxidised and sequestered in the HMW disulphide and it could be that this is enough to maintain cell viability in response to oxidative stress while maintaining some Cdc34 activity. Indeed by decreasing but not abolishing the expression of *CDC34* or *UBA1* cells show an increased resistance to diamide (Figure 3.7).

Multiple factors are involved in controlling cell cycle commitment. Interestingly, recent work studying the pheromone-dependent G1 phase arrest demonstrated that multiple and distinct pathways including transcriptional repressors and CKIs all contribute to a robust G1 phase arrest under these conditions (Pope and Pryciak, 2013). Hence, it was possible that Cdc34 oxidation and increased Sic1 stability were only one aspect of the arrest and that there were multiple pathways involved. Indeed, deletion of *SIC1* does not completely abolish the G1 phase delay in response to diamide suggesting that a further Sic1-independent mechanism may be required for diamide-induced G1 phase arrest (Doris *et al.*, 2012). Thus, in this chapter the potential contribution of other G1 to S phase regulatory pathways in oxidative stress-dependent cell cycle control will be investigated.

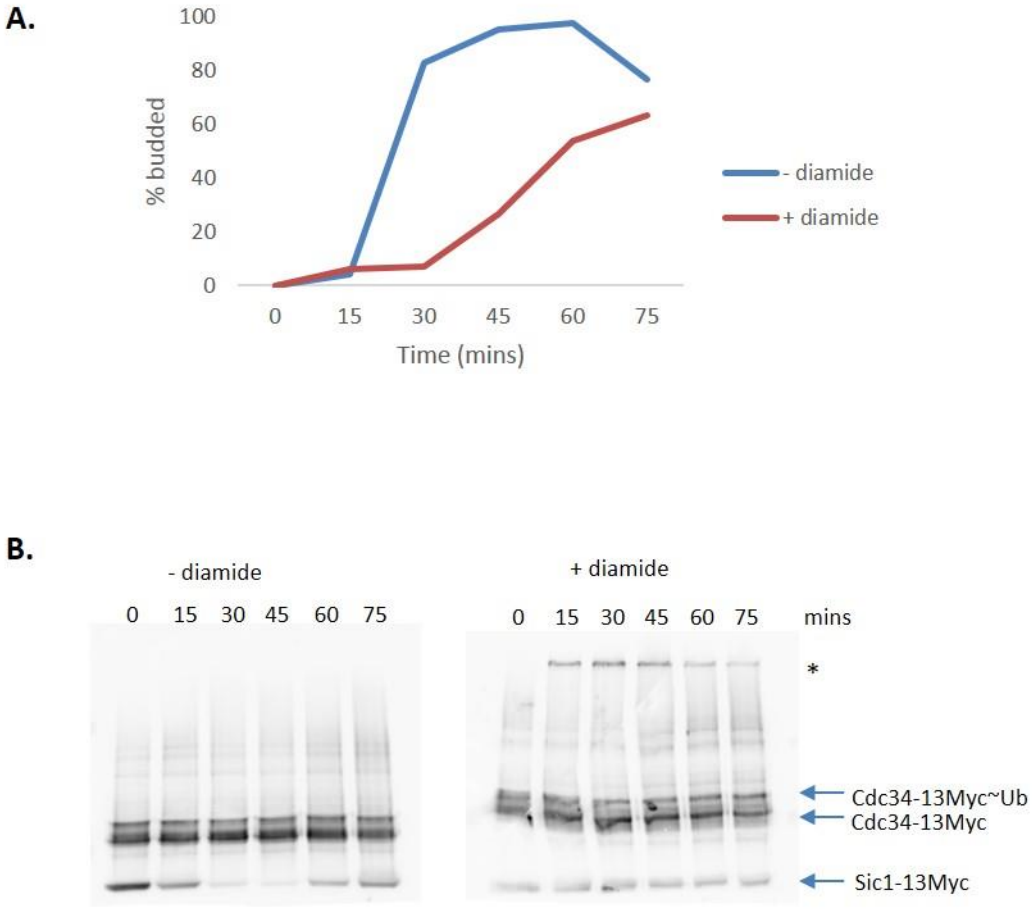


Figure 4.1 Cdc34 forms a HMW complex associated with an increase in Sic1 stability after diamide treatment. Cells expressing Cdc34-13Myc and Sic1-13Myc from the normal chromosomal locus (KD256) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell samples were taken after release for [A] budding and [B] western blot analysis. [B] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Myc antibodies. (* denotes HMW complex).

4.2 Rad53 is not activated by diamide

Activation of the DNA damage checkpoint can lead to cell cycle arrest at different points in the cell cycle including G1 phase (Allen *et al.*, 1994). Oxidising agents differ in their ability to activate the DNA damage checkpoint through Rad9, for example H_2O_2 has been shown to lead to a G2 phase Rad9-dependent cell cycle arrest while menadione leads to a G1 phase, Rad9-independent cell cycle arrest (Flattery-O'Brien and Dawes, 1998). The effector kinase Rad53 is phosphorylated upon checkpoint activation (Segurado and Tercero, 2009) for example in response to the DNA damaging agent hydroxyurea (HU) (Figure 4.2 A) and thus detection of phosphorylated Rad53 is used as an indicator of DNA damage checkpoint activation. Thus we analysed Rad53 phosphorylation during the cell cycle in diamide-treated and untreated cells. Cells were synchronised in G1 phase with α-factor and released into media in the presence or absence of diamide. Budding analysis confirmed that at the point of release the majority of cells were unbudded suggesting a successful G1 phase arrest (Figure 4.2 B). Western blot analysis using a Rad53 specific antibody showed no evidence of Rad53 phosphorylation during the cell cycle in either diamide-treated or untreated cells (Figure 4.2 C). These analyses suggest that the cause of the DNA damage checkpoint is not activated in response to diamide and is not involved in the G1 phase arrest observed under these conditions.

4.3 Met4 plays a role in G1 regulation in diamide-treated and untreated cells

Given that Sic1 is an inhibitor of G1 phase progression next the roles of two other inhibitors of G1 progression, the CKI Far1 and the transcription factor Met4 were investigated. Furthermore, Far1 and Met4 are also regulated by Cdc34 thus it was possible that the formation of the Uba1-Cdc34 HMW complex in response to diamide could also alter the stability/modification of these cell cycle regulators.

The Met4 transcription factor is kept inactive through ubiquitination mediated by Cdc34 and SCF^{Met30} but is deubiquitinated (Kaiser *et al.*, 2000) and activated in response to heavy metal stresses such as cadmium and arsenite (Yen *et al.*, 2005). Activation of Met4 leads to a Met4-dependent G1 phase cell cycle arrest although the mechanisms behind the Met4-dependent cell cycle arrest remain largely unknown. Deletion of *MET4* is able to supress the inviability of *met30* mutants suggesting that the essential role of SCF^{Met30} is to keep Met4 inactive (Patton *et al.*, 2000).

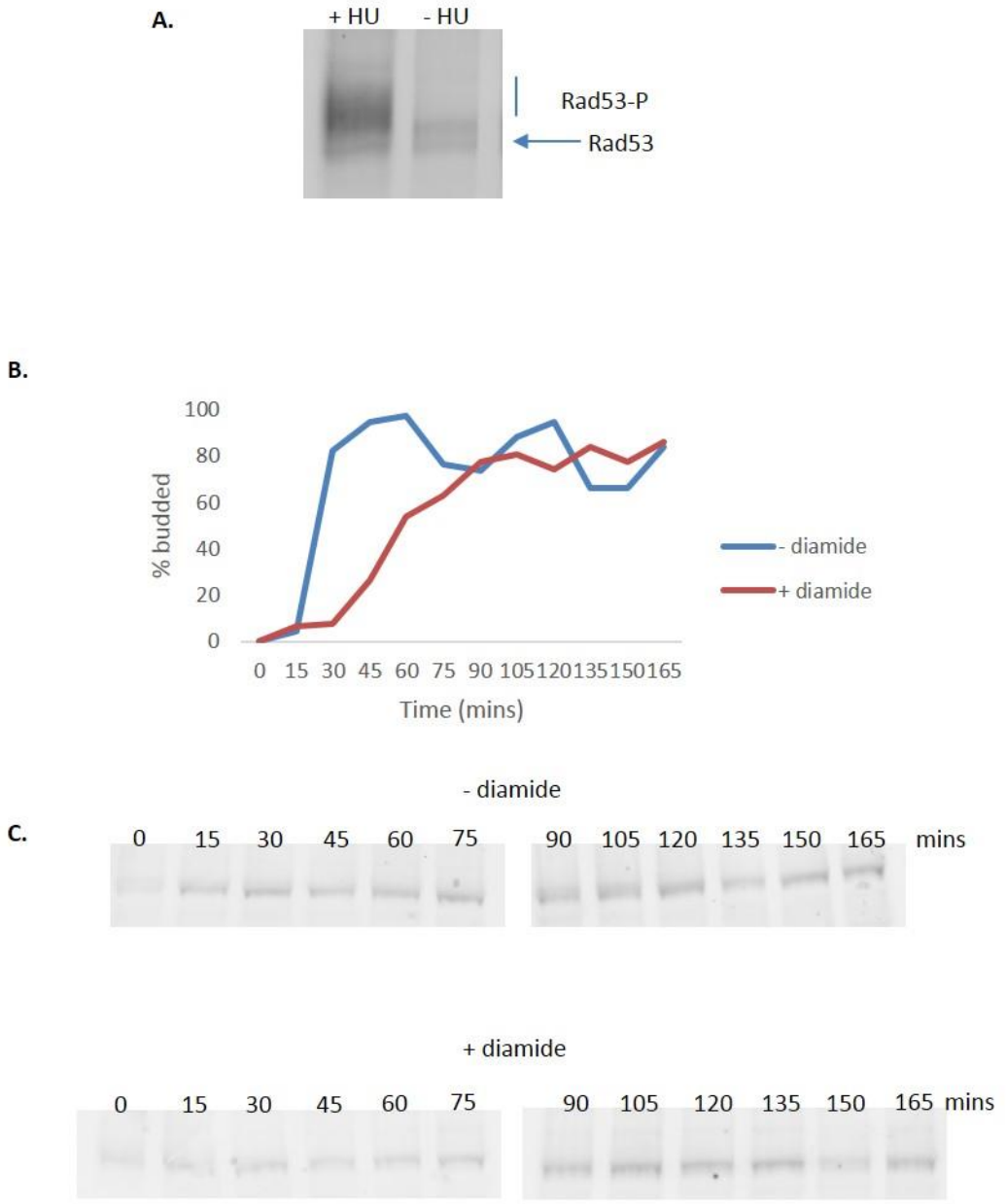


Figure 4.2 Rad53 is not activated during the cell cycle by diamide. [A] *gsh1*Δ (KD502) were treated with 100 mM HU for 3 hours. [B] and [C] Cells expressing Cdc34-13Myc and Sic1-13Myc from the normal chromosomal locus (KD256) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell samples were taken after release for [B] budding and [C] western blot analysis. [A] and [C] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Rad53 antibodies.

The role of Met4 in cell cycle regulation was first investigated in *met4*Δ cells. *met4*Δ cells were arrested in G1 phase with α-factor and released into media with or without diamide. A small proportion of budded cells and cells with greater than 1C DNA content were observed at the release from α-factor suggesting that *met4*Δ cells were not fully arrested in G1 phase (Figure 4.3 A and B). In *MET4* cells the population of budded cells increased rapidly between 15 and 30 minutes which coincided with a shift in the population of cells from 1C DNA content to 2C DNA content (Figure 4.3 A and B, *MET4*). In contrast, large proportions of *met4*Δ cells remained unbudded and with 1C DNA content after release from the α-factor block (Figure 4.3 A and B, *met4*Δ). Although preliminary, these analyses show that *met4*Δ cells release from the G1 phase, α-factor induced cell cycle arrest much slower than *MET4* cells suggesting that Met4 plays a role in G1 phase in unstressed conditions.

After release from the G1 phase arrest into diamide-containing media, *MET4* cells have an approximately 15 minute delay before the appearance of budded cells and movement from 1C to 2C DNA content (Figure 4.3 A and B, *MET4*). In contrast, a large proportion of *met4*Δ cells treated with diamide remained unbudded and with a 1C DNA content an hour after release from the α-factor arrest (Figure 4.3 A and B *met4*Δ). Taken together these data suggest that Met4 plays a role in the G1 phase arrest in both unstressed and stressed conditions. However, in contrast to *sic1*Δ cells which have a shorter G1 phase in response to diamide, *met4*Δ cells have a prolonged G1 phase arrest under these conditions.

As described previously, changes in Met4 ubiquitination regulate Met4 activity. Met4 has also been shown to exist in a phosphorylated form in the absence of *MET30* (Kaiser et al., 2000) and in response to cadmium and arsenite-induced heavy metal stress (Yen *et al.*, 2005). Thus we next investigated whether Met4 modification is altered during the cell cycle in diamide treated and untreated cells. Cells expressing Met4-18Myc from the normal chromosomal locus were arrested in G1 phase with α-factor and released in to media with or without diamide. Budding and DNA content analysis revealed that Met4-18Myc cells arrested similar to wild type cells in response to α-factor (Figure 4.4 A and B). High molecular weight Met4 containing bands were observed throughout the cell cycle in both diamide-treated and untreated cells (Figure 4.4 C). Importantly, in agreement with previous studies (Kaiser *et al.*, 2000) no change in the modification

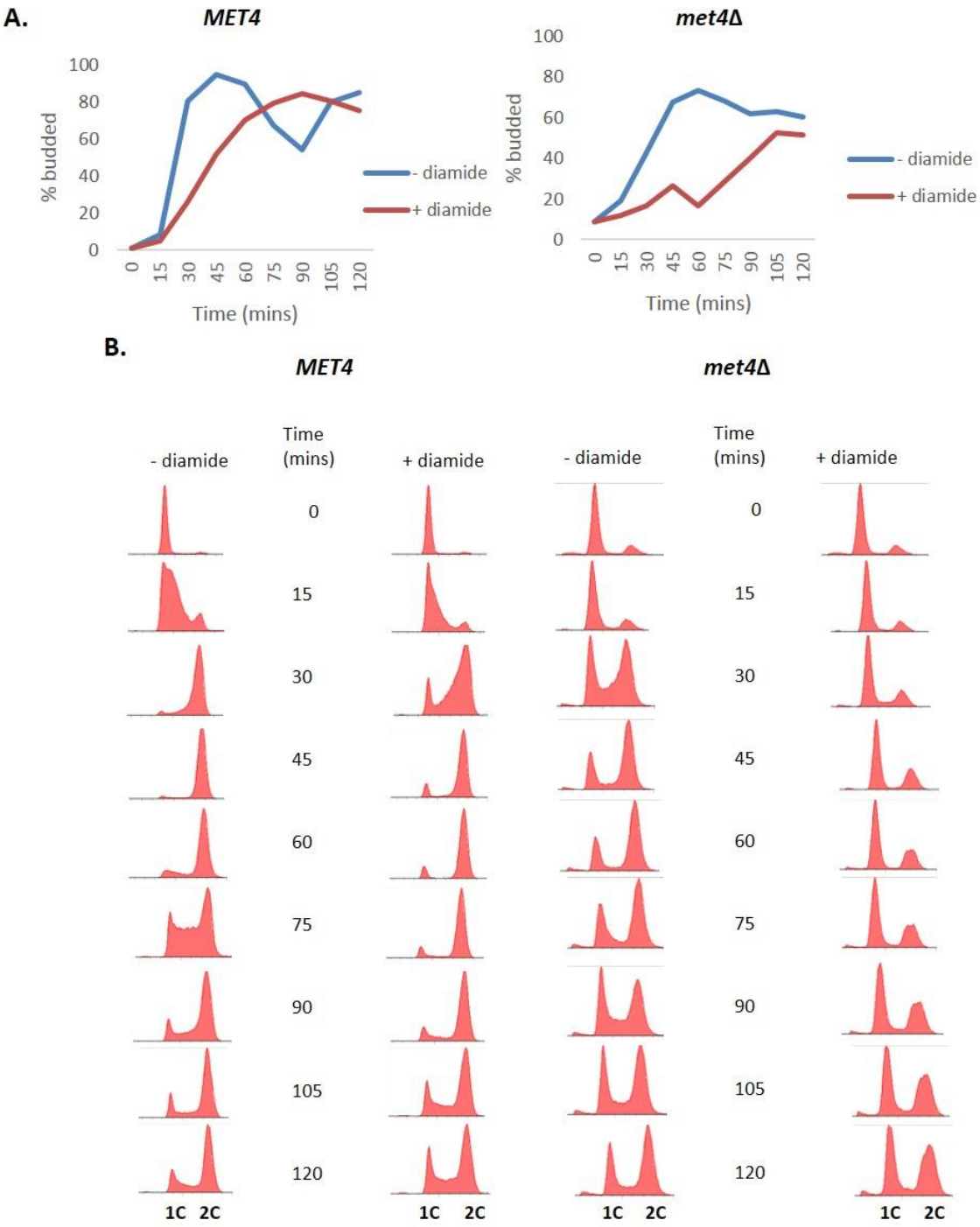


Figure 4.3 *MET4* is essential for the response of cells to diamide. *met4Δ* (KD117) and cells expressing Met4-18 Myc from the normal chromosomal locus (KD484) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell samples were taken after the release for [A] bud and [B] DNA content analysis. *met4Δ* results represent a single experiment.

pattern of Met4 was observed in untreated cells during the cell cycle (Figure 4.4 C). These results also show that Met4 modification is not detectably affected by diamide treatment (Figure 4.4 C). These analyses show no obvious change in Met4 modification during the cell cycle in either diamide-treated or untreated cells suggesting that activation of Met4 is not involved in the G1 phase arrest observed in response to diamide treatment.

4.4 The G1 phase diamide-induced delay does not lead to an increase in Far1 stability

Initial studies suggested that the role of Far1 in G1 phase arrest was through the inhibition of Cln2 in response to α-factor (Chang and Herskowitz, 1990). However, Far1 degradation has more recently been shown to be required for normal cell cycle progression (Fu *et al.*, 2003). *FAR1* is required for the G1 phase arrest in response to α-factor, thus we were unable to investigate the requirement of *FAR1* for the diamide-induced cell cycle delay using a *far1*Δ strain. As a downstream target of Cdc34, we investigated whether similar to Sic1, Far1 levels are also stabilised in response to diamide. A strain was successfully constructed which expressed *FAR1* from the normal chromosomal locus with the addition of 13 Myc epitopes. To ensure that the addition of 13 Myc epitopes to Far1 did not affect protein function, we first looked at the sensitivity of the Far1-13Myc strain to α-factor using halo tests (Figure 4.5 A). As expected, wild type *MATa* cells had their growth inhibited by α-factor, while *MATα* cells did not show any detectable sensitivity to α-factor. *MATa far1Δ* cells also did not show any detectable sensitivity to α-factor consistent with the essential role of Far1 for G1 phase arrest in response to the mating pheromone. In contrast, *MATa* Far1-13Myc cells, similar to wild type *MATa* cells, showed a clear zone of inhibition around the α-factor, suggesting that the addition of 13Myc epitopes to Far1 does not affect the function of the protein in its response to α-factor.

