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Bangor University
School of Biological Sciences

New roles of *S.pombe* Casein Kinase I epsilon (Hhp1) in DNA Replication Stress

Ph.D thesis 2015

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Supervision: Dr Thomas Caspari

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Dedication

To the memory of my young brother Ahmad and I wish, the God places him in his paradise...

To my parents, no one has ever been given more loving and unconditional support than I have been given by you...

To all my family members, borthers and sisters one by one...

I dedicate my work with my wholeheartedly feeling of thanks for all of you,

Salah Adam Mahyous Saeyd

Date: 28/ 07/ 2015

Acknowledgment

I would thank my supervisor, Dr Thomas Caspari for his appreciative supervision, kindness and cheerfulness, which surmounted all the obstacles during my project.

I would express my sincere thanks to my research committee, my internal examiner Professor Peter Golyshin for his valuable and kindly inputs on my research project, and the chairmen, Professor Chris Freeman for his efforts during the step parts of my project.

Many thanks for Dr Rolf Kraehenuehl for sharing his invaluable knowledge on sicence research.

I would appreciate the kindly help, daily presence and encouragement of the D2 and D7 laboratories postdoc colleagues who helpd me to solve some experimental technical problems.

Heartfelt thanks to my PhD colleague's for their daily presence and kind assistance. Many thanks for everyone have provided any assistance for me during this challenging time.

Summary

Schizosaccharomyces pombe Casein kinase 1 (Hhp1) is a dual-specific kinase phosphorylating serine and threonine residues as well as tyrosine side chains. *S. pombe* Hhp1 kinase is homologous to *Saccharomyces cerevisiae* Hrr25, *Drosophila* double-time (dbt), and mammalian Casein kinase 1-epsilon (CKI ϵ). CK1 enzymes regulate the circadian clock, Wnt (wingless) signalling, cell death, cell cycle progression and DNA repair. How one group of enzymes is able to execute so many functions is still poorly understood, especially because of the large number of isoforms and splice variants in mammalian cells. This study uses the fission yeast as a model to research the roles of CK1 in the response to broken DNA replication forks. *S.pombe* Hhp1 is closely related to human CKI ϵ and was previously implicated in DNA repair. A combination of genetic, biochemical and cell biological technologies revealed a novel role of Hhp1 kinase in the regulation of the DNA structure-specific endonuclease Mus81-Eme1. When DNA replication forks break in the presence of the topoisomerase 1 inhibitor camptothecin (CPT), Mus81-Eme1 acts on the broken chromosomes. Hhp1 is predicted to phosphorylate the regulatory subunit Emel jointly with the cell cycle regulator Cdc2 and the DNA damage checkpoint kinase Chk1. Genetic tests showed that all three kinases act in the same CPT-response pathway. The recreation of CK1 mutations of the circadian clock (*hhp1.R180C*, *hhp1.P49S*, and *hhp1.M82I*) and of CK1 mutations found in human breast cancers (*hhp1.L51Q*) in *S.pombe* Hhp1 revealed that different kinase activity levels are crucial for the regulation of DNA repair and cell cycle progression. While a limited drop in Hhp1 kinase activity, for example by widening its ATP binding site (methionine-84 to glycine), considerably delays the exit from a G2 arrest in the presence of damaged replication forks (CPT), it does not significantly impair DNA repair and cell survival. Only a dramatic drop in kinase activity, for example by replacing the active site residue lysine-40 with an arginine side chain, affects DNA repair and cell survival. How different kinase activity levels can have distinct biological outputs is discussed in the context of how CK1 recognises primed and acidic phosphorylation motifs.

Taken together, the outcomes of this work imply that the complex phenotypes of CK1 mutations in the circadian clock or in cancer cells are caused by mutations which impact differently on its kinases activity.

Abbreviations:

2D	Two dimensional
53BP1	Tumor suppressor p53-binding protein 1
5-FOA	5-Fluoroorotic acid
Aph	Aphidicolin
APS	Ammonium Persulfate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BamHI	Endonuclease <i>Bacillus</i> <i>amyloliquefaciens H</i>
BAX	Bcl-2-associated X protein
BER	Base Excision Repair
BGH	Bovine growth hormone (BGH) for polyadenylation signal
bp	base pair
BRCA2	breast cancer 2
BSA	Bovine Serum Albumin
CCDS	Consensus Coding Sequence
Cdc2	Cell division cycle 2
CDC25C	Cell division cycle 25C
CDK	Cyclin dependent kinases
cDNA	Complementary DNA
Cds1	CDP-diacylglycerol synthase 1
ChK1	Checkpoint kinase 1
ChK1S345ph	Checkpoint kinase 1-phosph Serin345
CMV	Cytomegalovirus
CPT	Camptothecin
Crb2	Crumbs homolog 2
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DTT	DL-Dithiothreitol
DDT	DNA Damage Tolerance
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSBs	Double strand breaks
dsDNA	double stranded Deoxyribonucleic acid
dsRNA	double stranded RNA
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene glycol-bis(2-aminoethylether)- N,N,N',N'-tetraacetic acid
EMM	Edinburg Minimal Media
Exo1	Exonuclease 1