Having established that the addition of an epitope tag did not appear to affect the function of Far1 we used this strain to investigate the stability of Far1 during the cell cycle in the presence and absence of diamide. Cells were arrested in G1 phase using α-factor and released in to media with or without diamide. As expected, Far1 levels were highest at the arrest point (time 0) in both diamide-treated and untreated samples

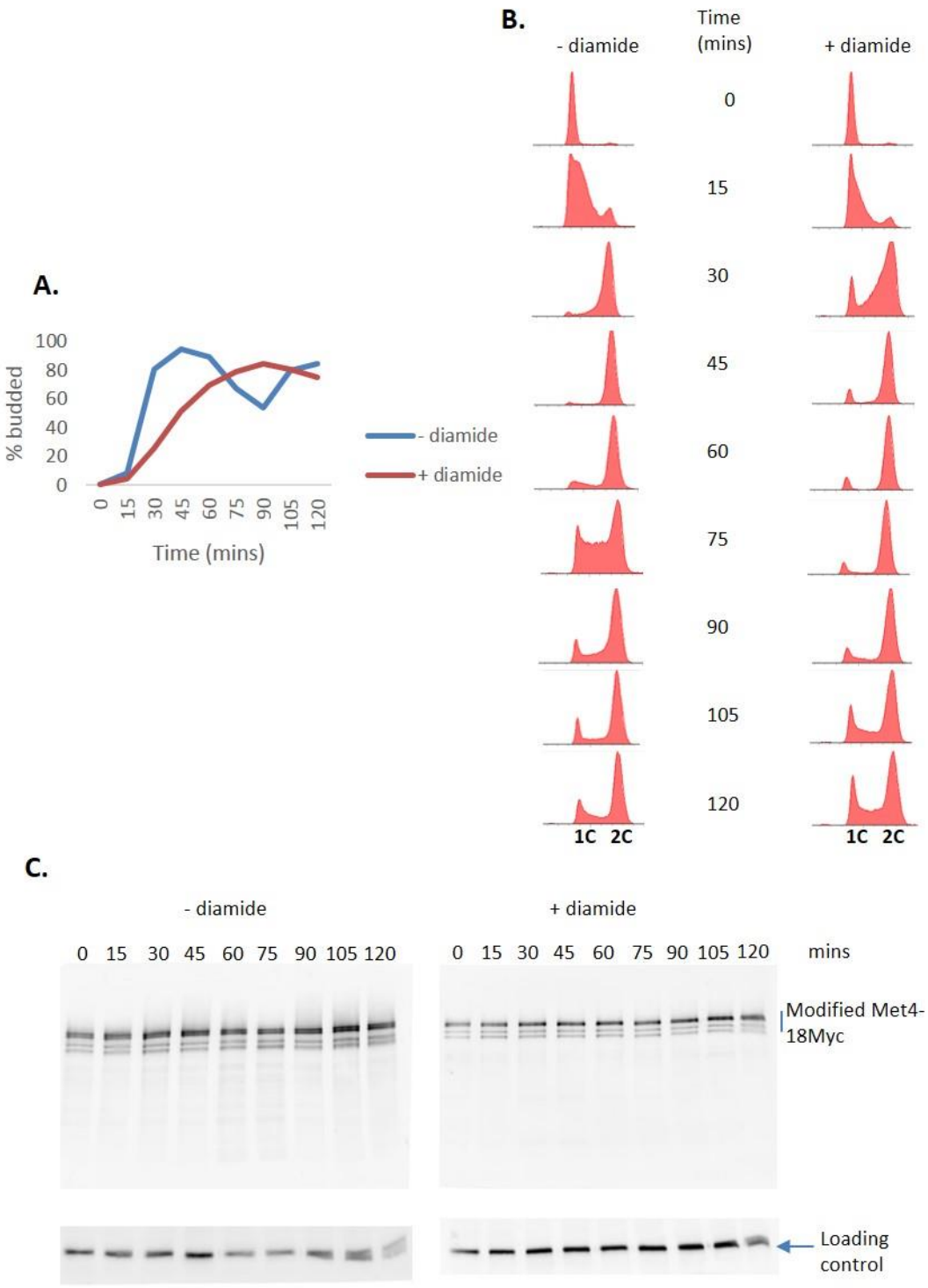


Figure 4.4 Met4 modification does not vary during the cell cycle in diamide-treated or untreated conditions. Cells expressing Met4-18 Myc from their normal chromosomal locus (KD484) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell samples were taken after the release for [A] budding, [B] DNA content and [C] western blot analysis. [C] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti Myc antibodies. Membrane was stripped and probed with anti-Hog1 antibodies (loading control).

(Figure 4.5 D). Far1 levels remained high 15 minutes after release in both samples but decreased between 15 and 30 minutes consistent with the DNA content analysis which shows the shift from a 1C DNA content population to a 2C population during this time (Figure 4.5 C). The cells treated with diamide show a proportion of cells remaining with 1C DNA content at the 30 minute time point (Figure 4.5 C) consistent with a diamide-induced G1 phase cell cycle delay. However, no increase in Far1 stability is observed in these cells (Figure 4.5 D). Interestingly, Far1 levels were shown to cycle throughout the cell cycle in both stressed and unstressed conditions (Figure 4.5 D), appearing in 1C DNA content populations (e.g. Figure 4.5 D, -diamide, 75 minutes) and decreasing in 2C populations. These results suggest that unlike Sic1, Far1 stability is not affected by the formation of the Uba1-Cdc34 HMW complex. This data supports previous work that Far1 has a role in the regulation of the normal cell cycle.

4.5 Ydj1 levels remain stable during the cell cycle in diamide-treated or untreated cells

Having explored two negative cell cycle regulators, next we explored Ydj1 a master upstream regulator of Start. Ydj1 is a DnaJ-related co-chaperone involved in the regulation of Cln3, the most upstream cyclin involved in the initiation of start (Figure 1.5). Cln3 is sequestered in the ER in early G1 through binding to the Hsp70 protein Ssa1. In late G1, Cln3 binding to Ssa1 is displaced by Ydj1 allowing the movement of Cln3 to the nucleus to initiate Start (Vergés *et al.*, 2007). Interestingly, Ssa1 has recently been found to be sensitive to oxidative stress (Wang *et al.*, 2012). Further preliminary analysis of Ydj1 in *S. cerevisiae* had revealed that similar to Cdc34, Ydj1 forms a HMW disulphide in response to diamide (Figure 4.6 A). Due to its role in Start initiation and its potential redox regulation, it was possible that Ydj1 levels/modification were altered during the normal cell cycle and following oxidative stress. Unfortunately, *ydj1*Δ cells are inviable in liquid media (Caplan and Douglas, 1991) and thus we were unable to use this strain for further analyses of the role of Ydj1 in the cell cycle. Therefore to test our hypothesis, cells expressing Ydj1-3HA from the normal chromosomal locus were arrested in G1 phase by α-factor and released into media in the presence or absence of

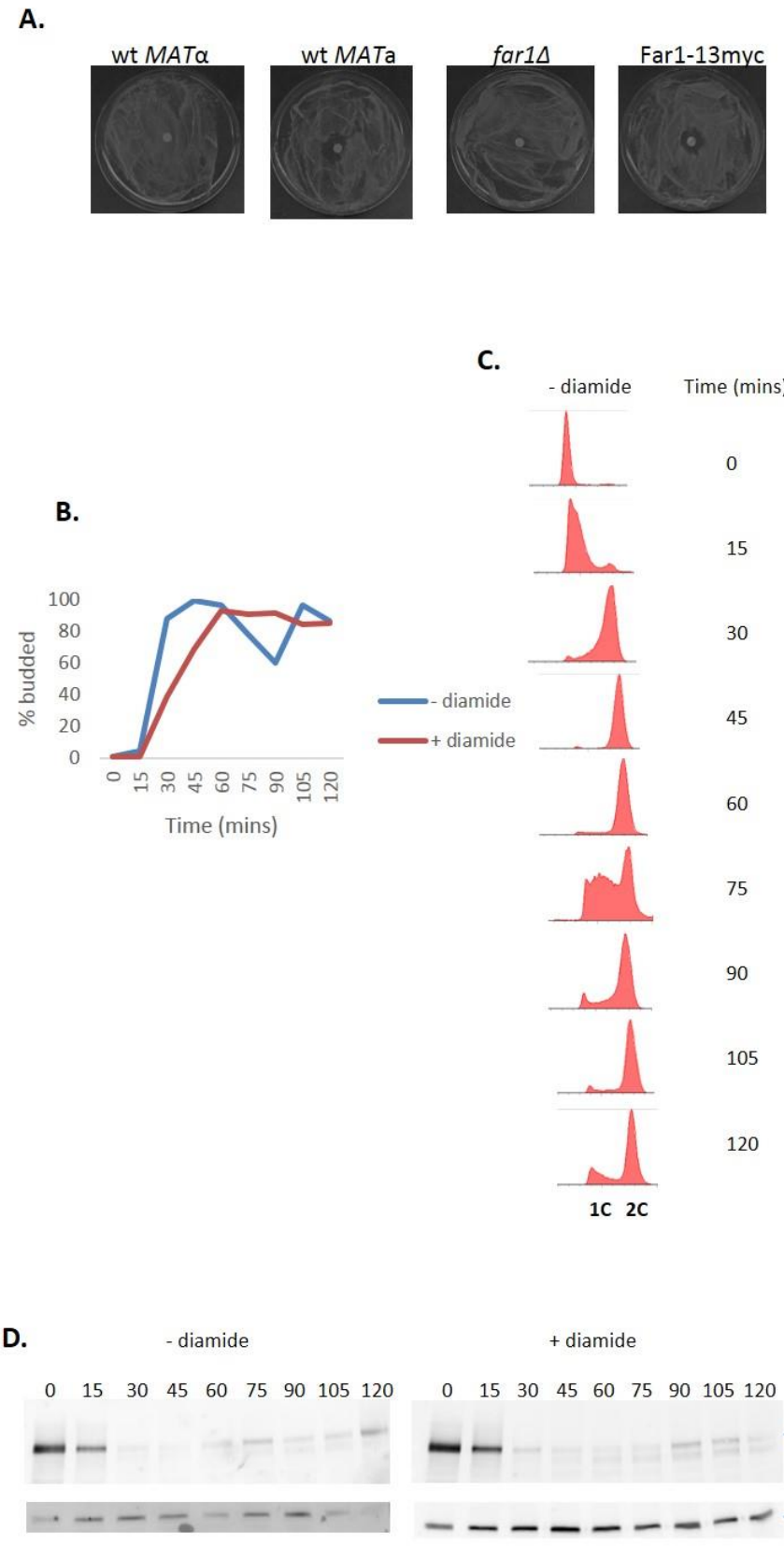

A.
wt MATα
wt MATa
far1Δ
Far1-13myc
B.
% budded
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120
Time (mins)
- diamide
+ diamide
C.
- diamide
Time (mins)
+ diamide
0
15
30
45
60
75
90
105
120
1C 2C
1C 2C
D.
- diamide
+ diamide
0 15 30 45 60 75 90 105 120
0 15 30 45 60 75 90 105 120
mins
Far1-13Myc
Loading control

Figure 4.5. Diamide-induced G1 phase delay does not lead to an increase in Far1 stability. [A] 100 µl of mid-log phase growing wild type *MATa* (KD66), *MATα* (KD65), *far1*Δ (ELR52) and cells expressing Far1-13Myc from the normal chromosomal locus (ELR75) were plated onto YPD and 10 µl of α-factor were spotted onto a disc in the centre of the plate. [B-D] Far1-13Myc expressing cells (ELR75) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell pellets were taken after the release for [B] budding, [C] DNA content and [D] western blot analysis. [D] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Myc antibodies. Membrane was stripped and probed with anti-Hog1 (loading control).

diamide. Budding and DNA content analysis confirmed that Ydj1-3HA cells arrested in response to α-factor treatment (Figure 4.6 B and C). Interestingly, despite the importance of Ydj1 in the regulation of Start, no differences were observed in the levels of Ydj during the cell cycle in diamide-treated or untreated cells (Figure 4.6 D). Furthermore, Ydj1 was not shown to form a HMW complex in diamide-treated cells under these conditions (Figure 4.6 D). The absence of a HMW complex in these cells may reflect a specific response of Ydj1 to different concentrations of diamide as cells were treated with 2 mM diamide in the cell cycle experiments (Figure 4.6 D) and 5 mM diamide in the preliminary experiments (Figure 4.6 A). Overall we see no obvious modification of Ydj1 or effect on Ydj1 protein stability during the cell cycle in diamide-treated or untreated cells

4.6 Rub1 is essential for cell cycle regulation in response to diamide

Previous studies have demonstrated a genetic linkage between SCF^{Cdc4} and the Rub1 pathway (Lammer *et al.*, 1998; Cope *et al.*, 2002). These studies have shown that *RUB1* and *CSN5* are critical in conditions where SCF^{Cdc4} is compromised. As we have demonstrated in Chapter 3, in response to diamide a proportion of Cdc34 is sequestered in a HMW complex and it could be that this is enough to compromise SCF^{Cdc4}. Furthermore, in Chapter 3 we showed evidence of the Rub1 pathway E1, Uba3 forming a HMW complex in response to diamide which may affect the movement of Rub1 through the conjugation pathway. Therefore the roles of the Rub1 pathway in the diamide-induced G1 phase arrest were explored.

As a dynamic modification, Rub1 is added and removed therefore we constructed two Rub1 pathway mutants, one where the gene encoding the modifier is deleted (*rub1*Δ) and one where Rub1 cannot be removed from its target substrate (*csn5*Δ). To confirm that these strains were correct we obtained a commercially available antibody directed towards Cdc53, a well-established Rub1 substrate and part of the SCF^{Cdc4} complex. Western blot analysis of Cdc53 in wild type cells showed the presence of two forms of Cdc53 (Figure 4.7 A). The two forms of Cdc53 were confirmed to be the modified and unmodified forms of Cdc53, as *rub1*Δ cells contained only the lower, unmodified form of Cdc53 while in *csn5*Δ cells Cdc53 was present only in the modified form (Figure 4.7 A).

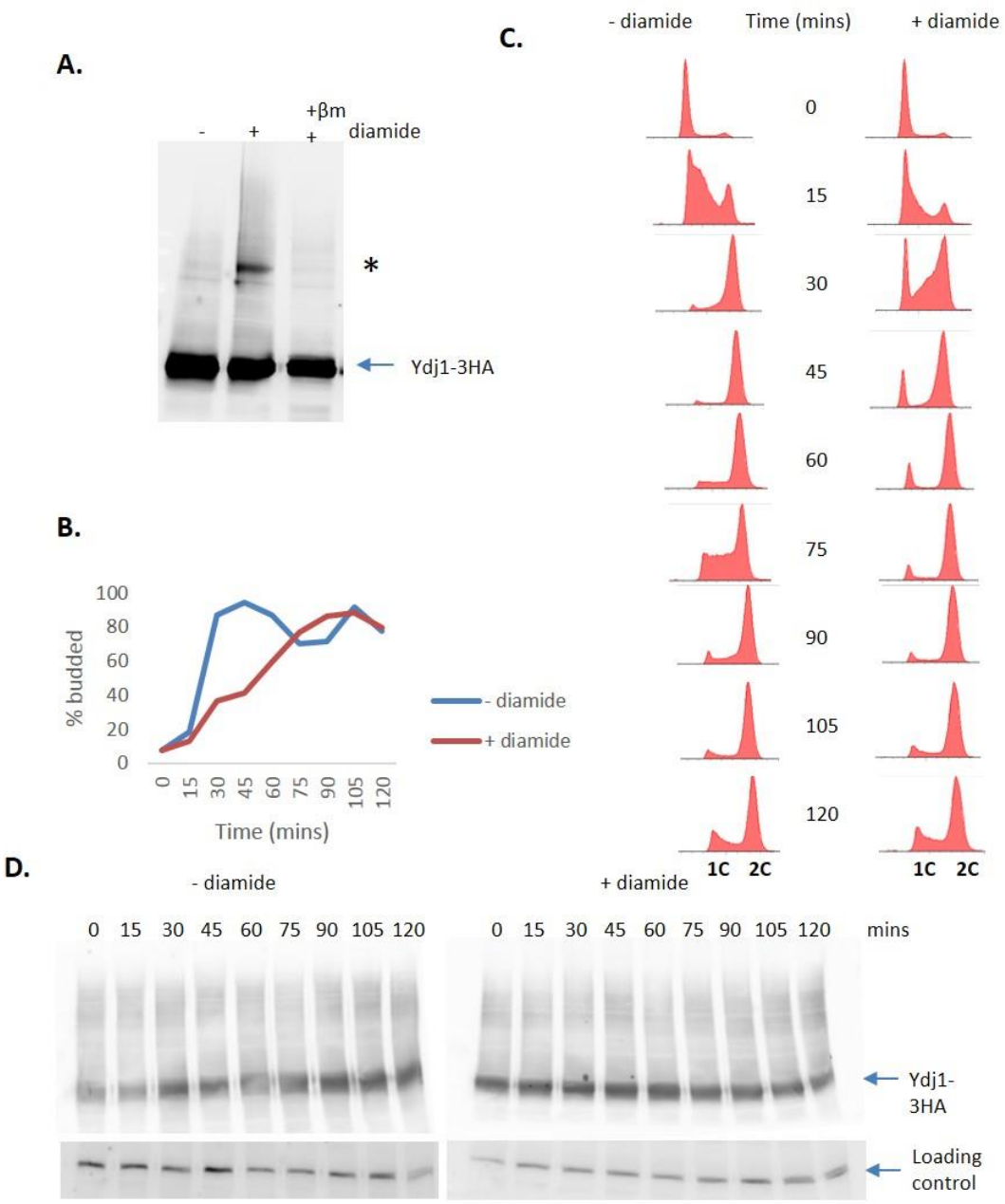


Figure 4.6 Ydj1 protein levels are stable during the cell cycle in diamide-treated and untreated cells. [A] Mid-log cells expressing Ydj1-3HA from the normal chromosomal locus (KD225) were treated with 5mM diamide for 30 minutes. * denotes HMW complex. [B-D] Cells expressing Ydj1-3HA (KD225) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell pellets were collected after the release for [B] budding, [C] DNA content and [D] western blot analysis. [D] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting with anti-HA antibodies. Membrane was stripped and probed with anti-Hog1 antibodies (loading control).

Importantly, in agreement with previous studies (Wee *et al.*, 2002), the majority of Cdc53 was present in the Rub1 modified form in wild type cells.

Having established that the antibody and strains were behaving as expected we next investigated the pattern of Rub1 modification to Cdc53 during the cell cycle in diamide-treated and untreated cells. Rub1 modification of Cdc53 is predicted to regulate its activity (Lammer *et al.*, 1998). Therefore it could be that Cdc53 modification by Rub1 is regulated during the cell cycle. Furthermore, as Cdc53 is part of the SCF^{Cdc4} complex along with Cdc34, it could be that under conditions when Cdc34 is weakened e.g. after diamide treatment that Cdc53 regulation may be affected. Thus we explored Cdc53 modification in diamide-treated and untreated cells. Wild type cells were synchronised in G1 phase using α-factor and released into media with or without diamide. G1 phase synchrony was confirmed by bud and DNA content analysis (Figure 4.7 B and C). At the G1 phase block, as in mid-log phase cells, Cdc53 was predominantly in the Rub1 modified form (Figure 4.7 D). Interestingly, there was no obvious change in Rub1 modification during the cell cycle in diamide-treated or untreated cells.

Deletion of *RUB1* or *CSN5* has been shown to have no discernible growth phenotypes unless the SCF^{Cdc4} is compromised (Lammer *et al.*, 1998; Cope *et al.*, 2002). Although no obvious differences were observed in Cdc53 Neddylation during the cell cycle it was possible that the Rub1 pathway was still playing an important role in cell cycle regulation. *rub1*Δ cells were synchronised in G1 phase using α-factor and released into media in the presence or absence of diamide. Budding and DNA content analysis confirmed that *rub1*Δ cells were able to arrest fully in G1 phase in response to α-factor (Figure 4.8 A and B *rub1*Δ). In the absence of diamide, *rub1*Δ cells released from the G1 phase block and commenced budding and DNA replication similar to *RUB1* cells (Figure 4.8 A and B compare *RUB1* and *rub1*Δ). The budding and DNA content analysis of *rub1*Δ cells also coincided with a decrease in Sic1 levels. Unexpectedly, budding and DNA content analysis revealed that *rub1*Δ cells displayed a prolonged G1 phase arrest following diamide treatment. In contrast to *RUB1* and untreated *rub1*Δ cells, a significant number of unbudded cells persisted when *rub1*Δ cells were treated with diamide (Figure 4.8 A). Furthermore, in agreement with these data, the proportion of *rub1*Δ cells with 1C DNA content at 30 minutes was much higher in *rub1*Δ cells

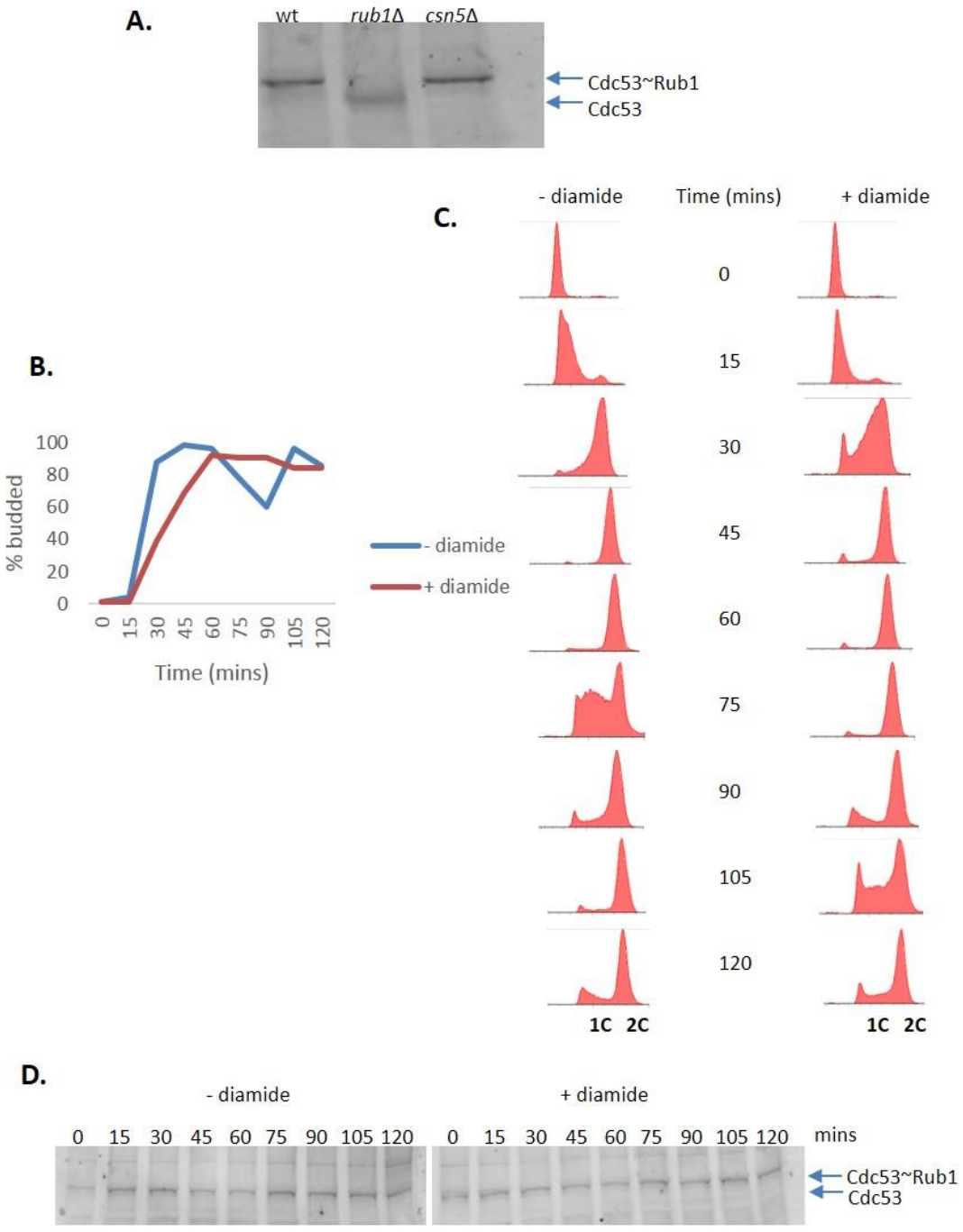
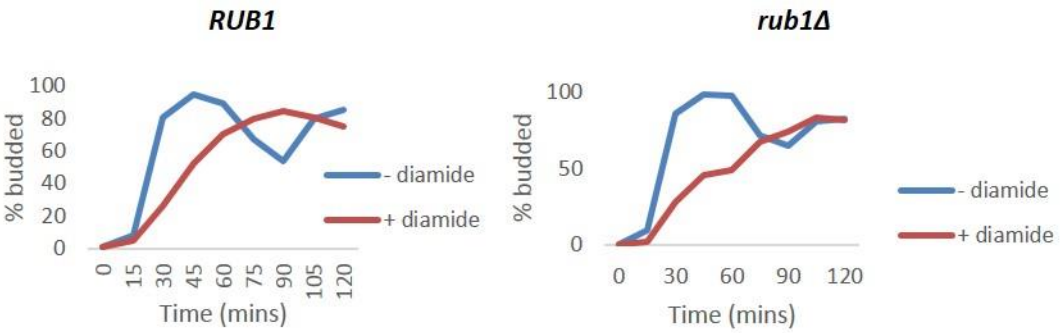
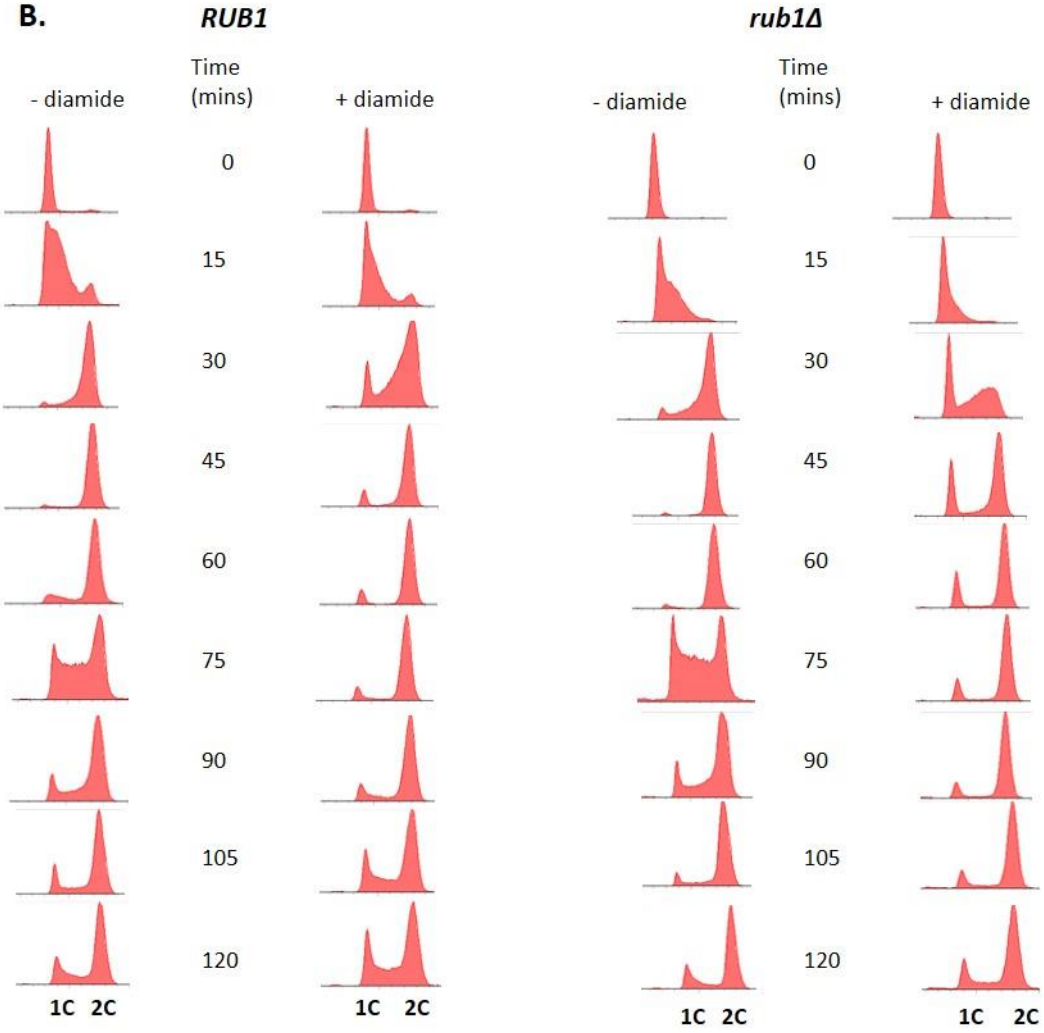


Figure 4.7 Rub1 modification of Cdc53 does not alter during the cell cycle in diamide treated or untreated cells. [A] Protein was extracted from mid-log phase growing wild type (KD66), *rub1*Δ (ELR49) or *csn5*Δ (ELR61) cells. [B-D] Cells expressing Far1-13Myc from the normal chromosomal locus (ELR75) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell pellets were collected after the release for [B] budding, [C] DNA content and [D] western blot analysis. [A] and [D] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Cdc53 antibodies.

A.


RUB1
rub1Δ
% budded
100
80
60
40
20
0
50
0 15 30 45 60 75 90 105 120
0 30 60 90 120
Time (mins)
- diamide
+ diamide

B.


RUB1
rub1Δ
- diamide
Time (mins)
+ diamide
0
15
30
45
60
75
90
105
120
1C 2C

C.

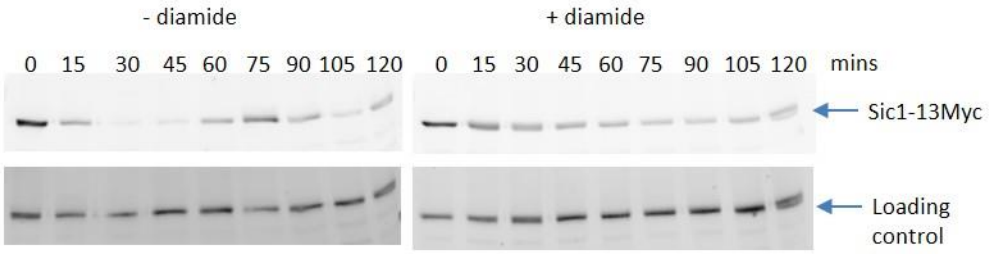


Figure 4.8 Rub1 is essential for cell cycle regulation in response to diamide. [A-C] *rub1Δ* cells expressing Sic1-13Myc from the normal chromosomal locus (ELR120) and Met4-18 Myc (KD 484) cells (*RUB1*) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell pellets were collected for [A] budding, [B] DNA content and [C] western blot analysis (*rub1Δ* only). [C] cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting with anti-Myc antibodies. Membranes were stripped and probed with anti-Hog1 antibodies (loading control).

compared to *RUB1* cells after diamide treatment (Figure 4.8 B compare *RUB1* and *rub1*Δ diamide). Western blot analysis also showed an increase in Sic1 stability at 30 minutes in diamide-treated *rub1*Δ cells compared to untreated *rub1*Δ cells (Figure 4.8 C). Moreover, a high population of diamide-treated *rub1*Δ cells had 2C DNA content from 45 minutes until the end of the experiment consistent with the also cells arresting in G2 phase.

Having established that in the absence of *RUB1*, cells are able to traverse the cell cycle under unstressed conditions but arrest their cell cycle in both G1 and G2 phase in response to diamide treatment we next explored regulation of the cell cycle in the absence of *CSN5*.

*csn5*Δ cells were synchronised in G1 phase using α-factor and were released into media in the presence or absence of diamide. Budding and DNA content analysis confirmed that *csn5*Δ cells were arrested in G1 phase in response to α-factor (Figure 4.9 A and B). Interestingly, untreated *csn5*Δ cells released from the G1 phase arrest marginally faster than *CSN5* cells as demonstrated by a higher proportion of cells with 2C DNA content 15 minutes after α-factor release (Figure 4.9 B, *CSN5* compared to *csn5*Δ). In contrast to *rub1*Δ cells, budding and DNA content analysis revealed that diamide-treated *csn5*Δ cells released from the G1 phase arrest quicker than *CSN5* cells (Figure 4.9 A and B). The increased proportion of *csn5*Δ, diamide-treated cells with 1C DNA content at 90 minutes also revealed that in contrast to *rub1*Δ cells, *csn5*Δ cells enter a second cell cycle. These data suggest that *csn5*Δ cells respond to diamide in a similar manner to *CSN5* cells.

Taken together, these analyses show that Cdc53 exists predominantly in its modified form throughout the cell cycle in both diamide-treated and untreated conditions. In agreement with these observations, *csn5*Δ cells where Cdc53 exists only in its modified form, display a similar cell cycle response to diamide to *CSN5* cells and may actually release from the G1 phase arrest faster than *CSN5* cells. In contrast, *rub1*Δ cells where Cdc53 remains unmodified show a prolonged G1 and G2 phase arrest in response to diamide compared to *RUB1* cells. These results suggest that Cdc53 modification is essential for cell cycle regulation in response to diamide.

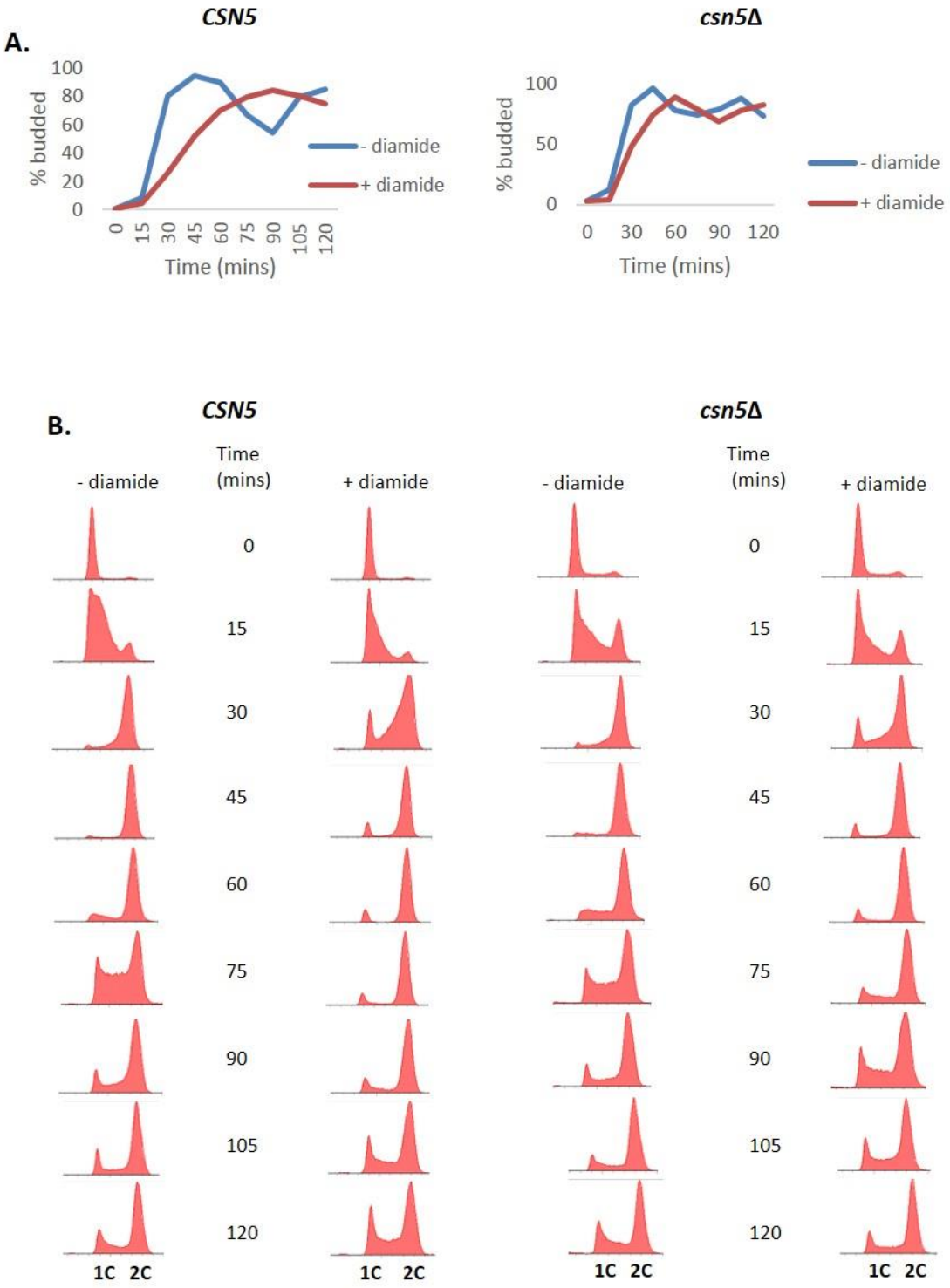


Figure 4.9 *CSN5* is required for the response to diamide. *csn5*Δ (ELR61) and Met4-18 Myc (KD 484) cells (*CSN5*) were synchronised in G1 phase with α-factor and then released from the block into YPD media in the presence or absence of 2 mM diamide. Cell pellets were collected after the release for [A] budding and [B] DNA content analysis.

4.7 Discussion

Previous work from our lab had shown that formation of the Uba1-Cdc34 HMW complex coincided with an increase in the stability of Sic1. However, deletion of *SIC1* did not completely alleviate the diamide-induced cell cycle arrest suggesting the involvement of other, Sic1-independent pathways in the G1 phase arrest. In this chapter we have investigated a number of Cdc34-dependent and independent pathways that may play a role in the diamide-induced G1 phase arrest. Importantly, we have identified that the Rub1 pathway is essential for the cellular response to diamide. In the absence of *RUB1*, cells were shown to arrest in both G1 and G2 phase of the cell cycle in response to diamide. Interestingly, here we show that Cdc53 a key target of Rub1 modification and an important cell cycle regulator exists predominantly in the Rub1 modified form throughout the cell cycle in both diamide-treated and untreated cells and that cells which have Cdc53 only its Rub1 modified form (*csn5*Δ) are able to release from the G1 phase diamide-induced arrest similar to *CSN5* cells and traverse the cell cycle. These results suggest that Rub1 modification of Cdc53 is important for the cellular response to diamide. The involvement of two Cdc34-dependent G1 phase regulators, Far1 and Met4 in the diamide-induced G1 phase arrest were also investigated. Here, we show that although Met4 ubiquitination patterns do not change during the cell cycle or in response to diamide treatment, Met4 plays a role in normal cell cycle regulation and in the regulation of the cell cycle in response to oxidative stress.

Cdc34 oxidation is coincident with an increase in Sic1 stability however deletion of *SIC1* does not lead to a complete release from the diamide-induced cell cycle arrest. Alongside Sic1, two other cell cycle inhibitors Far1 and Met4 are also regulated by Cdc34. As with Sic1, the CKI Far1 is also targeted for degradation by Cdc34 and SCF^{Cdc4}. The role of Far1 has traditionally been thought of as solely in response to mating pheromone where Far1 inhibits Cdc28-Cln complexes to bring about a G1 phase arrest prior to mating (Chang and Herskowitz, 1990). Far1 has also been linked to cell cycle regulation in the mitotic cell cycle (Fu *et al.*, 2003) and in agreement we have shown that Far1 accumulates at G1 in response to α-factor and during the subsequent G1 phases after release from pheromone block (Figure 4.5 D, 75 minutes – diamide). Far1 is essential for the cellular response to α-factor (Figure 4.5 A) therefore we were unable to investigate the G1 phase arrest in response to α-factor in *far1*Δ cells. However, using

cells expressing Far1-13Myc we saw no evidence of an increase in Far1 stability after diamide treatment (Figure 4.5 D). As both Sic1 and Far1 are ubiquitinated by SCF^{Cdc4} these data imply the involvement of another regulatory mechanism. As discussed in Chapter 3 only a small proportion of Cdc34 is oxidised which could reflect that only Cdc34 in a specific location is oxidised. Specific localisation of Cdc34, Sic1 or Far1 could explain the increased stability of Sic1 but not Far1 although both are targeted for ubiquitination through SCF^{Cdc4}. Interestingly, Far1 degradation has also been shown to be regulated by the AAA ATPase, Cdc48 (Fu *et al.*, 2003). Cdc48 has been shown to participate in many cellular activities including the recognition and transport of polyubiquitinated proteins to the 26S proteasome and the gene encoding Cdc48 is essential in *S. cerevisiae*. A study using a strain whereby Cdc48 was degraded in a temperature-dependent manner was used to study the relationship between Cdc48 and Far1. This study found that after Cdc48 had been degraded cells were unable to overcome α-factor-induced G1 phase arrest due to an inability to degrade Far1. This study demonstrates a way that Far1 may be regulated differently to Sic1 which may contribute to the differences in their stability in response to diamide.

The Met4 transcription factor is ubiquitinated through Cdc34 and SCF^{Met30} which keeps it in an inactive state. In conditions such as a drop in cellular methionine or in response to heavy metal stress, Met4 ubiquitination is inhibited and deubiquitination of Met4 converts it to an active transcription factor. Full activation of Met4 leads to the expression of genes required for synthesis of sulphur-containing amino acids (Lee *et al.*, 2010) and a cell cycle arrest (Yen *et al.*, 2005). Here, although no changes in Met4 modification were observed (Figure 4.4 C) we show a role for Met4 in cell cycle regulation in both diamide-treated and untreated cells (Figure 4.3).

The 70 kDa heat shock proteins (Hsp70s) are a family of highly conserved molecular chaperones, essential for viability whose activity is regulated by the Hsp40 chaperones (Walsh *et al.*, 2004). In *S. cerevisiae*, the upstream cyclin/Cdk complex, Cdc28-Cln3 is sequestered at the ER until late G1 phase by binding to the Hsp70 chaperone Ssa1. In late G1, a surplus of the Hsp40 protein Ydj1 leads to it displacing Cln3 in binding to Ssa1 allowing Cln3 to move to the nucleus and initiate Start (Figure 1.5) (Vergés *et al.*, 2007). Interestingly, Ssa1 has been shown to contain two redox-sensitive cysteine residues which are hypersensitive to thiol-reactive compounds. Inactivation of Ssa1 by oxidation

was shown to lead to activation of the heat shock response by alleviating the repression of Ssa1 on Hsf1, a transcription factor that mediates the heat shock response (Wang *et al.*, 2012). Our preliminary data had suggested that Ydj1 itself was also susceptible to oxidation by diamide, forming a HMW complex (Figure 4.6 A). It was therefore possible that this upstream cell cycle regulator was involved in the cellular response to diamide. Due to the inviability of *ydj1*Δ cells in liquid media (Caplan and Douglas, 1991) the importance of Ydj1 for the diamide-induced cell cycle arrest could not be investigated. However, the stability and modification of Ydj1 during the cell cycle in diamide-treated and untreated cells were analysed using a strain expressing Ydj1-3HA. Interestingly, in contrast to mid-log cells, no HMW complex was seen in α-factor arrested cells in response to diamide (Figure 4.6 D). The lack of HMW complex in α-factor synchronised cells could be due to the lower concentration of diamide used in these experiments, suggesting the HMW complex formation is dose-dependent. Furthermore, the preliminary Ydj1 results were performed on Yjd1-3HA cells in mid-log phase that were exposed to the higher concentration of diamide for 30 minutes before cell pellets were collected. Therefore, the difference in the two results could be due to diamide concentration, length of time that the cells were treated with diamide and/or the point in the cell cycle when diamide was added to the culture. Interestingly, despite being a major regulator of Start, no changes in the protein levels of Ydj1 were observed during the cell cycle in either diamide-treated or untreated cells suggesting that Start is not regulated directly through Ydj1 availability. Another cell cycle activator, the transcription factor Swi6, has been shown to be sensitive to oxidation. Swi6 forms part of the MBF and SBF transcription factors which control the expression of genes involved in morphogenesis (SBF) and DNA replication (MBF). Swi6 was shown to be oxidised on Cys404 in after exposure of cells to lipid hydroperoxide leading to a G1 phase cell cycle delay and down regulation of G1 cyclin expression (Chiu *et al.*, 2011). These results demonstrate another pathway upstream of Cdc34/Sic1 that may regulate the G1 phase diamide-induced cell cycle arrest.