FANCD2	Fanconi Anaemia Complementation group D2
GAPDH	Glyceralaldehydephosphate-dehydrogenase
GFP	Green Fluorescent Protein
GGR	Global Genome Repair
H2AX	H2A histone family, member X
H2O2	Hydrogen peroxide
H3S10ph	Histon H3 phospho- serin10
HCl	Hydrochloric acid
HR	Homologous Recombination
HU	hydroxyurea
IPTG	Isopropylthio-β-galactoside
KAc	Potassium acetate
kDa	Kilo Dalton
LBA	Luria Bertani Agar
LiOAc	Lithium acetate
LiOAc/TE	Lithium acetate/ Tris-EDTA
MAPK	Mitogen-Activated Protein Kinase
MDC1	Mediator of DNA damage checkpoint protein 1
ME	Malt Extract
MEA	Malt Extract Agar
MMC	mitomycin C
MMR	Mismatch Repair
MMS	Methyl methane sulfonate
mRNA	Messenger ribonucleic acid
NBS1	Nibrin
NCBI	National Center for Biotechnology Information database
NEB	New England Biolabs
NER	Nucleotide Excision Repair
NH4OAc	Ammonium acetate
NHEJ	Non Homologous End Joining
nu	nucleotides
ORF	Open Reading Frame
P21WAF1/CIP1	Cyclin-dependent kinase inhibitor 1
p38 MAPK	p38 mitogen-activated protein kinases
p53	Tumor suppressor p53
PBS	phosphate- buffered saline
PCR	Polymerase Chain Reaction
PEG/LiOAc/TE	Polyethylene glycol standard/ Lithium acetate/ Tris-EDTA
PEG4000	Polyethylene glycol standard
PEM	PIPES/ EGTA/ MgCl2
PEMS	PIPES/ EGTA/ MgCl2/Sorbitol
PER2	Period circadian protein homolog 2
PFA	Paraformaldehyde

PIPES	piperazine-N,N'-bis(ethanesulfonic acid)
RASV	Representative Alternative Splice Variant
Rb	Retinoblastoma protein
RFP	Red Fluorescent Protein
RMCE	recombinase-mediated cassette exchange
RNA	Ribonucleic acid
RNA Pol III	RNA polymerase III
ROS	Reactive oxygen species
rpm	Revolutions per minute
Rqh1	RecQ type DNA helicase I
S.pombe	Schizosaccharomyces pombe
SCE	Sister chromatid exchange
SDS	Sodium dodecyl sulfate
SDSA	Synthesis-dependent strand annealing
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
ssDNA	Singl stranded Deoxyribonucleic acid
TAE	Tris-Acetate-EDTA
TCA	Trichloroacetic acid
TCR	Transcription-Coupled Repair
TE	Tris-EDTA
Tel1	Telomere I
TEMED	Tetramethylethylenediamine
Tet	Tetracyclin
TLS	Translesion synthesis
UV	Ultraviolet
UVB	Ultraviolet B
V	Volt
w/v	Weight/ Volume
Wt	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
YEA	Yeast Extract Agar
YEL	Yeast Extract Liquid

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Chapter 1: Introduction and Project Aims

1. Introduction

This project aims to use the model organism *Schizosaccharomyces pombe* to understand how mutations in *CKI ϵ* promote cancer formation, neurological disorder and cell cycle abnormalities. Mutations of conserved amino acids, which were found in human and fly *ckI*, will be created in *S.pombe* *Hhp1*, and their effects on cell cycle regulation and the DNA damage response will be studied.

1.1. Background

CkI is a highly conserved serine/threonine kinase found in all eukaryotic cells (4). CKI enzymes have important roles in regulation of several cellular processes such as, cell division and cell differentiation in mammals (1, 384, 560). CKI activities may be affected via various factors like extracellular stimulants, different subcellular localisation, distinct interaction partners and autophosphorylation (1). CKI play an important role in the circadian clock in mammals (e.g. mouse and Syrian hamster) (1), also in *Drosophila* and yeast (e.g. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) (1, 5). Interestingly, there is a suggested link between the cell division cycle and the circadian clock in cancer growth (6), and cancer and neurodegenerative diseases may result from mutations in *CKI* genes (1, 384, 560).

1.1.2. The CKI isoforms

The isoforms of CKI kinases are well characterized in different species including the fission yeast *Schizosaccharomyces pombe* (10, 13), the budding yeast *Saccharomyces cerevisiae* (9, 13), the fruitfly *Drosophila* (1) and mammals (1). There are seven mammalian CKI isoforms: CKI α , CKI β , CKI γ 1, CKI γ 2, CKI γ 3, CKI δ and CKI ϵ . Some of them have splice variants. For example: CKI ϵ has three splice products CKI ϵ 1, CKI ϵ 2, CKI ϵ 3, and CKI α has also three splice variants CKI α L, CKI α S, and CKI α LS (1, 8, 14). The different CKI α isoforms have different activities, different subcellular localisations and biochemical properties (1, 384). *Schizosaccharomyces pombe* cell contains five *CKI* genes: *Hhp1*, *Hhp2*, *CKI-1*, *CKI-2* and *CKI-3* (90). *CKI* (1-3) are linked to vesicular trafficking and cytokinesis, whereas *Hhp1* and *Hhp2* are involved in cell cycle progression and DNA repair (130). *Saccharomyces cerevisiae* has four *CKI* genes: *Hrr25*,

CKI1, *CKI-2* and *CKI-3* (12, 15). Mammalian *CKI* isoforms function in Wnt signalling, circadian rhythms, DNA repair and transcription (5, 7, 11, 384).

1.1.3. Substrates and Key Regulatory Proteins

CKI substrates are characterized by the canonical consensus sequence S/T (P)-X1-X2-S/T. Unlike most other kinases, CKI enzymes are constitutively active and are normally regulated by a priming kinase which phosphorylates the N-terminal serine or threonine residue (Figure: 1.1.4.1; Figure: 1.1.4.2; Figure: 1.1.4.3; Figure: 1.1.4.4) within the consensus sequence (125, 126). This requirement of a priming phosphorylation by another kinase restricts CKI activity to a hierarchical phosphorylation cascade (1). Marin *et al.* (128) and Graves *et al.* (123) showed that acidic amino acids can substitute for the N-terminal phosphorylation event (3). Notably, non-canonical motifs like the sequence SLS (serine-leucine-serine) could be identified by CKI when accompanied with a complex of acidic amino acid residues on the C-terminal side of the phosphoacceptor site. Phosphorylation of non-canonical motifs in the human proteins NF-AT and β -catenin by CKI, is between 15 and 25 fold less efficient than motifs that are primed by a phospho-amino acid (21). CKI phosphorylate substrates may not depend on the consensus sequence and may phosphorylate substrates dependent on the tertiary structure of the substrate (1, 116). While CKI enzymes normally phosphorylate only serine and threonine residues, *Xenopus* CKI α , *S.pombe* Hhp1 and *S.cerevisiae* Hrr25 do also phosphorylate tyrosine residues *in vitro* and *in vivo* (12). Table 1.1.3 gives an overview of known *CKI* substrates (384).

Functional groups	CK1 substrates
Cytoskeleton-associated proteins, adhesion factors, and scaffolding proteins	Myosin, troponin, ankyrin, spektrin 3, filamin, vinculin, neurofilamentary proteins, dynein, α - β -tubulin, microtubule-associated protein (MAP)1A, MAP 4, stathmin, tau, keratin17, desmolein, annexin II, centaurin- α (p42IP4), neural cell-adhesion molecule (NCAM), E-cadherin, RhoB, myelin basic protein (MBP), kinesin-like protein 10A (KLP10A), lectin L-29, galectin-3, end binding 1 (EB1), Sid4, connexin-43, metastasis suppressor 1 (MTSS1), and Hsp79 and Hsp90
Receptors	β -Subunit of the insulin-receptor, TNF α -receptor, muscarin M3-receptor, Ste2p (α -factor-receptor), Ste3p (α -factor-receptor), platelet derived growth factor (PDGF) receptor, retinoid X receptor (RXR), low density lipoprotein-related receptor protein (LRP) 6, type I interferon receptor (IFNAR1), estrogen receptor α (ER α), amplified in breast cancer 1 (AIB1), calmodulin (CaM), and Ror2
Membrane transporters	Erythrocytes anion transporter, uracil permease (<i>Saccharomyces cerevisiae</i>), translocase of the outer mitochondrial membrane 22 (Tom22), and α -T663-hENaC
DNA-/RNA-associated proteins	Non-histone chromatin proteins, RNA polymerase I and II, topoisomerase II α , Star-poly(A) polymerase (Star-PAP), Rec8, DNA methyl-transferase (Dnmt1), TAR DNA-binding protein of 43 kDa (TDP-43), DEAD-box RNA helicase DDX3, Ubiquitin-like, with PHD, and RING finger domains 1 (UHRF1)
Ribosome-related proteins	15 kDa, 20 kDa, 35 kDa, L4, L8, L13, ribosomal protein S6 (rpS6), and ENP1/BYSL and LTV1
Transcription and splice factors	p53, cyclic AMP responsive element modulator (CREM), Swi6, nuclear factor of activated T-cells (NFAT), serine/arginine-rich (SR) proteins, T-cell factor (Tcf)3, brain and muscle Arnt-like protein (BMAL) 1, cryptochrome 1 (CRY), β -catenin, armadillo, SMAD 1–3 and 5, osmotic response element-binding protein (OREBP), cubitus interruptus (Ci), forkhead box G1 (FoxG1), SNAIL, tafazzin (TAZ), yes-associated protein (YAP), proliferator-activated receptor γ co-activator 1 α (PGC-1 α), <i>Drosophila</i> Myc (d-Myc), cyclic AMP response element-binding protein (CREB), Sre1N (yeast sterol regulatory element-binding protein homolog), and NF κ B (nuclear factor “kappa-light-chain-enhancer” of activated B-cells) subunit p65
Translation factors	Initiation factors (IF) 4B, 4E
Viral proteins	Simian virus 40 large T-antigen (SV40 T-Ag), hepatitis C virus non-structural 5A (NS5A), human cytomegalovirus ppUL44, Pox semilantent hordeivirus triple gene block 1 (TGB1), Kaposi sarcoma-associated herpesvirus latency associated nuclear antigen (LANA), and yellow fever virus methyl-transferase
Kinases and phosphatases	Cyclin-dependent kinase 5 (Cdk5), protein kinase C (PKC), protein kinase D2 (PKD2), cell division cycle 25 (Cdc25), and PH domain and leucine rich repeat protein phosphatase 1 (PHLPP1)
Inhibitors and modulators	Inhibitor 2 of PPA 1, dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32), disheveled, mammalian period circadian protein (mPER), adenomatous polyposis coli (APC), Bid, protein kinase C potentiated myosin phosphatase inhibitor of 17 kDa (CPI-17), nm23-H1, 14-3-3 proteins, MDM2, MDMX, FREQUENCY (FRQ), WHITE COLLAR-1 (WC-1), CARD containing MAGUK protein (CARMA1)/caspase recruitment domain (CARD11), SLR1, endogenous meiotic inhibitor 2 (Emi2), Chk1-activating domain (CKAD) of claspin, PER2, protein S, Rap guanine nucleotide exchange factor 2 (RAPGEF2), and Sprouty2 (SPRY2)
Enzymes (miscellaneous)	Acetyl-CoA carboxylase, glycogen synthase, yeast endoprotease Ssy5, and neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4)
Vesicle- and trafficking-associated proteins	SV2, β 3A- and β 3B-subunit of the AP-3 complex, snapin, and ceramide transfer protein (CERT)
Receptor-associated proteins	Fas-associated death domain (FADD), receptor interacting protein 1 (RIP1)
Factors of neuro-degenerative diseases	Presenilin-2, tau, β -secretase, parkin, and α -synuclein
Metastatic tumor antigens	Metastatic tumor antigen 1, short form (MTA1s)