In many eukaryotes Neddylation has been shown to play important roles in the cell cycle and to be linked to G1 phase regulation. However, in contrast to many other eukaryotes the Rub1 pathway is not essential in *S. cerevisiae* but has previously been genetically linked to G1 phase regulation. Deletion of *RUB1* or *CSN5* alongside temperature

sensitive mutations in components of the SCF^{Cdc4} complex exacerbates the phenotypes associated with the single temperature sensitive mutants (Lammer *et al.*, 1998; Cope *et al.*, 2002). The necessity for the Rub1 pathway when the SCF^{Cdc4} complex is compromised is thought to be due to the inability of the cell to cycle Rub1 on and off Cdc53 as *cdc53* and *cdc34* temperature sensitive mutants display synthetic lethality. Cdc53 is a cullin which acts as a scaffold in the SCF E3 ubiquitin ligase complex and Rub1 modification of Cdc53 is thought to positively regulate the activity of the SCF ligases (Liakopoulos *et al.*, 1998). Here, we have shown that the Rub1 pathway is essential for cell cycle regulation in response to diamide. In the absence of *RUB1*, cells were shown to have a prolonged G1 phase arrest after diamide treatment followed by a G2 phase arrest (Figure 4.8 B). Interestingly, in contrast to *rub1*Δ, cells lacking *CSN5* showed a similar cell cycle arrest and release profile to wild type cells after diamide treatment (Figure 4.9). The role of Neddylation in the diamide-induced cell cycle arrest remains unclear as modification of Cdc53 appeared to be unaffected after diamide treatment. However, as Cdc53 is predominantly in the Rub1 modified form during the cell cycle in both diamide-treated and untreated cells, similar to the constantly modified Cdc53 that is found in *csn5*Δ cells it could be speculated that Cdc53 must be Neddylated for cell cycle progression in response to diamide. The Rub1 pathway has recently been shown to regulate the cell cycle in response to DNA damage (Guenole *et al.*, 2013). *rub1*Δ or *ubc12*Δ cells treated with the DNA damaging agent camptothecin arrested in G2/M phase with a similar DNA content to *rub1*Δ cells after exposure to diamide.

Here we have identified several new contributors and/or regulators of the cell cycle in response to diamide. It would be interesting to further investigate whether the pathways identified in this study are also involved in the regulation of the cell cycle in response to other oxidising agents and whether they are conserved in other organisms. Interestingly, diamide is mainly cleared by the glutathione rather than the thioredoxin system (Muller, 1996). Furthermore, loss of *GSH1* leads to a G1 phase cell cycle arrest by unknown mechanisms (Spector *et al.*, 2001). Hence, we next investigated the role of the regulatory pathways identified here in the cellular response to glutathione depletion.

Chapter 5: Glutathione and the cell cycle

5.1 Background

Glutathione is a highly conserved, abundant antioxidant which is essential for normal growth in eukaryotes. Deletion of *GSH1*, encoding the rate-limiting step in glutathione synthesis leads to glutathione auxotrophy which can be fully rescued by the addition of reduced or oxidised glutathione to the media. Glutathione auxotrophy can also be partially rescued by addition of the reducing agent DTT (Spector *et al.*, 2001) suggesting that the cellular requirement for glutathione is at least in part due to its role as an antioxidant. Indeed, this conclusion is further supported by the increased sensitivity of *gsh1*Δ cells to certain oxidising agents (Grant *et al.*, 1996).

Interestingly, the essential role of Gsh1 has also been linked to cell cycle progression. In particular depletion of glutathione from *gsh1*Δ cells leads to cell cycle arrest, predominantly in G1 phase, after approximately 7 generations and a loss of viability after 3-4 days (Spector *et al.*, 2001). However, the mechanisms behind how and why *gsh1*Δ cells arrest in G1 phase in response to glutathione depletion have not been investigated. In Chapter 4 we investigated the role of a number of Cdc34-dependent and independent pathways in the diamide-induced G1 phase delay. Here, we will examine whether these same pathways are also involved in the G1 phase arrest observed after glutathione depletion.

5.2 Glutathione depletion leads to cell cycle arrest

It had previously been shown that *gsh1*Δ cells in the BY strain background arrest in G1 phase of the cell cycle after approximately 7 generations of growth in glutathione depleted media (Spector *et al.*, 2001). To confirm that *gsh1*Δ cells displayed the same G1 phase arrest in the W303 strain background, *gsh1*Δ cells were grown to mid-log phase in minimal media containing 1 mM glutathione (SD +GSH), washed twice in sterile water, resuspended in minimal media lacking glutathione (SD –GSH) and were grown continuously in mid-log for 16 hours (approximately 7 generations). DNA content analysis confirmed that after 16 hours of growth in media lacking glutathione, *gsh1*Δ cells had arrested with 1C DNA content consistent with the cells being arrested in G1

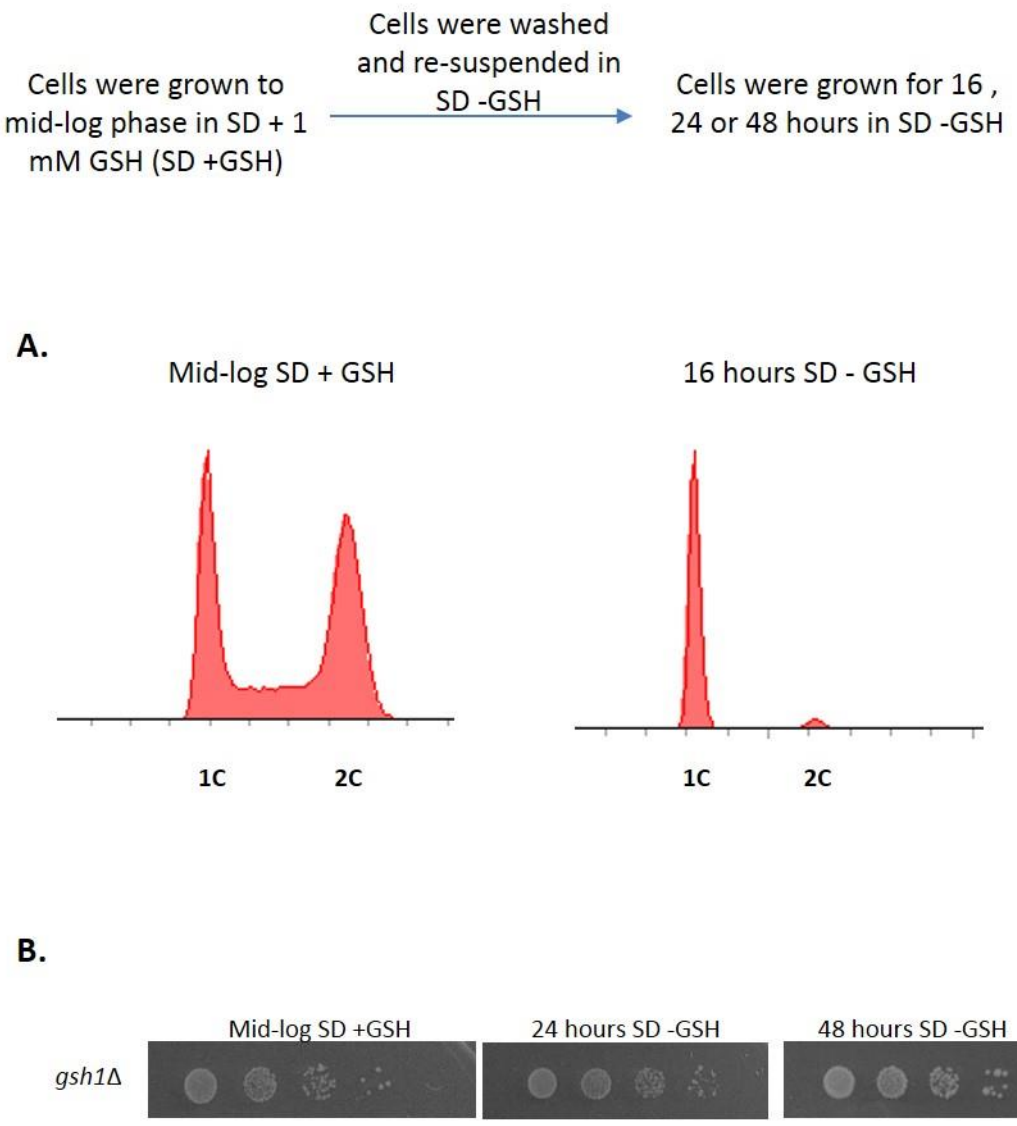


Figure 5.1 Glutathione depletion leads to G1 phase arrest after approximately 7 generations. Cells were grown to mid-log phase in minimal media supplemented with 1 mM glutathione (SD +GSH), washed twice in water and re-suspended in media lacking glutathione (SD –GSH). Cells were grown continually in mid-log phase in SD –GSH for approximately 7 generations. [A] After growth for 16, 24 or 48 hours pellets were taken for DNA content analysis. [B] For viability assays 10-fold serial dilutions of cells grown for the indicated time in the indicated media were spotted on to SD+GSH plates and incubated at 30°C for 2-3 days.

phase (Figure 5.1 A). We hypothesised that the G1 phase arrest of *gsh1*Δ cells following glutathione depletion may be important for cell viability under these conditions at least in the short term. *gsh1*Δ cells grown for 24 and 48 hours in SD –GSH showed no loss of viability when grown on SD +GSH (Figure 5.1 B). These results suggest that the G1 phase arrest observed following glutathione depletion may indeed allow these cells to maintain viability. Having confirmed that our assay mirrored previous results and that the loss of *GSH1* results in similar cell cycle arrest profiles in different strain backgrounds we next investigated the mechanisms underlying the G1 phase arrest.

5.3 Rad53 is not activated in response to the glutathione depletion-induced cell cycle arrest

The G1 phase cell cycle arrest of glutathione-depleted cells suggested that glutathione may be essential for completion of DNA replication. The DNA damage checkpoint is activated when DNA damage causes replication forks to stall. Rad53 acts as an effector kinase in the DNA damage checkpoint and becomes phosphorylated when the checkpoint is activated (Segurado and Tercero, 2009). For example, treatment of cells with hydroxyurea (HU) which blocks initiation of DNA replication leads to phosphorylation of Rad53 and activation of the DNA damage checkpoint (Figure 5.2 and (Segurado and Tercero, 2009). Given that cells arrest in G1 phase in response to glutathione depletion it was possible that Rad53 was also activated under these conditions leading to cell cycle arrest. However, in contrast to HU treatment, Rad53 was not phosphorylated in *gsh1*Δ cells following either 16 hours or 24 hours of glutathione depletion (Figure 5.2). Hence, these results suggest that the DNA damage checkpoint is not required for glutathione depletion-induced G1 phase cell cycle arrest.

5.4 Met4 is not essential for the glutathione depletion-induced G1 phase cell cycle arrest

Met4 is a transcription factor which regulates the transcription of genes involved in the synthesis of sulphur containing molecules such as methionine and cysteine and is essential for the regulation of *GSH1* gene expression in response to glutathione depletion (Wheeler *et al.*, 2002). Significantly, Met4 has been linked to the regulation of G1 phase progression suggesting a potential role in the glutathione depletion-induced cell cycle arrest (Patton *et al.*, 2000).

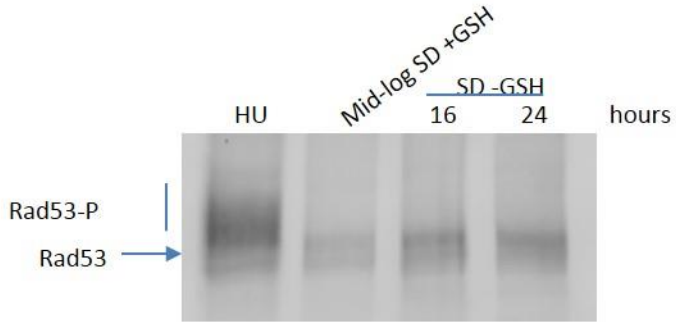


Figure 5.2 The cell cycle checkpoint kinase Rad53 is not phosphorylated by glutathione depletion. *gsh1Δ* (KD502) cells were treated with 100 mM hydroxyurea (HU) for 3 hours to activate Rad53 or were grown to mid-log phase in SD+GSH, washed twice in water, resuspended in SD –GSH and grown continuously in SD-GSH for the indicated time. Cell lysates were prepared under non-reducing conditions to preserve phosphorylation, separated by SDS-PAGE and analysed by western blotting using anti-Rad53 antibodies.

Previous studies have shown that ubiquitination of Met4 directed by SCF^{Met30} retains Met4 in an inactive form. However, under conditions known to activate Met4, for example, heavy metal stress, a reduction of ubiquitination is observed corresponding to activation of the transcription factor (Yen *et al.*, 2005). Hence, to investigate the relationship between the regulation of Met4 and glutathione depletion, a *gsh1*Δ strain expressing Met4-18Myc was created. Glutathione was depleted from *gsh1*ΔMet4-18Myc cells using the protocol described in Figure 5.1 and protein extracts obtained. Significantly, western blot analysis showed a loss of the slower motility forms of Met4-18Myc consistent with activation of the transcription factor (Figure 5.3 A).

To assess the potential role of Met4 in the glutathione depletion-induced cell cycle arrest, the percentage of budded cells in *gsh1*Δ and *gsh1*Δ*met4*Δ cultures grown in media lacking glutathione was examined. Significantly, this analysis revealed that both *gsh1*Δ and *gsh1*Δ*met4*Δ cultures had mainly unbudded cells suggesting that the cells had arrested in G1 phase (Figure 5.3 B). However, to confirm this conclusion DNA content analysis was performed on the arrested cells to examine whether DNA replication had occurred. This analysis revealed that following glutathione depletion *gsh1*Δ*met4*Δ cultures have a predominantly 1C DNA peak, confirming that these cells had indeed arrested in G1 phase (Figure 5.3 C). Furthermore, consistent with these data *gsh1*Δ and *gsh1*Δ*met4*Δ cells displayed no loss of viability after 24 and 48 hours of growth in media lacking glutathione (Figure 5.3 D). These data therefore suggest that whilst Met4 may be activated in *gsh1*Δ cells in an attempt to restore glutathione levels, Met4 appears to have no major role in the glutathione depletion-induced cell cycle arrest.

5.5 The CKI Sic1 and not Far1 is essential for G1 phase arrest following glutathione depletion

Interestingly, both Sic1 and Far1 are targeted for degradation by ubiquitination through the E2 Cdc34 and the ubiquitin ligase (E3) SCF^{Cdc4}. Given the relationship between oxidation of Cdc34, increased Sic1 stability and the regulation of the cell cycle by oxidative stress (Chapter 3, (Doris, 2008)) it was possible that Sic1 and/or Far1 may be required for the glutathione depletion-induced G1 phase arrest. To examine this possibility *gsh1*Δ*far1*Δ and *gsh1*Δ*sic1*Δ cells were grown in the absence of glutathione

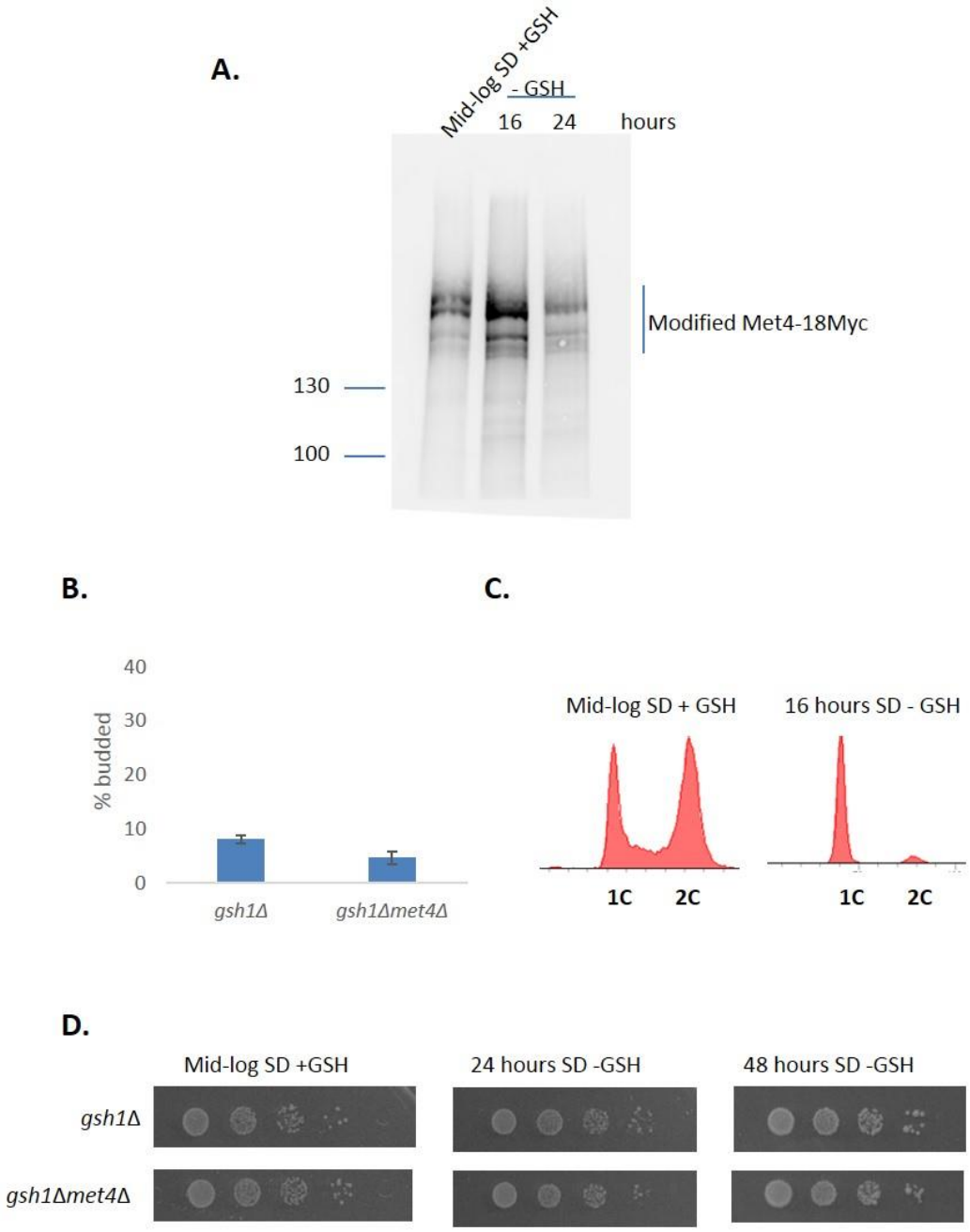


Figure 5.3 Met4 is not essential for the glutathione depletion-induced G1 phase arrest. [A] *gsh1*Δ cells expressing Met4-18Myc (ELR102) were grown until mid-log phase in SD+GSH, washed twice in water, re-suspended in SD-GSH and pellets taken at the indicated times. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting with anti-Myc antibodies. [B], [C] and [D] *gsh1*Δ (KD502) and *gsh1*Δ*met4*Δ (KD118) were grown as described in [A] and pellets collected at the indicated time for bud counts [B] and DNA content analysis [C]. [D] for viability assays, 10-fold serial dilutions of cells grown for the indicated time in the indicated media were spotted on to SD +GSH plates and incubated at 30°C for 2-3 days.