Table 1.1.3: Known CKI substrates (384).

1.1.4. CKI Expression and Activity Regulation

Active CKI isoforms are monomeric kinases and were identified in many different tissues and organisms (97). Activation or modulation of CKI isoforms is influenced by several factors, such as the level of insulin in cells, treatment with γ -irradiation and viral transformation (74, 96). The activity of CKI α declines in nervous cells and red blood cells (erythrocytes) when phosphatidylinositol-4, 5-biphosphate (PIP2) concentrations increase in the membrane (108, 109, 110). Kinase-substrate interactions may be influenced by the subcellular localisation and compartmentalisation (1). In yeast, activation of CKI can be regulated by their subcellular position which brings the kinase and substrate together. For example, *S.cerevisiae* CKI localises to the plasma membrane to which the kinase binds via a lipid anchor (9, 10). Vancura *et al.* (10) showed that *S.cerevisiae* deletion mutant in the nuclear kinase *hrr25* is complemented by the membrane-associated CKI isoform when the isoprenyl-lipid anchor to the plasma membrane is removed. Conversely, deletion mutants of the membrane bound CKI isoform are complemented by substitution of the nuclear localisation signal (NLS) of the Hrr25 kinase by an isoprenylation site (10, 113).

10	20	30	40	50	60
MALDLRIGNK	YRIGRKIGSG	SFGDIYLGTN	VVSGEEVAIK	ESTRAKHPC	LEYEYRVYRI
70	80	90	100	110	120
LSGGVGIPFV	RWEGVECDYN	AMVMDLLGPS	LEDLFNFCNR	KFSLKTVLLD	ADQLISRIEF
130	140	150	160	170	180
IHSKSEFLHRD	IKPDNFLMGI	GKRGNQVNII	DFGLAKKYRD	HKTHLHIBYR	ENKNLTGTAR
190	200	210	220	230	240
YASINTHLGI	EQSRRDDLES	LGYVLVYFCR	GSLPWQGLKA	TKKQKYEKI	MEKKISTPTE
250	260	270	280	290	300
VLCRGFPQEF	SIYLNTRSL	RFDDKPDYAY	LRKLFRLDFC	RQSYEFDYMF	DWTLKRKTQQ
310	320	330	340	350	360
DQQHQQLQQ	QLSATPQAIN	PPPERSSFRN	YQKQNFDEKG	GDINTTVPVI	NDPSATGAQY
INRPN					

Figure: 1.1.4.1: Amino acid sequence and domains of Hhp1 in *S.pombe* (source: <http://www.pombase.org/spombe/result/SPBC3H7.15.>) The mutated amino acids are highlighted. Domains: kinase domain (11aa-279aa); ATP binding site (17aa-40aa); HIPYR = microtubule binding domain; TTKQKY = nuclear localisation domain.

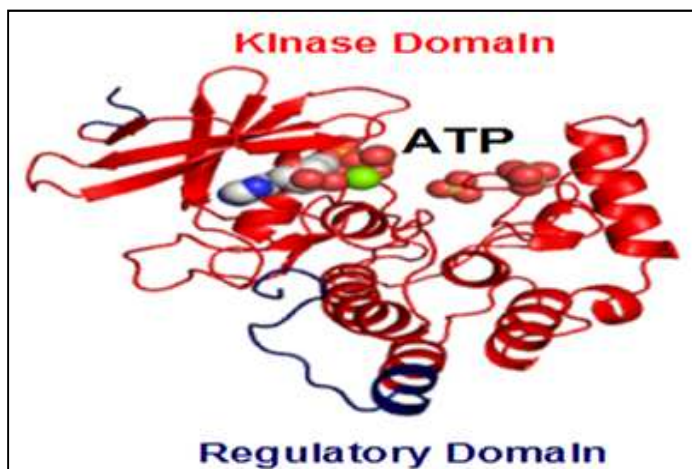


Figure: 1.1.4.2: Crystal structure of *S.pombe* Cki1 (CK1) kinase. The ATP molecule in the active site and one manganese and one sulfate ion are shown. The kinase domain (11aa-279aa) is shown in red, the C-terminal regulatory domain is shown in blue. Please note that the C-terminal 148aa are missing from the structure. (PDB ID: 1CSN, Source: Polyview3D).

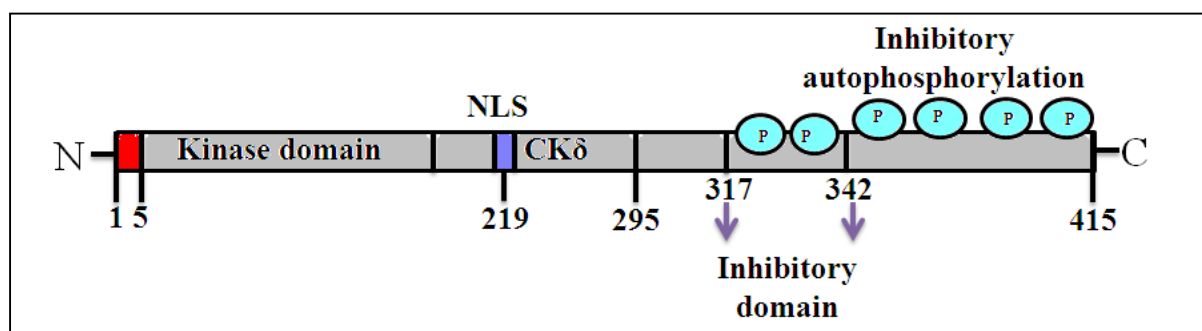


Figure: 1.1.4.3: Structure of human CKI δ (1). An N-terminal domain of 5-amino acid is followed by the kinase domain (5aa-295aa). The C-terminal domain starts from amino acid 317 to 415. NLS = nuclear localisation domain.

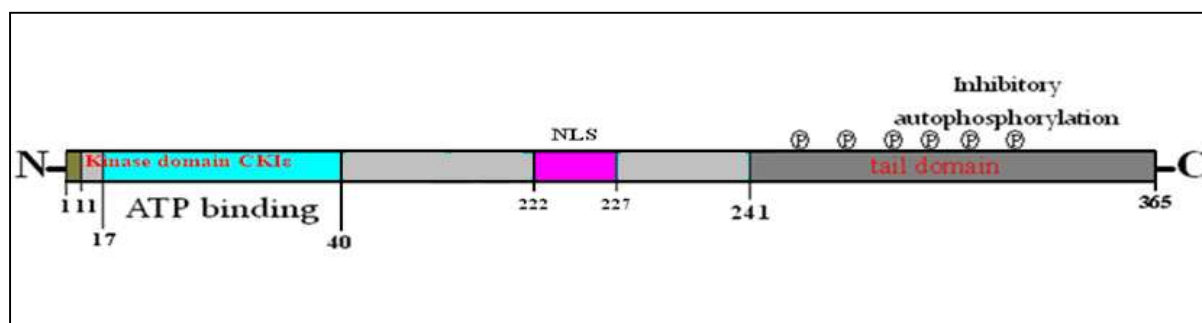


Figure: 1.1.4.4: Structure of human CKI ϵ . The N-terminal domain has 11 amino acids and is followed by the kinase domain (11aa-241aa) with the ATP binding domain located between 17aa and 40aa. The C-terminal domain starts from amino acid 241 to 365. NLS = nuclear localisation domain.

Another important regulatory mechanism is the inhibitory autophosphorylation of CKI isoforms that affects their activity. The subcellular localisation of human CKI δ relies on its kinase activity (120) and its inhibition by autophosphorylation (Figure: 1.1.4.3) in its C-terminal domain (114, 117, 123, 124) and also in its kinase domain (115). The C-terminal domains of human Cki δ , ϵ and γ 3 are large and may work as pseudosubstrates inhibiting the kinase activity (117, 122, 123,

124). Interestingly, Carmel *et al.* (114) and Cegielska *et al.* (116) observed an increase in kinase activity upon truncation of the C-terminal domain which supports the idea that the C-terminal domain blocks the activity of the N-terminal kinase domain. Gietzen and Virshup (115) stated that *in vivo* dephosphorylation of the autophosphorylation sites may happen by cellular phosphatases resulting in the activation of CK1ε. Swiatek *et al.* (119) managed to enhance the activity of human CKIε in the Wnt-signaling pathway by reducing CKIε autophosphorylation, which is normally observed in neostriatal neurons when the metabotropic glutamate receptors are activated. In this case, dephosphorylation of CK1ε by phosphatase PP2B activates the enzyme (20). As shown in Figure 1.1.4.4, *S.pombe* Hhp1 is most closely related to human CK1ε/δ, as CKIα carries an insertion of 28 amino acids in its kinase domain. While the N-terminal kinase domains are highly related (Hhp1: 11aa-279aa; CK1ε: 11aa-241aa; CKIδ: 5aa-295aa) (Figure 1.1.4.3; Figure 1.1.4.34; Figure 1.1.4.5), the homology is much reduced in the C-terminal regulatory domains.