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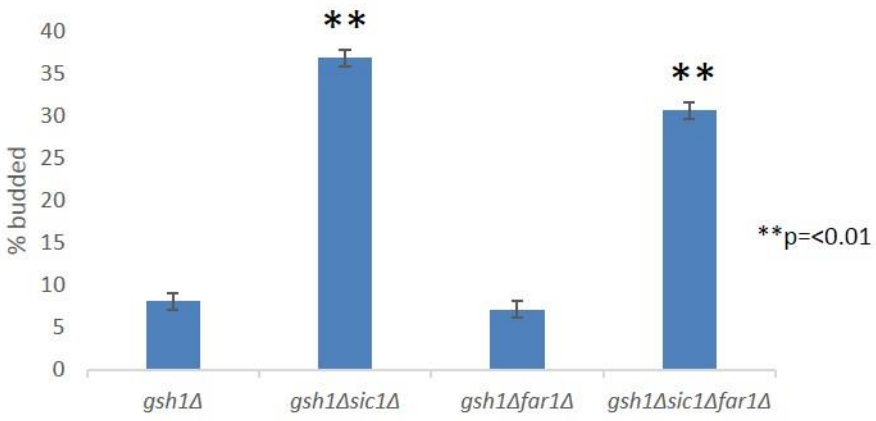
and the budding analysed. As observed in *gsh1*Δ*met4*Δ, the percentage of unbudded *gsh1*Δ*far1*Δ and *gsh1*Δ were similar (Figure 5.4 A). Furthermore, DNA content analysis of these cells showed that both *gsh1*Δ and *gsh1*Δ*far1*Δ cells arrested with similar levels of 1C DNA (Figure 5.4 B). However, in contrast, similar analysis of *gsh1*Δ*sic1*Δ revealed a much higher percentage of budded cells (Figure 5.4 A) and consistent with these data a much higher proportion of cells with a greater that 1C DNA content (Figure 5.4 B). It was possible that *SIC1* compensates for the loss of *FAR1* in *gsh1*Δ*far1*Δ cells. To test this hypothesis, a triple *gsh1*Δ*sic1*Δ*far1*Δ strain was constructed and analysed in glutathione-depleted conditions as described in Figure 5.1. As can be seen, *gsh1*Δ*sic1*Δ and *gsh1*Δ*sic1*Δ*far1*Δ budding and DNA content analysis were very similar (Figure 5.4 A and B). Taken together, and in agreement with previous, similar studies (Doris, 2008), these results suggest that Sic1, not Far1 or Met4 is essential for glutathione depletion-induced G1 phase arrest.

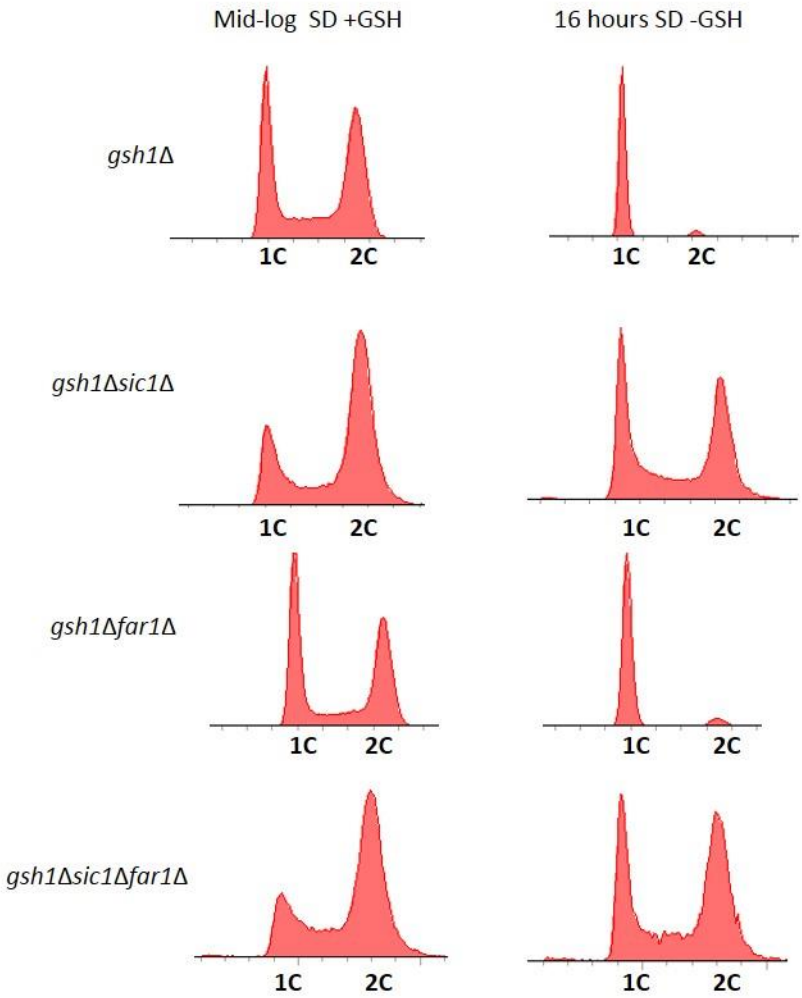
The data above suggests that Sic1 is required for the G1 phase arrest associated with glutathione depletion. During G1 phase, the role of Sic1 is to block the activity of the G1 cyclins Clb5/6, preventing entry into S phase. Furthermore, Sic1 is stabilised in response to oxidative stress and this is linked to G1 phase cell cycle delay (Chapter 3). Hence to examine the relative roles of Sic1 and Far1 stability in response to glutathione depletion a *gsh1*Δ strain was constructed expressing either Sic1-13Myc or Far1-13Myc from their normal chromosomal locus and western blot analysis was performed on these cultures after glutathione depletion. Consistent with the genetic analysis of *SIC1* and *FAR1* in the G1 phase arrest associated with loss of *GSH1* function, Sic1-13Myc levels but not Far1-13Myc levels were found to be increased in response to glutathione depletion (Figure 5.4 C).

As we had previously shown that *gsh1*Δ cells do not lose viability following glutathione depletion (Figure 5.1 B) we proposed that the glutathione depletion-induced G1 phase arrest is a protective mechanism to maintain viability. Hence the viability of *gsh1*Δ*sic1*Δ, *gsh1*Δ*far1*Δ and *gsh1*Δ*sic1*Δ*far1*Δ cells was investigated following growth for 24 and 48 hours in media lacking glutathione. Consistent with our hypothesis, *gsh1*Δ*sic1*Δ and *gsh1*Δ*sic1*Δ*far1*Δ but not *gsh1*Δ or *gsh1*Δ*far1*Δ displayed a similar loss of viability after 24 and 48 hours of growth in media lacking glutathione (Figure 5.4 D, upper panel).

A.


40
35
30
25
20
15
10
5
0
% budded
**
**
**p=<0.01
gsh1Δ
gsh1Δsic1Δ
gsh1Δfar1Δ
gsh1Δsic1Δfar1Δ

B.


Mid-log SD +GSH
16 hours SD -GSH
gsh1Δ
gsh1Δsic1Δ
gsh1Δfar1Δ
gsh1Δsic1Δfar1Δ
1C
2C

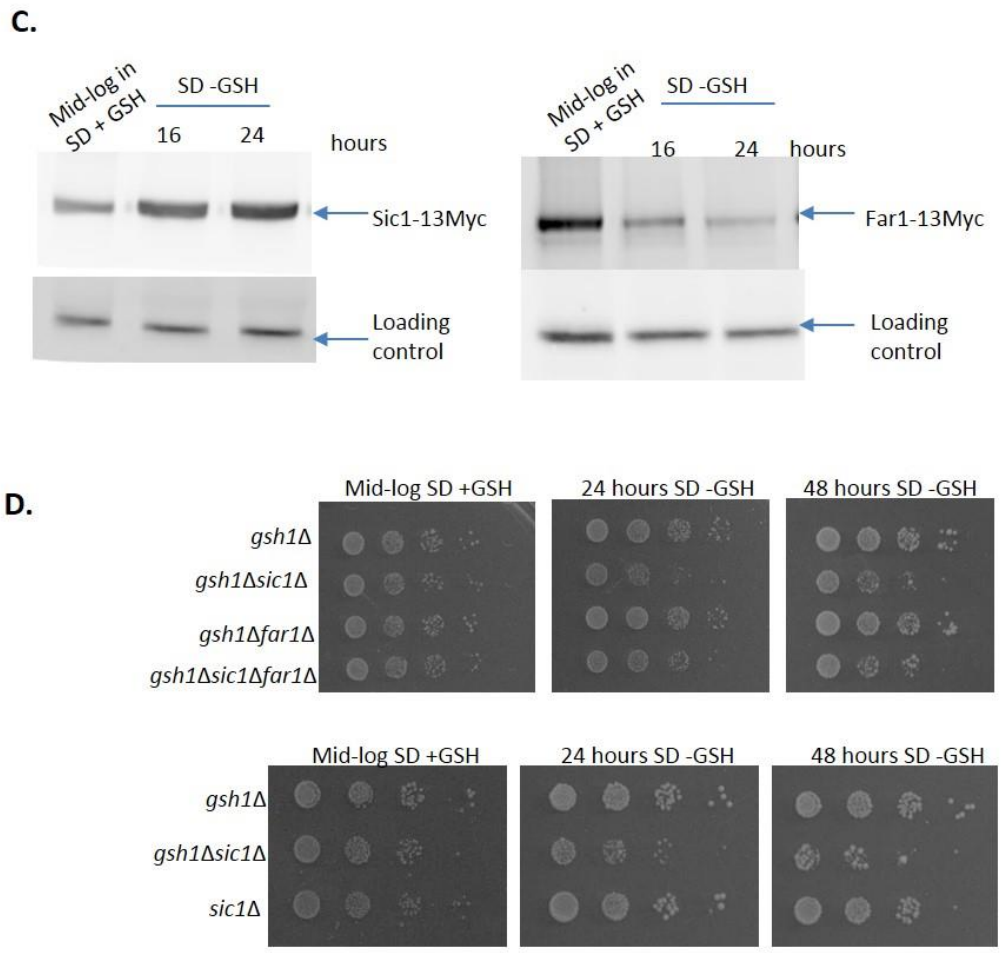


Figure 5.4 Sic1 but not Far1 is required for cell cycle arrest and viability following glutathione depletion. [A] and [B] *gsh1*Δ (KD502), *gsh1*Δ*sic1*Δ (KD125), *gsh1*Δ*far1*Δ (ELR58), and *gsh1*Δ*sic1*Δ*far1*Δ (ELR76) cells were grown until mid-log phase in SD +GSH, washed twice in water and re-suspended in SD –GSH. Pellets were taken after 16 hours for bud counts [A] and DNA content analysis [B]. [C] *gsh1*Δ cells expressing Sic1-13Myc (KD294) or Far1-13Myc (ELR77) from the normal chromosomal locus were grown to mid-log phase in SD +GSH, washed twice in water, re-suspended in SD-GSH and pellets were taken at the indicated time. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Myc antibodies. Membranes were stripped and probed with anti-Hog1 antibodies (Loading control). [D] 10-fold serial dilutions of *gsh1*Δ (KD502), *gsh1*Δ*sic1*Δ (KD125), *gsh1*Δ*far1*Δ (ELR58), *gsh1*Δ*sic1*Δ*far1*Δ (ELR76) and *sic1*Δ (KD19) cells grown as described for [A] and [B] for the indicated times were spotted on to SD +GSH plates and incubated at 30°C for 2-3 days.

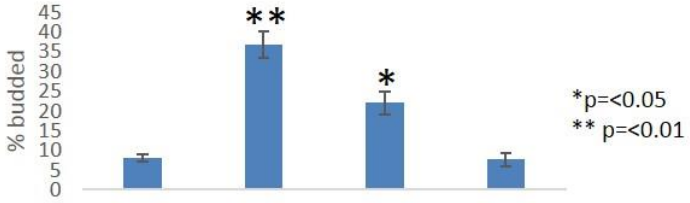
Importantly, the loss of viability did not occur in *GSH1sic1*Δ cells, indicating that the phenotype is associated with glutathione depletion (Figure 5.4 D, lower panel).

These results show that Sic1 but not Met4 or Far1 is required for the glutathione depletion-induced G1 phase cell cycle arrest and, moreover, that this G1 phase arrest is essential to maintain viability.

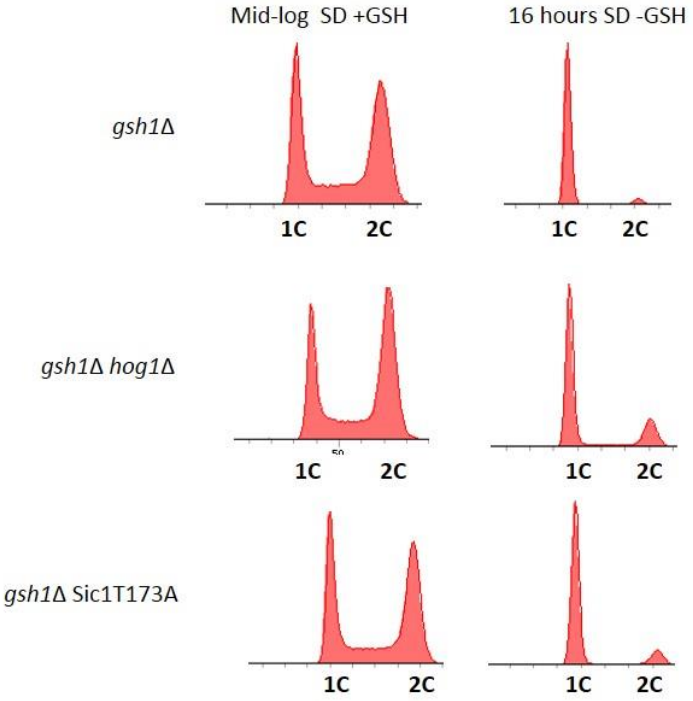
5.6 The MAPK Hog1 is important for the response of cells to glutathione but through a mechanism that does not involve Sic1

Previous studies have shown that the Hog1 MAPK is required for the cellular response to high osmolarity and a range of other stresses including H_2O_2 (Haghnazari and Heyer, 2004). Moreover, Sic1 is required for G1 phase cell cycle arrest in response to osmotic stress and Hog1 phosphorylation of Sic1 on threonine 173 has been directly linked to this G1 phase arrest (Escoté *et al.*, 2004). The results above revealed the importance of Sic1, but not Far1 in the response of the cell to glutathione depletion. Hence, given that both these CKIs are targeted by Cdc34/SCF^{Cdc4} it was possible that Sic1 was regulated by glutathione depletion via a Hog1-dependent, Cdc34-independent mechanism. To investigate the role of Hog1 in the glutathione depletion-induced G1 phase arrest a *gsh1*Δ*hog1*Δ strain was obtained and the budding analysed. Interestingly, in contrast to *gsh1*Δ cells, *gsh1*Δ*hog1*Δ cells displayed a significant increase in budded cells following glutathione depletion (Figure 5.5 A). Furthermore, consistent with the increase in budding, a small but reproducible increase in the number of cells with greater than 1C DNA content was also detected (Figure 5.5 B). The increased population of *gsh1*Δ*hog1*Δ cells with buds or with greater than 1C DNA content were however not as high as those detected in *gsh1*Δ*sic1*Δ cell populations (compare Figure 5.5 A and B with Figure 5.4 A and B). Nevertheless, these data raised the possibility that a component of Sic1 regulation by glutathione depletion may involve Hog1-dependent phosphorylation of Sic1 on threonine 173. Indeed, *gsh1*Δ*hog*1Δ cells display a lesser but detectable decrease in viability compared with *gsh1*Δ*sic1*Δ in glutathione depleted conditions (Figure 5.5 C) raising the possibility that Sic1 stability was being affected. To test the potential role of threonine 173 phosphorylation, a *gsh1*Δ strain expressing Sic1 with a substitution of threonine to alanine (Sic1T173A) from its normal chromosomal locus was

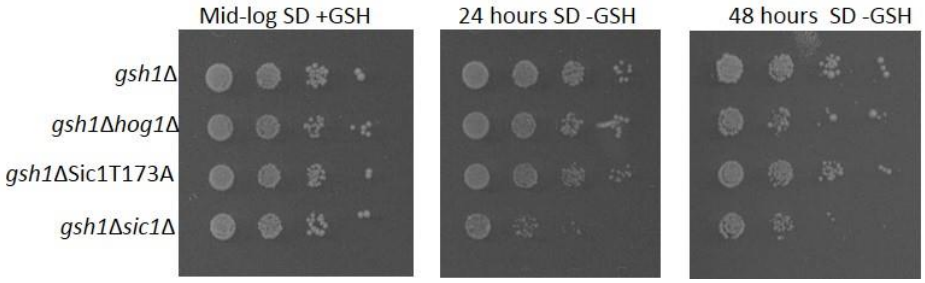
A.


% budded
45
40
35
30
25
20
15
10
5
0
**
*
*p=<0.05
** p=<0.01

B.


Mid-log SD +GSH
16 hours SD -GSH
gsh1Δ
gsh1Δ hog1Δ
gsh1Δ Sic1T173A
1C
2C

C.


Mid-log SD +GSH
24 hours SD -GSH
48 hours SD -GSH
gsh1Δ
gsh1Δhog1Δ
gsh1ΔSic1T173A
gsh1Δsic1Δ

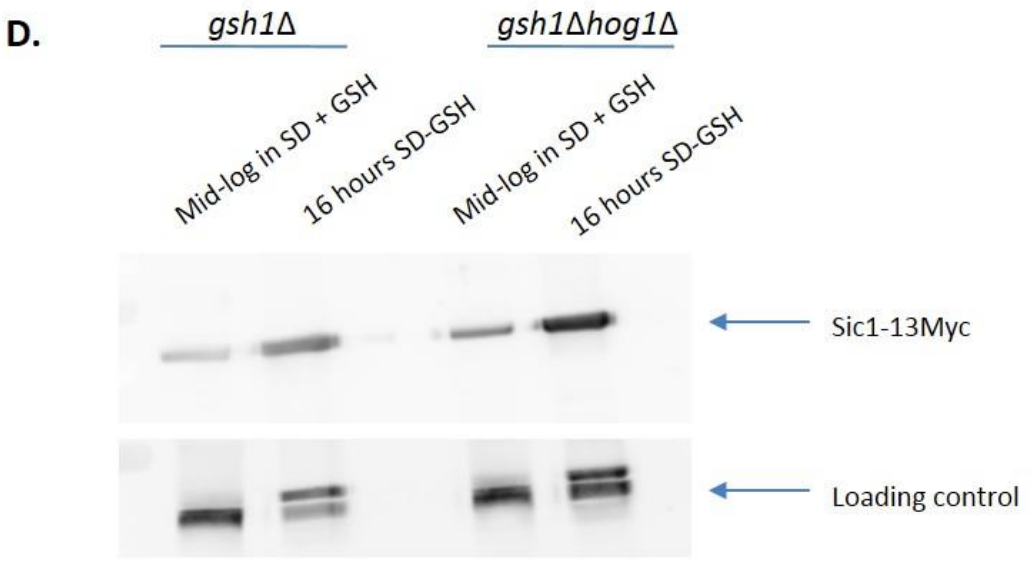


Figure 5.5 The MAPK Hog1 is important for the response of cells to glutathione depletion through a mechanism that does not involve Sic1. [A] Bud counts, [B] DNA content analysis and [C] viability assays were performed on *gsh1*Δ (KD502), *gsh1*Δ*hog1*Δ (KD192) and *gsh1*Δ cells expressing Sic1 with a T173A mutation from the normal chromosomal locus (*gsh1*ΔSic1T173A)(KD183) grown until mid-log in SD+GSH, washed twice in water and re-suspended in SD-GSH. [C] For viability assays 10-fold serial dilutions of the indicated strains were grown for the indicated time in SD –GSH and then spotted on to SD +GSH and incubated at 30°C for 2-3 days. [D] *gsh1*Δ (KD294) and *gsh1*Δ*hog1*Δ (KD192) expressing Sic1-13Myc from the normal chromosomal locus were grown to mid-log in SD +GSH, washed twice in water and re-suspended in SD-GSH. Pellets were taken at the indicated times. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Myc antibodies. The membrane was stripped and probed with anti-tubulin antibodies (Loading control).

obtained. This mutation prevents Sic1 phosphorylation at this site and had previously been shown to prevent the osmotic stress-induced G1 phase cell cycle delay (Escoté *et al.*, 2004). In contrast to *gsh1*Δ*hog1*Δ cells, bud and DNA content analysis following glutathione depletion revealed that *gsh1*ΔSic1T173A cells behaved similar to *gsh1*Δ cells (Figure 5.5 A and B). Moreover, consistent with these observations, in contrast to *gsh1*Δ*hog1*Δ and *gsh1*Δ*sic1*Δ, *gsh1*ΔSic1T173A cells displayed similar viability to *gsh1*Δ cells following glutathione depletion (Figure 5.5 C).

Although there is no evidence that Hog1-dependent phosphorylation of threonine 173 of Sic1 is important for glutathione depletion-induced G1 phase arrest it was possible that Sic1 stability may be targeted by Hog1 via another mechanism. To test whether Hog1 is required for the increase in Sic1 stability following glutathione depletion, *gsh1*Δ*hog1*Δ cells expressing Sic1-13Myc from the normal chromosomal locus were obtained. Significantly, western blot analysis of *gsh1*ΔSic1-13Myc and *gsh1*Δ*hog1*ΔSic1-13Myc cells following glutathione depletion revealed no detectable drop in Sic1-Myc levels in cells lacking *HOG1* (Figure 5.5 D).

Hence taken together these results suggest that in contrast to osmotic stress, Sic1 is regulated in a Hog1-independent manner in cells depleted of glutathione. However, Hog1 does appear to be required for proper cell cycle arrest and retention of viability after glutathione depletion by an unknown mechanism(s).