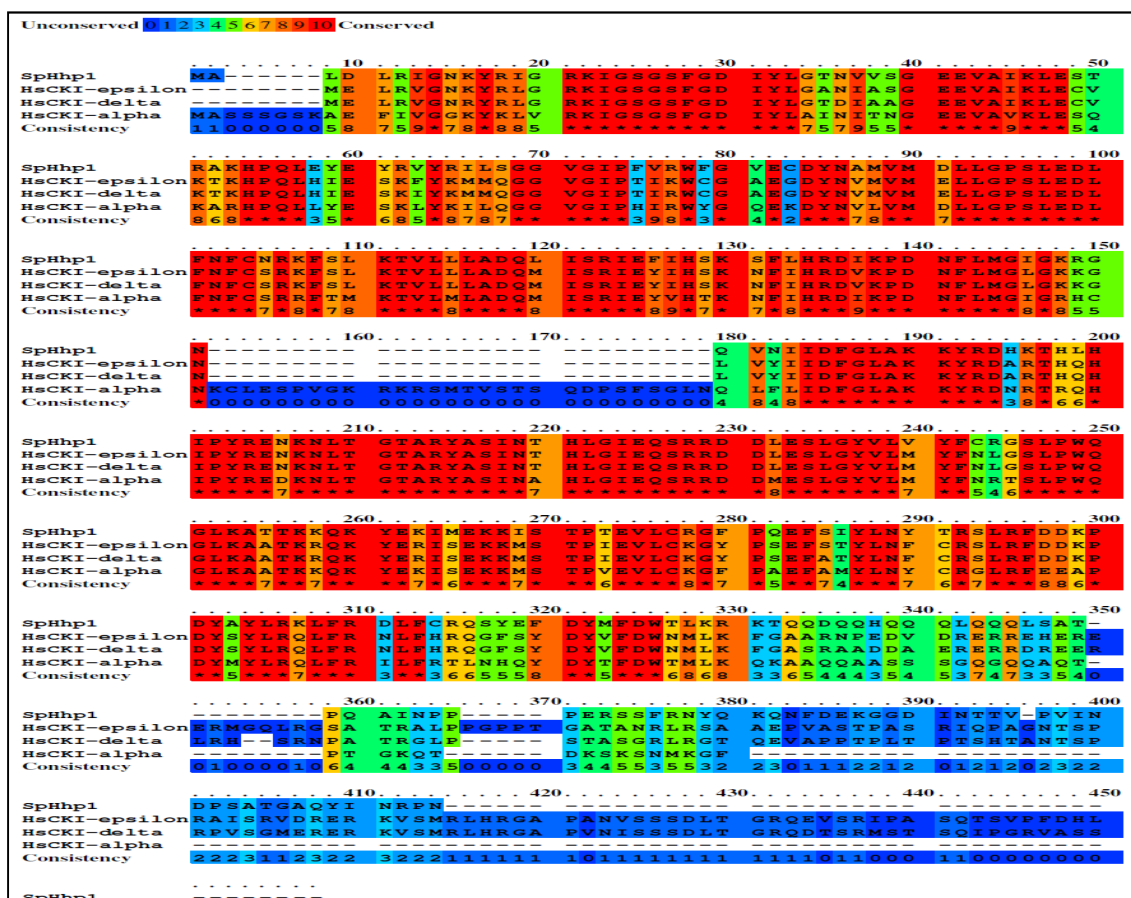


Figure 1.1.4.5: Alignment of *S.pombe* Hhp1 with human CKI-alpha, delta and epsilon. (source: PRALINE sequence alignment tool; available at: <http://zeus.few.vu.nl/jobs/0fa74434fdb50c270e6fdec103c18c5c/>; accessed 13 October 2015). Human CKIε: NP_689407.1, CKIδ: P48730.2, CKIα: NP_001020276.1, and SPHhp1: CAA20311.1.

1.1.5. *S. pombe* Hhp1 and *S. cerevisiae* Hrr25

The gene products of *hhp1* and *hhp2* encode the fission yeast *Schizosaccharomyces pombe* homologs of CKI ϵ and CKI δ , respectively. Both proteins are closely related to Hrr25 kinase in the budding yeast *Saccharomyces cerevisiae* (15). Like the human CK1 enzymes, all yeast CK1s are protein serine/threonine kinases, but unlike the human kinases, they also phosphorylate tyrosine residues (12). Moreover, both Hhp1 and Hrr25 kinases are involved in the response to genotoxic damage (102), and *S. pombe* Hhp1 is suggested to have a role in DNA repair especially when chromosomes break upon ionising radiation or when DNA is methylated (15, 91, 92, 94, 96). The biological roles of Hhp1 in DNA repair and cell cycle regulation are still enigmatic. The role of Hhp2 is currently unknown, but genetic studies indicate that the kinase acts as a back-up enzyme for Hhp1. While deletion of the *hhp2* gene on its own has no phenotype, the *hhp1.hhp2* double mutant is more DNA damage sensitive than the *hhp1* single deletion (15).

The alignment shown in Figure 1.1.5.1 reveals the greatest degree of divergence between *S.pombe* Hhp1 and Hhp2 in the C-terminal regulatory domain. Interestingly, some of the highly conserved amino acids like D24 in Hhp1, which is present in human CKI- α , CKI- δ and CKI- ϵ (Figure 1.1.4.5) is replaced by an uncharged glutamine (Q25) in Hhp2. This implies that there are differences in the kinase activity of both Hhp enzymes in fission yeast.

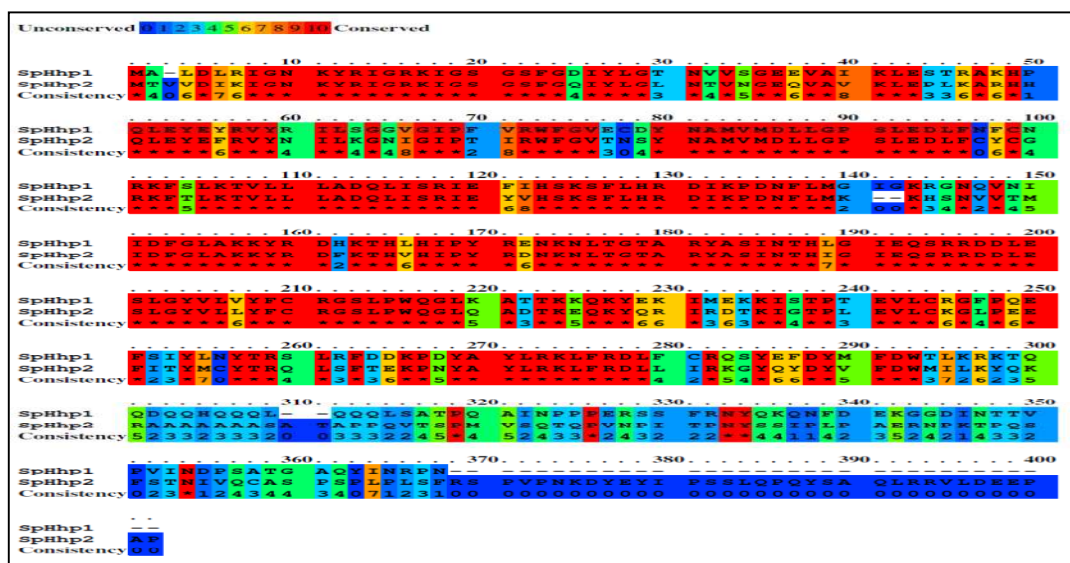


Figure: 1.1.5.1: Alignment of *S.pombe* Hhp1 and Hhp2. (source: PRALINE sequence alignment tool; available at: <http://zeus.few.vu.nl/jobs/f49a99ed96971c208165d210b3c5ca0b/>; accessed 13 October 2015). SPHhp1: CAA20311.1, and SPHhp2: CAB16883.1.

S.cerevisiae Hrr25 plays a second role in the response to DNA damage by up-regulating gene expression by phosphorylating the transcription factor Swi6 (Figure: 1.1.5.2). Swi6 is known as a cell cycle regulated transcription factor, which works to activate gene expression in the G1 phase of cell cycle (9). It serves as a transcription factor by communicating with different DNA binding partners. Swi6 forms the SBF complex when interacting with Swi4. This complex is activated in G1 by the Cdc28 (CDK1) cell cycle regulator to promote gene expression after its binding to *MCB* DNA motives in gene promoters (137). *MCB* elements were discovered in many promoters of cell cycle regulated genes like *Cdc9* and *Pol1* and *Rnr* genes, and also in genes required for DNA repair *Cdc9*, *Pol1*, *Rnr1*, *RAD51* and *Rad54*.

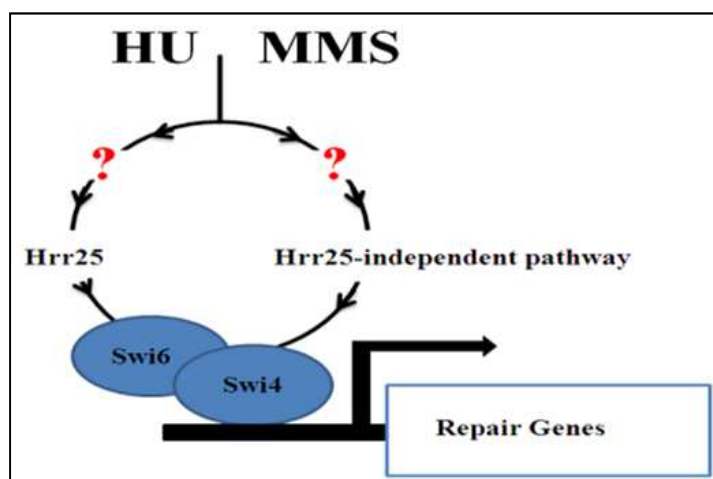


Figure: 1.1.5.2: DNA repair pathway. Role of Hrr25 kinase and Swi4-Swi6 (SBF) in transcriptional response to DNA damage. Hrr25 phosphorylate Swi6 in the response to DNA damage. A Hrr25-independent pathway could act through Swi4. Both pathways active SBF to promote transcription of repair genes (9).

CKI kinases are also involved in microtubule related processes like mitosis and vesicle transport. For example, CKI activity in vertebrates regulates the position of the mitotic spindle and localises to the centrosome (84). *S.cerevisiae* Hrr25 plays important roles in meiosis regulating monopolar spindle attachment and sister chromatid separation (84, 138, 139). Location of CKI to the centrosome and spindle microtubule may be authorized by phosphorylation catalyzed by the cell cycle regulator Cdc2 (CDK1) kinase activity of these components together may initiate a sequential or hierarchical cascade of phosphorylation events required for spindle assembly and chromosomal separation.