5.7 Cdc34 protein levels decrease in response to glutathione depletion

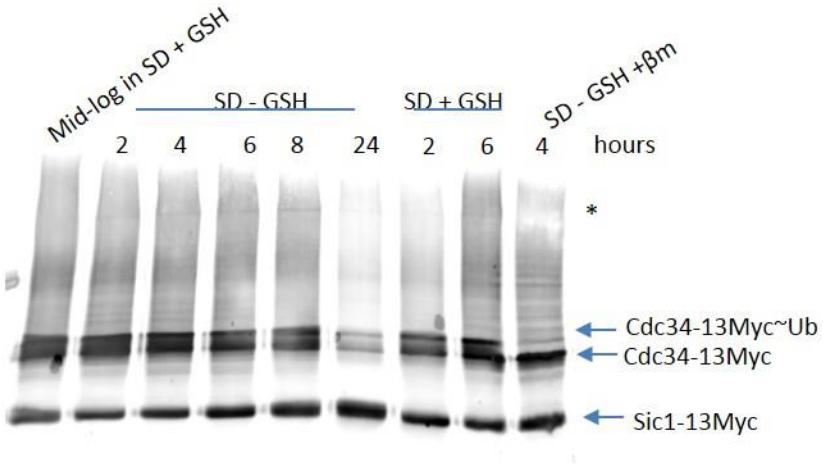
Our studies above reveal the importance of Sic1 in glutathione depletion-induced G1 phase arrest and furthermore, that the mechanism of regulation is independent of Hog1. As we described in Chapter 3, the ubiquitin pathway E2, Cdc34 is specifically sensitive to oxidation by diamide, a drug that oxidises glutathione. In addition, treatment of cells with the drug buthionine sulfoximine (BSO) which specifically inhibits Gsh1 also stimulates oxidation of Cdc34 (Doris *et al.*, 2012) suggesting that glutathione levels are coupled to Cdc34 activity. Hence, it was possible that glutathione depletion triggers oxidation of Cdc34, preventing degradation of Sic1, leading to G1 phase arrest. To test this hypothesis *gsh1*Δ cells expressing Cdc34-13Myc and Sic1-13Myc from their normal chromosomal locus were depleted of glutathione. Interestingly, after 2 hours of growth in media lacking glutathione, Cdc34 did indeed form a HMW, β-mercaptoethanol

sensitive complex (Figure 5.6 A*). Although the amount of Cdc34 involved in this disulphide was far less than observed when cells are exposed to diamide the timing of the appearance of this band and its selective abundance more closely mimics the effects of BSO treatment (Doris *et al.*, 2012). However, unexpectedly, the overall levels of Cdc34 decreased significantly after 24 hours of growth in media lacking glutathione (Figure 5.6 A). Furthermore, this decrease in the level of Cdc34-13Myc was coincident with the increased levels of Sic1, suggesting the two events are linked (Figure 5.6 A). Interestingly this reduction in Cdc34 levels is rapidly reversed upon addition of reduced glutathione to the media and, importantly, coincides with a reduction in Sic1 protein levels (Figure 5.6 A). To demonstrate that the reduction in Cdc34 protein levels was a specific response to the lack of glutathione and not due to the arrest of the cells in G1 phase, *gsh1*ΔCdc34-13Myc Sic1-13Myc cells were synchronised in G1 phase by α-factor and Cdc34-13Myc and Sic1-13Myc protein levels analysed by western blotting (Figure 5.6 B). Despite detecting an increase in the levels of Sic1-13Myc, no decrease in the levels of Cdc34-13Myc were observed in α-factor arrested cells suggesting that the regulation of Cdc34 protein levels after glutathione depletion is indeed a specific response. Next, to establish whether the reduction in Cdc34 protein levels was due to a decrease in *CDC34* transcript levels, *CDC34* mRNA levels in mid-log phase *gsh1*Δ cells growing in glutathione replete media were compared with the levels of *CDC34* mRNA in *gsh1*Δ cells growing in media lacking glutathione for 16 hours by qRT-PCR (Figure 5.6 C). *ARN2* transcript levels were used as a positive control as the expression of this gene has been shown to be upregulated in response to glutathione depletion (Ayer *et al.*, 2010). Significantly, mRNA analysis revealed that there was no decrease in *CDC34* transcript levels after glutathione depletion while *ARN2* transcript levels increased as predicted (Figure 5.6 C). Thus, this data suggests that the decrease in Cdc34 protein levels is regulated via a protein translation and/or stability mechanism(s). Collectively, these results have revealed a new mechanism of Cdc34 regulation that is specifically linked to glutathione depletion and not oxidative stress responses or cell cycle regulation.

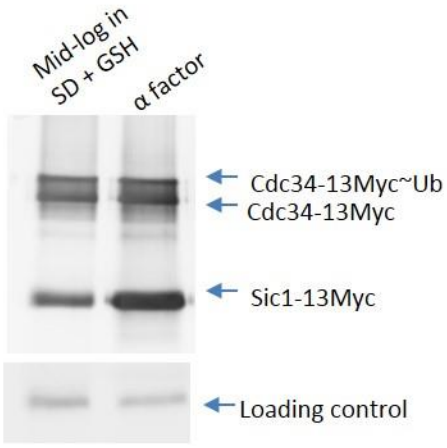
5.8 Budding is inhibited by the Rub1 pathway following glutathione depletion

Our data above revealed that Cdc34 protein levels are reduced following glutathione depletion. Cdc34 normally acts with different E3 complexes consisting of different F-box proteins e.g. Cdc4 and Grr1 and scaffold proteins such as Cdc53 and Rbx1. Hence it was

A.


Mid-log in SD + GSH
SD - GSH
SD + GSH
SD - GSH +βm
2 4 6 8 24 2 6 4 hours
*
Cdc34-13Myc~Ub
Cdc34-13Myc
Sic1-13Myc

B.


Mid-log in SD + GSH
α factor
Cdc34-13Myc~Ub
Cdc34-13Myc
Sic1-13Myc
Loading control

C.

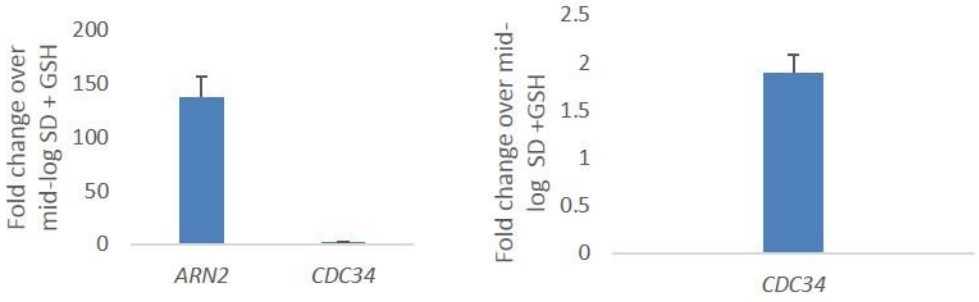
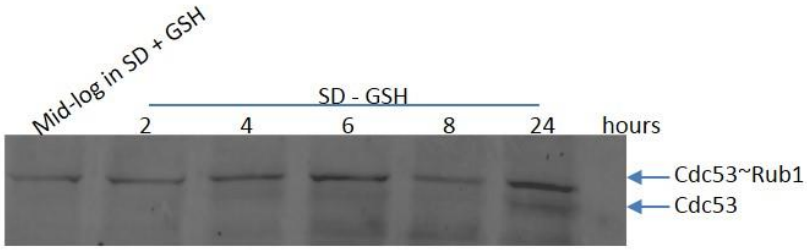

Fold change over mid-log SD + GSH
200
150
100
50
0
ARN2
CDC34
Fold change over mid-log SD +GSH
2.5
2
1.5
1
0.5
0
CDC34

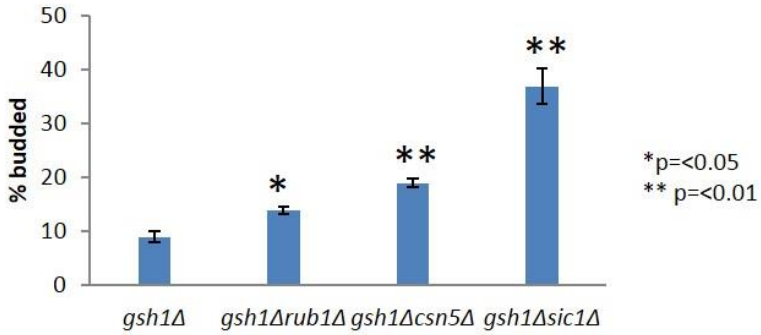
Figure 5.6 Cdc34 protein levels decrease in response to glutathione depletion. [A] *gsh1*Δ cells expressing Cdc34-13Myc and Sic1-13Myc from their normal chromosomal locus (KD 294) were grown to mid-log in SD +GSH, washed twice in water and re-suspended in SD-GSH. After 24 hours of growth in SD –GSH cells were washed twice in water and re-suspended in SD +GSH. Cell pellets were taken at the indicated times. [B] Mid-log phase growing *gsh1*ΔCdc34-13Myc Sic1-13Myc (KD294) cells grown in SD +GSH were synchronised in G1 phase by α-factor and cell samples taken before α-factor addition and at the G1 phase arrest. [A] and [B] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Myc antibodies. +βm sample had β-mercaptoethanol added to loading dye. [B] Membrane was stripped and probed with anti-Hog1 antibodies (Loading control). [C] RNA was extracted from mid-log phase *gsh1*Δ (KD502) cells growing in SD +GSH and *gsh1*Δ grown in SD –GSH for 16 hours (as described in [A]) and subjected to qRT-PCR to detect *CDC34* and *ARN2* transcripts. qPCR was normalised to *ACT1* and fold change over mid-log phase *gsh1*Δ cells grown in SD +GSH is shown. *CDC34* is the mean of 6 biological repeats and *ARN2* of 3 biological repeats. Error bars represent standard error (SE).

possible that the levels of proteins such as Cdc53 may also be reduced following glutathione depletion. To examine this possibility, Cdc53 protein levels were examined by western blot analysis following glutathione depletion (Figure 5.7 A). In contrast to Cdc34 no significant decrease in Cdc53 protein levels were detected after glutathione depletion. Hence, the effect of glutathione depletion on Cdc34 protein levels appears to be specific to this enzyme. Interestingly, however, the pattern of Rub1 modification of Cdc53 (Neddylation) was changed. In particular, whilst Cdc53 was predominantly Rub1 modified in mid-log cells, a proportion of Cdc53 was in the unmodified form in glutathione-depleted cells (Figure 5.7 A). To explore the potential role of the Rub1 pathway further, *gsh1*Δ*rub1*Δ and *gsh1*Δ*csn5*Δ strains which prevent or stimulate Rub1 modification of Cdc53 (Chapter 4) were constructed. Interestingly, deletion of *RUB1* and *CSN5* both led to a significant increase in the number of budded cells after glutathione depletion (Figure 5.7 B). Moreover, consistent with the previous result that the deNeddylation of Cdc53 is important for the G1 phase arrest, loss of *CSN5* had a bigger effect on budding (Figure 5.7 B). In contrast to *gsh1*Δ*sic1*Δ cells, neither loss of *RUB1* or *CSN5* had any major effect on the numbers of cells with a greater than 1C DNA content (Figure 5.7 C), suggesting that the major regulatory role of the Rub1 pathway in glutathione-depleted cells is to prevent budding and not DNA replication. The role of the Rub1 pathway was also examined using viability assays. These data revealed that whilst *gsh1*Δ*sic1*Δ cells lose viability after 24 hours of glutathione depletion, *gsh1*Δ*rub1*Δ and *gsh1*Δ*csn5*Δ cells do not show the same loss of viability even after 48 hours. Although the data reveal that deNeddylation of Cdc53 occurs in glutathione-depleted cells and the importance of deNeddylation in prevention of budding under these conditions, it was possible that Neddylation was regulating budding independent of Cdc53. However, in our attempts to construct a *gsh1*Δ strain expressing epitope-tagged Cdc53 we found that ~20% of cells remained budded after 16 hours of growth in glutathione depleted media. Thus, these data suggest that Cdc53 Neddylation patterns are linked to regulation of budding following glutathione depletion.

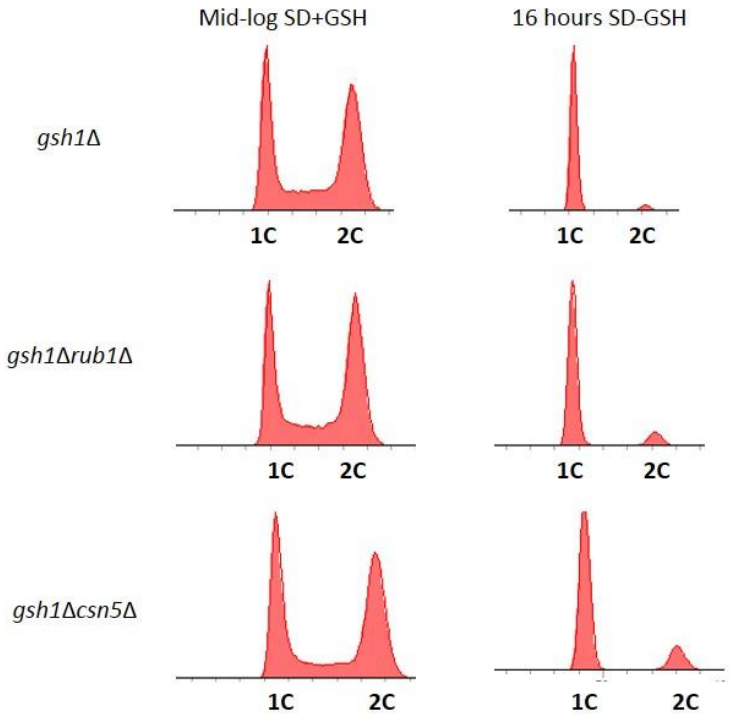
A.


Mid-log in SD + GSH
SD - GSH
2
4
6
8
24
hours
Cdc53~Rub1
Cdc53

B.


50
40
30
20
10
0
% budded
*
**
**
*p=<0.05
** p=<0.01
gsh1Δ
gsh1Δrub1Δ
gsh1Δcsn5Δ
gsh1Δsic1Δ

C.


Mid-log SD+GSH
16 hours SD-GSH
gsh1Δ
gsh1Δrub1Δ
gsh1Δcsn5Δ
1C
2C

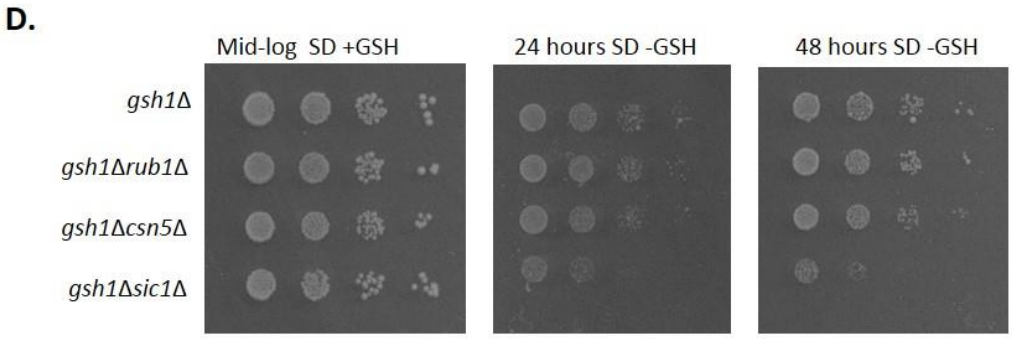


Figure 5.7 Budding is inhibited by the Rub1 pathway following glutathione depletion. [A] *gsh1*Δ (KD502) cells were grown to mid-log in SD +GSH, washed twice in water and re-suspended in SD –GSH. Cell pellets were taken at the indicated times. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Cdc53 antibodies. [B] Bud counts, [C] DNA content analysis and [D] viability assays were performed on *gsh1*Δ (KD502), *gsh1*Δ*rub1*Δ (ELR 60), *gsh1*Δ*csn5*Δ (ELR72) and *gsh1*Δ*sic1*Δ (KD125) grown to mid-log in SD +GSH, washed twice in water and re-suspended in SD-GSH. [D] For viability assays, 10-fold serial dilutions of each strain were spotted on to SD +GSH and incubated at 30°C for 2-3 days.

Taken together these results indicate that Cdc53 protein levels are not reduced following glutathione depletion indicating a specific regulation of Cdc34. However, Neddylation is also affected by glutathione depletion and this is linked to prevention of budding. Given the specific role of Neddylation in budding rather than DNA replication, comparison of the viability data for *gsh1*Δ*sic1*Δ, *gsh1*Δ*hog1*Δ, *gsh1*Δ*rub1*Δ and *gsh1*Δ*csn5*Δ suggests that inhibition of DNA replication in glutathione-depleted cells is the important action to prevent cell death.

5.9 Regulation of Ydj1 is important for the response of cells to glutathione depletion

Our preliminary analyses had revealed that Ydj1 forms a HMW, β-mercaptoethanol-sensitive complex when treated with 5 mM diamide (Figure 4.6). These data suggested that the Ssa1/Ydj1/Cln3 pathway is also regulated by oxidative stress. Hence the regulation of Ydj1 was explored under conditions of glutathione depletion. First a *gsh1*Δ strain expressing Ydj1-3HA from the normal chromosomal locus was constructed and western blot analysis performed on *gsh1*ΔYdj1-3HA following glutathione depletion (Figure 5.8 A). In contrast to diamide treatment, no HMW Ydj1 complex was observed after glutathione depletion, however, similar to Cdc34, Ydj1 protein levels significantly decreased 24 hours after glutathione depletion. Indeed, consistent with the effect on Cdc34, no decrease in the protein levels of Ydj1 were observed in α-factor blocked cells (Figure 5.8 B). Furthermore, similar to *CDC34*, *YDJ1* transcript levels were not reduced in glutathione depletion arrested cells (Figure 5.8 C). These results suggest that the regulation of Ydj1 is important for the response of cells to glutathione depletion.

To further investigate the role of Ydj1 regulation in the response of cells to glutathione depletion, a *gsh1*Δ strain where Ydj1 was tagged at the N-terminus with 3HA epitopes and expressed from the normal chromosomal locus but from the strong GAL promoter rather than its normal promoter was obtained (Ydj1-3HA GAL). Comparison of Ydj1-3HA levels expressed from the normal chromosomal locus from its own promoter (*gsh1*ΔYdj1-3HA) or the *GAL1* promoter (*gsh1*ΔYdj1-3HA GAL) in cells grown in galactose-containing media revealed that the *GAL1* regulated protein was present at higher levels (Figure 5.9 A, compare the two mid-log SD +GSH lanes). Furthermore, growth of these cells in glutathione-depleted media showed that as expected, protein levels of Ydj1-3HA expressed from its normal promoter decreased after 16 and 24 hours

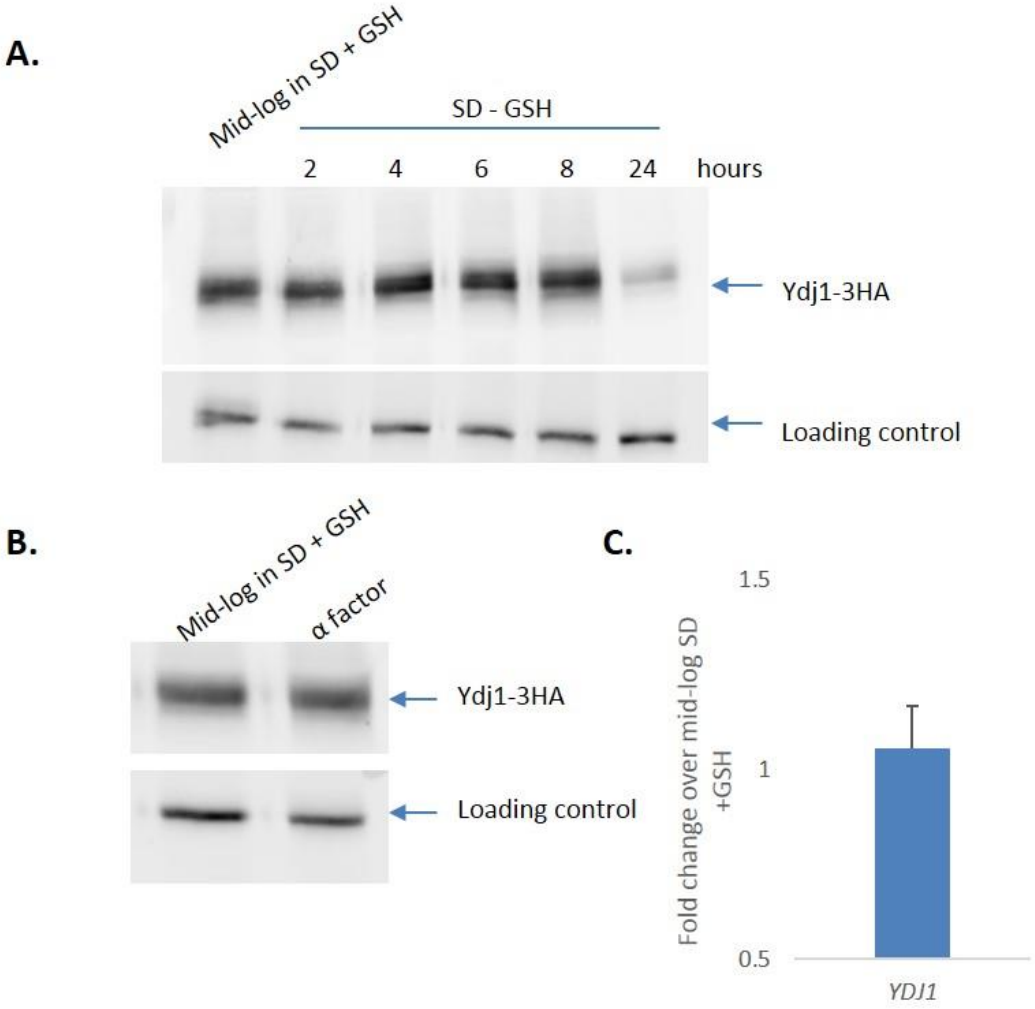
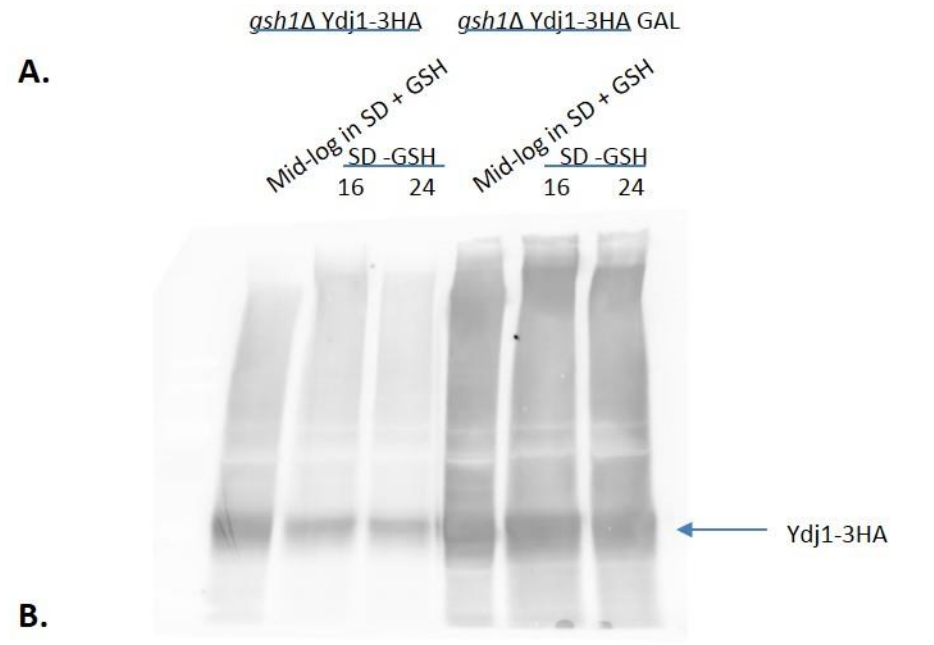


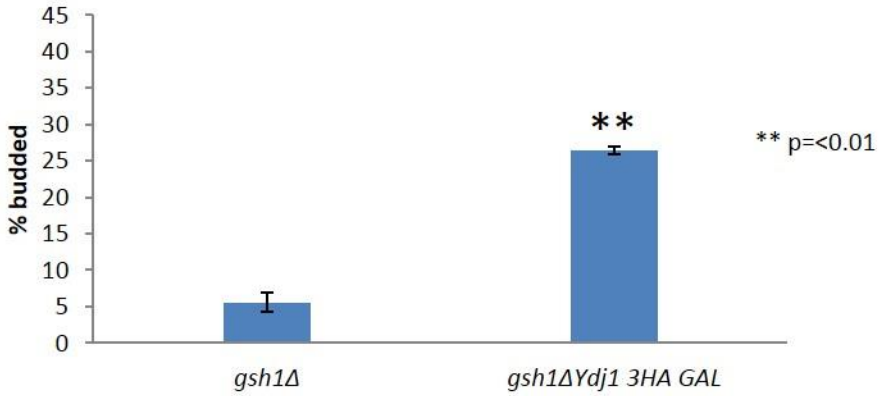
Figure 5.8 Ydj1 protein levels but not RNA levels decrease in response to glutathione depletion. [A] *gsh1*Δ cells expressing Ydj1-3HA from the normal chromosomal locus (ELR71) were grown to mid-log in SD +GSH, washed twice in water and re-suspended in SD +GSH. Pellets were collected at the indicated times. [B] Mid-log phase growing *gsh1*ΔYdj1-3HA(ELR71) cells grown in SD +GSH, were synchronised in G1 phase by α-factor and cell samples taken before α-factor addition and at the G1 phase arrest. [A] and [B] Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-HA antibodies. The membrane was stripped and probed with anti-Hog1 antibodies (Loading control). [C] RNA was extracted from mid-log phase *gsh1Δ* (KD502) cells and *gsh1Δ* cells grown in SD –GSH for 16 hours and subjected to qRT-PCR to detect *YDJ1* transcripts. qPCR was normalised to *ACT1* and fold change over mid-log *gsh1*Δ cells grown in SD +GSH is shown. *YDJ1* is the mean of 3 biological repeats. Error bars represent SE.

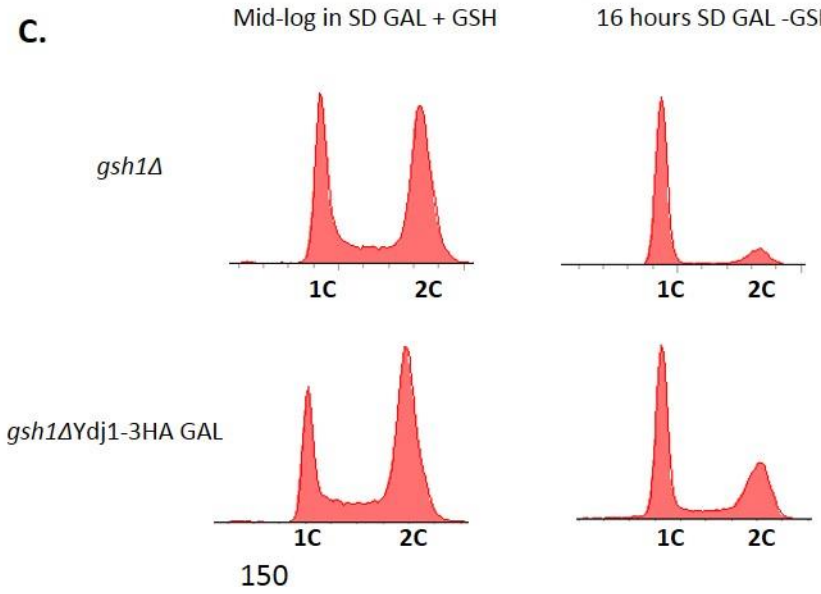
while Ydj1-3HA under the control of the *GAL1* promoter did not show the same decrease in protein levels (Figure 5.9 A). Having established that *GAL1*-dependent expression of Ydj1 maintains higher levels of the protein in glutathione-depleted conditions we next investigated the potential effects of such expression on budding, DNA content and viability. Budding and DNA content analysis indicated that *gsh1*Δ cells displayed similar profiles when grown in galactose containing media (SD +GAL) versus glucose containing media (SD) (Compare Figure 5.9 B and C and 5.5 A and B). Interestingly, similar analysis of *gsh1*Δ cells expressing Ydj1-3HA from the *GAL1* promoter revealed high levels of budded cells and cells with greater than 1C DNA content (Figure 5.9 B and C).

Next the effect of Ydj1 overexpression and lack of G1 phase arrest on viability following glutathione depletion was investigated. The viability of *gsh1*Δ, *gsh1*Δ*sic1*Δ and *gsh1*ΔYdj1-3HA GAL that had been grown in galactose-containing media lacking glutathione for 24 and 48 hours was examined by plate assays (Figure 5.9 D). Interestingly, comparison of growth of mid-log phase *gsh1*Δ and *gsh1*ΔYdj1-3HA GAL cells plated on to glucose-containing media (represses *GAL1*-dependent expression) revealed that cells expressing *YDJ1* from the *GAL1* promoter show a loss of viability compared to cells expressing *YDJ1* from its normal promoter (Figure 5.9 D, mid-log SD GAL +GSH). Interestingly, the difference in growth was rescued by exposure of both strains to glutathione-depleted conditions (Figure 5.9 D). This further supports the hypothesis that Ydj1 function is inhibited under these conditions and is important for the cellular response to glutathione depletion. Unfortunately, attempts to assess the effect of overexpression of Ydj1 on the response of cells to glutathione depletion were hampered by the poor growth of *gsh1*Δ cells in galactose-containing media. Nevertheless, these viability assays provide some evidence that inhibition of Ydj1 has an important role in the response of cells to glutathione depletion.

Taken together these data suggest that Ydj1 and Cdc34 may be regulated by similar mechanisms in oxidative stress and glutathione-depleted conditions. In particular, both proteins form HMW disulphide complexes following specific oxidative stress and the translation/stability of both proteins is downregulated in response to glutathione depletion.


A.
gsh1Δ Ydj1-3HA
gsh1Δ Ydj1-3HA GAL
Mid-log in SD + GSH
SD -GSH
16
24
Mid-log in SD + GSH
SD -GSH
16
24
Ydj1-3HA
B.


45
40
35
30
25
20
15
10
5
0
% budded
**
** p=<0.01
gsh1Δ
gsh1ΔYdj1 3HA GAL


C.
Mid-log in SD GAL + GSH
16 hours SD GAL -GSH
gsh1Δ
1C
2C
1C
2C
gsh1ΔYdj1-3HA GAL
1C
2C
1C
2C

150

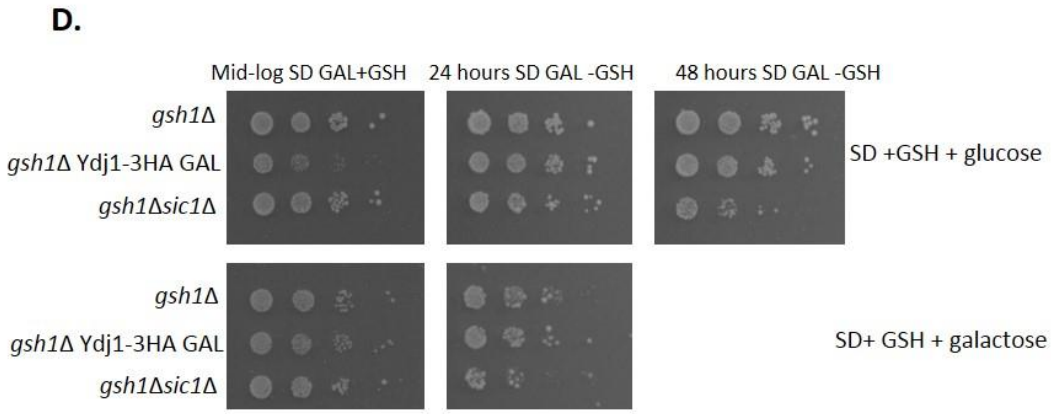


Figure 5.9 Overexpression of Ydj1 prevents the glutathione depletion-induced G1 phase arrest. [A] *gsh1Δ*Ydj1-3HA (ELR71) and *gsh1Δ*Ydj1-3HA GAL (KD231) were grown to mid-log in SD +GAL +GSH, washed twice in water and re-suspended in SD +GAL –GSH. Cell pellets were taken at the indicated times. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-HA antibodies. [B] Bud counts, [C] DNA content analysis and [D] viability assays were performed on *gsh1Δ* (KD502), *gsh1Δ*Ydj1-3HA (ELR71) and *gsh1Δ* Ydj1-3HA GAL (KD231) grown until mid-log in minimal media supplemented with 2% galactose and 1mM GSH (SD GAL +GSH), washed twice in water and re-suspended in SD GAL –GSH. [D] For viability assays, 10-fold serial dilutions of the indicated strains, grown for the indicated times were spotted on to SD GAL +GSH or SD +GSH +glucose and incubated at 30°C for 2-3 days

5.10 Rad53 is activated in glutathione-depleted *gsh1*Δ*sic1*Δ cells

The results described above suggest that increased DNA content, possibly signifying DNA replication, rather than budding is important for the loss of viability observed in *gsh1*Δ*sic1*Δ cells following glutathione depletion. An obvious hypothesis to explain these observations is that DNA damage/aberrant DNA replication occurs in *gsh1*Δ*sic1*Δ cells that cannot arrest DNA replication after glutathione depletion. To test this hypothesis the activation of the DNA damage checkpoint was assessed in *gsh1*Δ*sic1*Δ cells following glutathione depletion. As described above, *gsh1*Δ cells do not show activation of Rad53 following glutathione depletion (Figure 5.2). Significantly, some Rad53 activation was observed in *gsh1*Δ*sic1*Δ cells in glutathione-depleted conditions, suggesting that DNA damage was occurring in these cells (Figure 5.10). The observed activation of Rad53 is not as great as seen in HU-treated cells but nevertheless is consistent with the model that Sic1 is required to prevent DNA replication and DNA damage in glutathione-depleted conditions.

5.11 Discussion

Glutathione has long been recognised for its role as a highly abundant cellular antioxidant essential for viability in all eukaryotes studied. In *S. cerevisiae*, *gsh1*Δ cells grown in the absence of exogenous glutathione arrest their cell cycle in G1 phase by a previously unknown mechanism (Spector *et al.*, 2001). In this chapter we have shown that the glutathione depletion-induced G1 phase arrest is regulated by a number of different pathways. Importantly, Sic1 was found to play an essential role in the cell cycle arrest in response to glutathione depletion, likely by preventing DNA replication-induced DNA damage. Indeed inhibition of DNA replication appeared to be vital to maintain viability in the absence of glutathione. Cdc34 is a regulator of Sic1 stability and significantly Cdc34 protein levels decrease in the absence of glutathione. Furthermore, an important regulator of Start, Ydj1, was also found to display a decrease in its protein levels after glutathione depletion. The mechanism behind the regulation of Cdc34 and Ydj1 protein levels is not clear but appears to be due to changes in translation and/or protein stability. The Hog1 MAPK is an important regulator of the cell cycle in response to osmotic stress, targeting Sic1 by direct phosphorylation (Escoté *et al.*, 2004). However, although Hog1 was shown to be important for the response of cells to

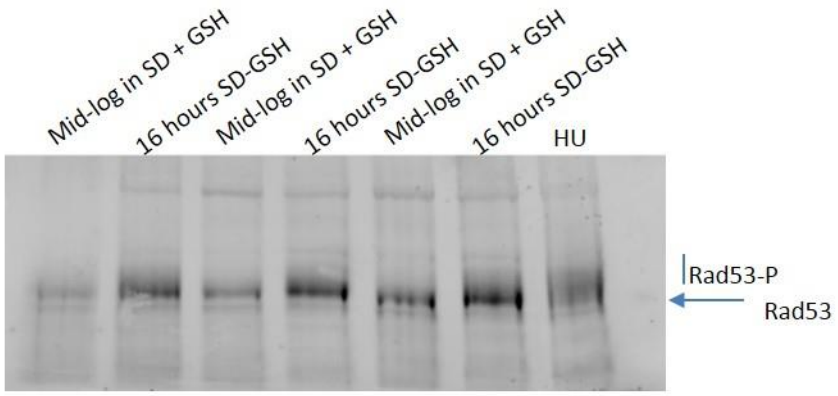


Figure 5.10 The cell cycle checkpoint kinase Rad53 is activated in *gsh1Δsic1Δ* cells following glutathione depletion. *gsh1Δsic1Δ* (KD125) cells were grown to mid-log phase in SD +GSH, washed twice in water, re-suspended in SD –GSH and grown for 16 hours. Cell lysates were prepared under non-reducing conditions, separated by SDS-PAGE and analysed by western blotting using anti-Rad53 antibodies. Western blot shows 3 biological repeats.

glutathione depletion, this mechanism appears to be Sic1-independent. Finally, the Ubl Rub1 has poorly defined roles in *S. cerevisiae* but the studies presented here revealed that altered Rub1 modification of Cdc53 is linked to prevention of budding in glutathione depletion-induced cell cycle arrest.

Previous studies revealed that different oxidising agents affect cell cycle progression by different mechanisms and at different points in the cell cycle. For example, H_2O_2 leads to a G2/M phase, Rad9-dependent arrest (Flattery-O'Brien and Dawes, 1998) while menadione leads to a G1 phase, Rad9-independent arrest (Nunes and Siede, 1996). Here, in agreement with a recent study (Hatem *et al.*, 2014) we have shown that glutathione depletion does not lead to activation of Rad53 indicating that the associated G1 phase arrest is not due to activation of the DNA damage checkpoint.

In response to decreased levels of glutathione, *GSH1* gene expression is induced by the transcription factor Met4 (Wheeler *et al.*, 2002). Regulation of Met4 has also been shown to be important for the transition of G1 to S phase of the cell cycle (Patton *et al.*, 2000). Thus we thought that Met4 might be important for the G1 phase arrest observed following glutathione depletion. However, as we have shown here although the pattern of Met4 ubiquitination does alter after glutathione depletion consistent with its activation, in the absence of *MET4*, *gsh1*Δ cells are still able to arrest in G1 phase.

Previous work from our lab had shown that Sic1 plays an important role in the cellular response to oxidative stress. Indeed, in the absence of *SIC1*, cells were shown to be unable to arrest in G1 phase in response to diamide (Doris *et al.*, 2012). Here we have shown that Sic1 is essential for G1 phase arrest in response to glutathione depletion. Moreover, here we have shown that Sic1 is necessary for viability and the inhibition of both budding and DNA replication under conditions of glutathione depletion.

Sic1 is regulated differently in response to specific cell cycle stresses. For example, in response to osmotic stress Sic1 was shown to be phosphorylated directly by Hog1, and this phosphorylation was required for G1 phase arrest in response to osmotic stress (Escoté *et al.*, 2004). In this study although we find that Hog1 is important for the G1 phase arrest in response to glutathione depletion we find no evidence that the role of Hog1 under these conditions is the direct phosphorylation of Sic1.

In Chapter 3, we showed that in response to diamide, Cdc34 and Uba1 form a HMW complex. Here, although we detect a HMW Cdc34 containing complex early after glutathione depletion our key observation is that the levels of Cdc34 protein drop after 24 hours growth in glutathione depleted media. Interestingly, the drop in Cdc34 protein levels coincides with an increase in the stability of Sic1. While the mechanisms behind the observed decrease in Cdc34 protein levels are unclear here we have shown that Ydj1, another major regulator of Start is also regulated in a similar manner. Importantly, proper regulation of Ydj1 was shown to be important following glutathione depletion as overexpression of Ydj1 drives both budding and DNA replication although not to the same extent as observed in *gsh1*Δ*sic1*Δ cells.

A decrease in protein levels can arise by a number of different mechanisms. For example, a decrease in transcription or translation or a change in protein stability or degradation can all lead to a decrease in the levels of a protein. We have shown that the decrease seen in Cdc34 and Ydj1 protein levels does not coincide with a decrease in their transcript levels which is in agreement with data from a global microarray of *gsh1*Δ cells after glutathione depletion (Ayer *et al.*, 2010). Unfortunately time constraints have prevented us from establishing whether the decrease in Cdc34 and Ydj1 protein levels is at the level of translation or protein stability. Translation is highly regulated in *S. cerevisiae* in response to H_2O_2. For example in response to H_2O_2 the number of ribosomes located at upstream open reading frames (uORFs) increases (Gerashchenko *et al.*, 2012). uORFs are located immediately upstream of the main gene sequence and are known to modulate gene expression. One consequence of translation from a uORF is the addition of an N-terminal extension to the translated protein, which can act as a signal peptide directing a fraction of the protein to another cellular compartment. Interestingly, Ydj1 was shown to have an N-terminal extension after H_2O_2 treatment and has been predicted to exist in more than one cellular compartment. In our study, the construction of the *gsh1*Δ Ydj1-3HA GAL strain is likely to have disrupted any uORFs and thus could prevent Ydj1 localisation to another cellular compartment in response to glutathione depletion. It would therefore be interesting to investigate whether the localisation of Ydj1 is important for its regulation and for its role in the cellular response to glutathione depletion. Furthermore, a study looking at the role of glutathione in the cellular response to oxidative stress showed that while wild type cells and to a lesser

extent *gsh1*Δ cells grown in glutathione replete media abolish their translational activity after H_2O_2 treatment the levels of translation did not alter between *gsh1*Δ grown with exogenous glutathione and *gsh1*Δ depleted for glutathione in a manner similar to the present study (Hatem *et al.*, 2014). These results suggest that the decrease in Cdc34 and Ydj1 protein levels observed in this study may therefore be due to changes in protein stability after glutathione depletion rather than problems with transcription or translation.

The Neddylation pathway has been linked to oxidative stress in mammalian cells but its role is unclear in *S. cerevisiae* (Kumar *et al.*, 2007). Genetic studies have demonstrated a link between Rub1 and SCF^{Cdc4} (Lammer *et al.*, 1998) however, the function of Rub1 has remained largely unclear. Here we have shown that Rub1 modification of Cdc53 changes with glutathione depletion suggesting that deNeddylation is important for the G1 phase arrest. Indeed the absence of *CSN5* shows a bigger effect on budding than the absence of *RUB1*. Interestingly, although a significant increase in the number of budded cells is observed in a *gsh1*Δ*csn5*Δ culture there is not a corresponding increase in DNA replication or a loss of cell viability. Thus these results suggest that DNA replication and not budding is linked to cell viability. The specific Rub1 target under these conditions is unclear however the fact that an epitope-tagged version of Cdc53 prevents budding inhibition suggests that Cdc53 is the target. It is not known whether glutathione depletion blocks the Rub1 E1/E2 or whether deNeddylation is upregulated or both but it would be interesting to investigate whether the levels of the Rub1 pathway E2, Ubc12 are affected as is seen with Cdc34.

Recently, the essential role of glutathione in *S. cerevisiae* has been linked to its role in the synthesis of iron-sulphur (Fe-S) clusters rather than as an antioxidant (Kumar *et al.*, 2011). A number of observations suggested that the major role of glutathione was not the maintenance of the cellular redox status. In *S. cerevisiae* the inactivation of either the thioredoxin pathway through deletion of both cytoplasmic thioredoxins (Muller, 1991) or the glutathione pathway through deletion of both glutaredoxins (Luikenhuis *et al.*, 1998) results in viable cells while inactivation of both pathways simultaneously causes cell death (Muller, 1996), suggesting a functional overlap between the two pathways. However, as shown here and in other studies, deletion of *GSH1* alone is inviable in the absence of exogenous glutathione and this loss of viability is not rescued

by anaerobic conditions suggesting that its role in reducing disulphide bonds is not the sole explanation for why *GSH1* is essential (Spector *et al.*, 2001). Indeed glutathione has been shown to be necessary for the maturation of cytosolic Fe-S clusters (Sipos *et al.*, 2002). Fe-S clusters are essential to sustain viability and are found in a large and functionally diverse range of proteins and enzymes and have been shown to be involved in processes such as electron transport, gene expression (Johnson *et al.*, 2005) and DNA replication (Netz *et al.*, 2012). The inclusion of Fe-S clusters in DNA replication proteins makes it tempting to speculate a connection between the role of glutathione in Fe-S cluster maturation and the activation of Rad53 that is observed in *gsh1*Δ*sic1*Δ cells following glutathione depletion. The thioredoxin pathway has also been shown to play a role in DNA replication through the reduction of ribonucleotide reductase (RNR). RNR plays an important role in DNA replication and repair by controlling the pool of deoxyribonucleoside triphosphates (dNTPs). In fact *trx1*Δ*trx2*Δ cells display a prolonged S-phase that is thought to be due to the impaired reduction of RNR in these cells (Camier *et al.*, 2007). Thus both the glutathione and thioredoxin antioxidant pathways have been linked to DNA replication.

It is not clear whether the G1 phase arrest seen in response to glutathione depletion is due to the redox role of glutathione or its role in Fe-S cluster maturation but we have shown evidence here that a successful arrest of the cell cycle and particularly DNA replication is vital for cells to survive in the absence of exogenous glutathione.

Chapter 6: Final Discussion

Regulation of the cell cycle in response to oxidative stress prevents damage to DNA and other cellular components. Many oxidising agents have been identified that arrest the cell cycle at different stages however, the mechanisms behind the cell cycle arrest observed under these conditions have remained largely unclear. Ubiquitin and the Ubl proteins are a highly conserved family of protein modifiers that play roles in a wide range of processes. The use of catalytic cysteine residues by enzymes in their conjugation and deconjugation pathways raised the possibility that modification of proteins by ubiquitin and Ubls are regulated by redox conditions. Thus the overall aim of this project was to investigate the role of the ubiquitin and the Ubl proteins in the regulation of the cell cycle in response to oxidative stress.

6.1 Summary of Results

Previous studies from our group had demonstrated that in *S. cerevisiae*, the ubiquitin conjugation pathway E1 (Uba1) and E2 (Cdc34) enzymes form a HMW disulphide complex in response to specific oxidising agents (Doris, 2008). Furthermore, this oxidation was found to be coincident with an increase in the stability of the CKI Sic1 and oxidative stress-induced G1 phase cell cycle arrest. However, the specificity of this redox-regulation of Cdc34 had not been explored. Here, we have expanded this initial study to show that Cdc34 is specifically more sensitive to diamide than many other ubiquitin pathway E2 enzymes examined and, moreover, forms a HMW complex independent of both strain background or epitope tag. Hence, Cdc34 is specifically more sensitive to oxidation than other ubiquitin pathway E2 enzymes. Furthermore, the importance of Cdc34 in the response of cells to oxidative stress was demonstrated from our analysis of a DAmP allele of *CDC34* which weakens the expression of *CDC34* and showed resistance to diamide.

Previous studies had demonstrated that diamide-induced G1 phase cell cycle arrest was not completely abolished in a *sic1*Δ mutant suggesting the involvement of other pathways in the cell cycle arrest (Doris, 2008). Here, we have expanded the investigation to examine the role of a number of Cdc34-dependent and independent pathways in oxidative stress-induced G1 phase arrest. Excitingly, our preliminary data shows that *met4*Δ mutants are slower to release from α-factor-induced G1 phase cell

cycle arrest and have a prolonged G1 phase arrest after treatment with diamide. These results, while preliminary, suggest a requirement for Met4 in both normal cell cycle progression and in the cell cycle arrest in response to diamide.

While several Rub1 substrates have been identified in *S. cerevisiae* the apparent lack of phenotypes observed in a *rub1*Δ mutant has meant that very little is known about what the Rub1 pathway does in this model organism. Here, we have uncovered a novel role for the Rub1 pathway in regulating cell cycle progression in response to diamide. In particular, our initial results suggest that the cycling of Rub1 modification on Cdc53 is important for cells to re-enter and traverse the cell cycle following diamide treatment.

Although previous work has demonstrated that *S. cerevisiae* cells arrest their cell cycle in G1 phase in response to glutathione depletion the mechanisms underlying this regulation of the cell cycle were largely unknown. Work described in this thesis has provided insight into the glutathione depletion-induced cell cycle arrest and has revealed that cell cycle arrest in response to glutathione depletion is multifaceted. For example, we have shown that Sic1 is essential for the G1 phase arrest following glutathione depletion and that in the absence of Sic1, cells show a loss of viability. These results demonstrated that G1 phase cell cycle arrest in response to glutathione depletion is required to maintain viability however the reasons behind the loss in viability were unknown. We find that in *gsh1*Δ*sic1*Δ cells, which are unable to arrest the cell cycle following glutathione depletion, the DNA damage checkpoint kinase, Rad53 is activated. In contrast, in *gsh1*Δ cells which do arrest, Rad53 is not phosphorylated. Our results therefore demonstrate that cell cycle arrest in response to glutathione depletion is required to prevent DNA-replication-induced DNA damage.

Similar to observations following diamide treatment, Cdc34 was demonstrated to form a HMW complex in cells depleted of glutathione. However, the amount of Cdc34 involved in the HMW complex was much smaller in response to glutathione depletion compared to that observed after diamide treatment. Excitingly, in contrast to oxidative stress-induced cell cycle arrest, the protein levels of Cdc34 were shown to decrease in response to glutathione depletion suggesting a different regulatory mechanism of Cdc34. Interestingly, the protein levels of Cdc53, which is part of the Cdc34-Ubiquitin conjugation pathway do not drop suggesting that this regulation is specific to Cdc34.

The levels of Ydj1 have previously been shown to play an important role in regulation of G1 phase of the normal cell cycle. Here, we have shown that Ydj1 levels were also decreased in response to glutathione depletion suggesting a link between Ydj1 and cell cycle regulation under these conditions. While the mechanisms underlying the observed decrease in Cdc34 and Ydj1 protein levels remain unknown, *CDC34* and *YDJ1* transcript levels are maintained in glutathione-depleted cells suggesting that Cdc34 and Ydj1 are regulated at the level of translation and/or stability.

Here, we have identified novel roles for the Rub1 pathway in both the diamide and glutathione depletion-induced G1 phase cell cycle arrests in *S. cerevisiae*. Interestingly, the role and regulation of the Rub1 pathway in the cell cycle arrest in response to these two different environments differ. In response to glutathione depletion, the proportion of unmodified Cdc53 increased suggesting that removal of Rub1 was important under these conditions. Indeed, while *gsh1*Δ*rub1*Δ and *gsh1*Δ*csn5*Δ cells both had a higher proportion of budded cells after glutathione depletion than *gsh1*Δ cells, *gsh1*Δ*csn5*Δ cells, which are unable to remove the Rub1 modification of Cdc53, had a higher proportion of budded cells than *rub1*Δ cells. Although *gsh1*Δ*rub1*Δ and *gsh1*Δ*csn5*Δ continued to bud after glutathione depletion, viability was maintained and DNA replication did not occur suggesting that the Rub1 pathway is involved in the inhibition of budding after glutathione depletion. In contrast to glutathione depletion, diamide treatment led to no obvious differences in the Cdc53 Neddylation pattern. However, *rub1*Δ cells displayed a prolonged G1 phase arrest and a G2 phase delay while *csn5*Δ cells were able to traverse the cell cycle similar to wild type cells.

In conclusion this thesis has addressed several aspects of cell cycle regulation in response to oxidative stress and glutathione depletion. We find that similar proteins are involved in the G1 phase cell cycle arrest in response to both diamide and glutathione depletion however, these proteins are regulated differently and by different mechanisms under these two conditions.

6.2 Implications for mammalian cells

Many of the regulatory proteins and pathways that we have identified as playing a role in the regulation of the cell cycle in *S. cerevisiae* in response to oxidative stress, and

glutathione depletion are conserved in mammalian cells. Thus these studies have potential implications for understanding cell cycle regulation in mammalian cells.

6.2.1 p27

$p27^{Kip1}$ is the mammalian functional homologue of Sic1. Similar to Sic1, $p27^{Kip1}$ acts as a CKI and negatively regulates CDK2 which is required for progression from G1 to S phase of the cell cycle. The levels of $p27^{Kip1}$ protein are high in quiescent cells and rapidly decrease upon mitogenic stimulation (Polyak *et al.*, 1994). Interestingly, like Sic1, $p27^{Kip1}$ is degraded by the proteasome following ubiquitination mediated by the mammalian homologue of Cdc34 and SCF^{Skp2}. Nuclear export of $p27^{Kip1}$ precedes its degradation by the 26S proteasome and requires CSN5 (Tomoda *et al.*, 2002). CSN5 contains a nuclear export sequence and is therefore able to act as an adaptor between $p27^{Kip1}$ and the export factor CRM1. In cells expressing a form of CSN5 lacking the nuclear export sequence an increase in nuclear $p27^{Kip1}$ levels and a decrease in cell proliferation was observed, suggesting that CSN5-dependent nuclear export of $p27^{Kip1}$ is necessary for cell cycle progression from G1 phase. Given the important role of $p27^{Kip1}$ as a negative cell cycle regulator it is not surprising that $p27^{Kip1}$ is downregulated in many cancers and is associated with poor prognosis in several different types of cancer (Catzavelos *et al.*, 1997).

$p27^{Kip1}$ transcription is regulated by the forkhead transcription factor Foxo3a (Medema *et al.*, 2000). In response to oxidative stress Foxo3a enters the nucleus and upregulates genes required for antioxidant responses and cell cycle arrest including $p27^{Kip1}$. Deacetylation of Foxo3a in response to oxidative stress enhances the induction of $p27^{Kip1}$ leading to a G1 phase cell cycle arrest (Wang *et al.*, 2007). Interestingly, scavenging of ROS can also lead to a G1 phase arrest in mammalian cells (Ibanez *et al.*, 2011). In particular, cells treated with catalase arrest in G1 phase and this is coincident with an increase in the levels of $p27^{Kip1}$ and a switch from cytoplasmic to nuclear localisation of $p27^{Kip1}$. Although the localisation of Sic1 in response to oxidative stress and/or glutathione depletion was not studied in this thesis, it would be interesting to explore whether the cellular localisation of Sic1 contributes to the regulation of G1 phase progression.

6.2.2 CDC34

The degradation of many cell cycle regulators by the ubiquitin-proteasome pathway has created great interest in targeting this pathway in anti-cancer drug therapy (Harper and King, 2011). Indeed a drug that specifically inhibits the human Cdc34 homologue (CDC34), has been identified as a potential therapeutic (Ceccarelli *et al.*, 2011). Levels of the CDC34 protein have been shown to be 3-4 times higher in T-cell lymphoblastic leukaemia than in normal T cells suggesting a direct role for CDC34 in this cancer (Eliseeva *et al.*, 2001). However, the most significant role of CDC34 in cancer progression is likely to be in its role, along with the CRLs, in the ubiquitination and subsequent degradation of cell cycle regulators such as $p27^{Kip1}$. The E2 and E3 enzymes of the ubiquitin pathway are responsible for target specificity, therefore by blocking E2 enzymes it is hoped that only specific pathways could be blocked. Indeed, specific inhibition of CDC34 was associated with a decrease in cell proliferation and the accumulation of $p27^{Kip1}$ (Ceccarelli *et al.*, 2011).

Unfortunately, mammalian studies were beyond the scope of this thesis. However, given the specificity of the oxidation of Cdc34 observed in *S. cerevisiae* it will be important to investigate the relative sensitivity of CDC34 in mammalian cells. In addition Cdc34 regulates both Sic1 and $p27^{Kip1}$ raising the possibility that cell cycle regulation through Cdc34 oxidation/protein levels is a conserved mechanism for responding to cellular conditions. If found to be conserved, the increased sensitivity of Cdc34 to oxidation may also be a route for specifically targeting Cdc34 as part of drug therapies (see Section 6.3).

6.2.3 DNJ3

As previously discussed, Ydj1 is a critical cell cycle regulator in *S. cerevisiae* and the levels of Ydj1 ensure correct cell size at Start. Indeed, overexpression of Ydj1 in *S. cerevisiae* leads to cells entering the cell cycle at a smaller size (Ferrezuelo *et al.*, 2012). A homologue of Ydj1, DNJ3 has been identified in mammalian cells (Edwards *et al.*, 1997). DNJ3 shares 42% sequence identity with Ydj1 and is able to rescue the temperature sensitivity of *ydj1*Δ *S. cerevisiae* cells. It is of particular interest that DNJ3 was originally identified in a genetic screen performed in *S. cerevisiae* to identify human cDNAs which, when overexpressed, were able to confer resistance to α-factor arrest. These results

suggests that DNJ3 may act to regulate the cell cycle in mammalian cells. Although the roles of DNJ3 have not been widely explored in mammalian cells DNJ3 has also been described as a negative regulator of tumour growth in a specific breast cancer model (Ohlsson *et al.*, 2001). In particular, in a breast cancer model where oestrogen inhibits cell growth, DNJ3 expression, along with one other gene was shown to increase after oestradiol treatment suggesting that DNJ3 had an anti-proliferative effect in these cells. These studies, although apparently contradictory, provide evidence that the human homologue of Ydj1 may act to regulate of cell cycle progression.

6.2.4 Nedd8 pathway

A number of studies have identified important roles for Nedd8 modification in cell cycle regulation in higher eukaryotes and furthermore an increase in Nedd8 conjugation has been observed in oral squamous cell carcinoma (Chairatvit and Ngamkitidechakul, 2007). *CSN5*/*Jab1* and *Uba3* deletion is embryonic lethal in mice (Tateishi *et al.*, 2001; Tomoda *et al.*, 2004) and *CSN5*/*Jab1* heterozygote mice have impaired cell proliferation related to upregulated $p27^{KIP1}$ (Tomoda *et al.*, 2004). The role of Nedd8 conjugation in the degradation of proteins involved in cell cycle progression has led to the generation of a novel anti-cancer drug, MLN4924 that specifically inhibits the Nedd8 E1 preventing movement of Nedd8 through the conjugation pathway. Interestingly, one of the ways that MLN4924 induces cellular apoptosis is through an increase in ROS production and treatment with N-acetylcysteine has been demonstrated to decrease the effectiveness of MLN4924 treatment in cultured cells (Swords *et al.*, 2010).

6.3 Implications for drug therapies

One common change observed in cancer cells is an increase in the production of cellular ROS compared to non-transformed cells. This difference in ROS levels between cancerous and non-cancerous cells is beginning to be explored as a potential therapeutic strategy to specifically target malignant cells. It has been predicted that the high levels of ROS detected in cancer cells renders them more susceptible than normal cells to further increases in cellular ROS thus triggering apoptosis. A number of screens have been performed to identify small molecules that increase ROS as potential low toxicity therapeutic agents (Trachootham *et al.*, 2006; Raj *et al.*, 2011). Piperlongumine and β-phenylethyl isothiocyanate (PEITC) were both identified as compounds that lead to

specific accumulation of ROS in cancer cells. Interestingly, both of these compounds lead to the accumulation of ROS by affecting the glutathione pathway. Piperlongumine interacts directly with glutathione-S transferase inhibiting its activity and also decreases the levels of GSH (Raj *et al.*, 2011). PEITC inhibits Gpx enzyme activity and promotes the export of GSH thus reducing its level within the cell (Trachootham *et al.*, 2006). Other studies have identified compounds that increase cellular ROS but do not lead to cell death unless cells are also treated with sub-lethal doses of the glutathione synthesis inhibitor BSO (Adams *et al.*, 2013). Together these studies demonstrate that approaches to selectively kill cancer cells based on their redox status may be particularly productive by specifically targeting cellular glutathione levels.

6.4 Outstanding questions based on this work

While this study has increased our knowledge of aspects of cell cycle regulation in response to diamide and glutathione depletion there are many outstanding questions that remain to be addressed.

The study described in this thesis has demonstrated that Cdc34 is specifically sensitive to oxidation compared to several other ubiquitin pathway E2s. However, the mechanism that underlies the increased sensitivity of Cdc34 to oxidation remains unknown. It would therefore be interesting to extend these studies using cell fractionation techniques to investigate whether a specific pool of Cdc34, in a particular cellular location undergoes oxidation. Cdc34 is the E2 enzyme for a number of different E3 enzymes and it may be that oxidation of a pool of Cdc34 localised in a particular cellular location may allow for inhibition of the ubiquitination of a set of Cdc34 substrates while maintaining the activity of others. The basis of the increases sensitivity of Cdc34 to oxidation compared to other E2 enzymes could also be investigated by creating strains containing point mutations of Cdc34 and analysing their oxidation. Ubiquitin is a reversible modification and its removal is catalysed by the DUB enzymes. To get a more complete picture of the regulation of the ubiquitin pathway in response to ROS further analyses on the DUB enzymes should be carried out to determine whether these enzymes also display differential sensitivity to oxidation.

This study has demonstrated two novel roles for the Rub1 pathway in cell cycle recovery following diamide treatment and in the prevention of budding in response to

glutathione depletion. Our results suggest that Cdc53 is the key substrate of Rub1 after glutathione depletion or treatment with diamide. However, an in depth analysis of the effect of the above conditions is required to fully understand how the Rub1 conjugation and deconjugation pathways are effected by diamide and glutathione depletion and the role of the Rub1 modifier in the observed G1 phase cell cycle arrests.

Results obtained during this study demonstrated that the cell cycle of *S. cerevisiae* is regulated by different mechanisms in response to diamide, a drug that oxidises glutathione, and following depletion of cellular glutathione. These results suggest that the cell cycle arrest observed in response to glutathione depletion is not simply due to an increase in the cellular ROS caused by the absence of this major antioxidant. Our results strongly suggest that the G1 phase cell cycle arrest observed following depletion of glutathione is required to prevent DNA damage. The essential role of glutathione has been predicted to be in its role in the maturation of Fe-S clusters not as a cellular antioxidant (Sipos *et al.*, 2002). Due to time constraints our study has been unable to establish whether the cell cycle arrest observed in response to glutathione depletion is due to the cells inability to synthesis Fe-S clusters or an indirect mechanism whereby Fe-S clusters are required for fully functioning DNA replication proteins.

6.5 Concluding remarks

While some preliminary investigations presented here and in mammalian cells have shown that the conjugation pathways of certain ubiquitin and Ubl modifiers undergo oxidation there has been no large scale investigation into the oxidation of these pathways. Given the roles of ubiquitin and Ubls in lots of cellular processes and their links with important human diseases such as cancer there is a need to investigate the oxidation of all components of ubiquitin and Ubl conjugation and deconjugation pathways under a range of different oxidative conditions and concentrations. This goes hand in hand with studies to identify all ubiquitin and Ubl targets in cells and the substrates of specific deconjugation pathways. Such studies are essential to build a full picture of what is going on in particular conditions of both oxidising agent and concentration. These studies are vital in understanding the linkage between ROS, ubiquitin/Ubls and their roles in specific processes and may potentially lead to the

identification of novel therapeutic targets that can specifically be targeted by their sensitivity to different ROS.

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Appendix

Work presented as part of this thesis was published as part of the following paper,

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