1.1.6. CKI kinase as Autonomous Timers

1.1.6.1. The Circadian Clock

"Circadian" has a Latin word. It is built of two parts: *circa* means around, and *diem* means day (31). The human circadian clock operates in a period of approximately 24 hours (34, 58, 61). Circadian rhythms are observed in plants, animals, fungi and bacteria. The circadian clock is triggered by light which reaches the brain via the optic nerve. Amongst the main outputs are the cell cycle, activity patterns (sleep/wake) and DNA repair. Deregulation of the clock affects the cell division cycle in diseases like cancer, delayed sleep phase syndrome (DSPS) and familial advanced sleep phase syndrome (FASPS) (34, 68, 72). The circadian clock contains three components: a signal transduction pathway integrating external signals with the rhythm time; a central oscillator that produces the circadian signal; and a signal transduction pathway that defines the outputs in several biology processes (1). Experiments conducted with *Drosophila*, *Neurospora*, *Synechococcus* and mice (Table: 1.1.6.1.1) led to the discovery of many clock genes: *period* (*per*), *timeless* (*tim*), *doubletime* (*dbt*), *clock* (*Clk/Jrk*) and *cycle* (*cyc*) in *Drosophila*; *frequency* (*frq*), *White Collar 1* (*wc-1*) and *White Collar 2* (*wc-2*) in *Neurospora*; *kaiA*, *kaiB* and *kaiC* in *Synechococcus* and *per1*, *per2*, *per3*, *tim*, *Clock* and *bmal1/mop3* in Mouse (32). Human genes include the *period genes* (*hPer1*), *hPer2*, *hPer3*, and *hDec1* (33).

These clock genes produce oscillators that lead to a regulated transcription loop which is regulated by positive and negative post-translational modifications. The main circadian transcription factors in mammals are CLOCK and BMAL1, which activate the transcription factors Period (*PER1-3*) and Cryptochrome (*CRY1-2*). Period and Cryptochrome act as inhibitors of BMAL-1 and Clock (Figure: 1.1.6.1.2) (1). BMAL-1 and Clock form a hetero-dimeric transcription factor that binds to *E-box* sequences in the promoter regions of *PER1-3*, *CRY1-2* and *PEV-ERBa* (1, 32).

Organisms	Clock genes names				
<i>Drosophila</i>	<i>per</i>	<i>tim</i>	<i>dbt</i>	<i>clk/jrk</i>	<i>cyc</i>
<i>Neurospora</i>	<i>frq</i>	<i>wc-1</i>	<i>wc-2</i>		
<i>Synechococcus</i>	<i>kaiA</i>	<i>kaiB</i>	<i>kaiC</i>		
Mice	<i>per1</i>	<i>per2</i>	<i>per3</i>	<i>clock</i>	<i>bmal1/mop3</i>
Human	<i>hPer1</i>	<i>hPer2</i>	<i>hPer3</i>	<i>hDec1</i>	

Table: 1.1.6.1.1: Clock genes names (1, 32, 33).

This interdependent loop results in the peak of BMAL1-Clock activity during the day and Per-Cry activity during the night. This cycle would stop after one round if it wouldn't be CKI δ/ϵ kinase which associates with the Period proteins to facilitate the inactivation of Clock and BMAL-1 by giving the Period proteins access to the nucleus as well as by initiating the degradation of the Period proteins (Figure: 1.1.6.1.2). The PER-CRY-CKI δ/ϵ complex translocates into the nucleus to block BMAL-1 and Clock. How one kinase can have such diverse outputs is yet unknown, but may be linked with differences in its activity levels or interaction partners.

In mammals, the transcription factor Clock interacts with the histone acetylase p300 which is required for the activation of *PER* and *CRY* transcription (54). Modification of PER and CRY by CKI δ/ϵ triggers their ubiquitination and destruction by the proteasome (55, 60). This phosphorylation was discovered in *Drosophila* (1), where the Doubletime protein (DBT) which is very similar to CKI ϵ in mammals phosphorylates DmPER proteins (62). CKI δ/ϵ phosphorylates also PER1-3 in mammals (55, 56).

In mammals, CKI δ/ϵ phosphorylation causes a conformation change in PER masking a nuclear localisation signal (58). CRY proteins bind to PER and protect the protein from phosphorylation so that the complexes of CRY-PER-CKI δ/ϵ can enter the nucleus (55, 56). This complex inhibits transcription of the genes *BMAL1* and *Clock* (Figure: 1.1.6.1.2). In addition, CKI δ/ϵ effects the clock by phosphorylating BMAL1 and CRY directly (1). However, PER2 may be able to stimulate transcription of *BMAL1* as well and causing another positive feedback loop (71). Since CKI δ/ϵ is a clock protein, its mutations could be linked with diseases. Mutations in *dbt* in *Drosophila* result in changes in both, the phosphorylation and stability of the Per proteins as reviewed by Knippschild *et al.* (1) and confirmed by Price *et al.* (64). A good example of a mutation in *CKI* is the *tau* mutation discovered in CKI ϵ of the Syria hamster. This mutation (R178C) removes a positively charged arginine residue that makes contact with the negatively charged ATP in the active site. As a consequence of this, CKI ϵ autophosphorylation changes reducing its kinase activity. This results in a shorter circadian rhythm (69). The mutated CKI ϵ (R178C) binds to PER and CRY but the kinase function is not high enough to phosphorylate PER, and PER becomes more stable and the circadian rhythm in hamster becomes shorter than normal reducing it to 22 hours instead of 24 hours (1, 51). The equivalent residue in SpHhp1 is R180 (Figure 1.1.6.1.1).

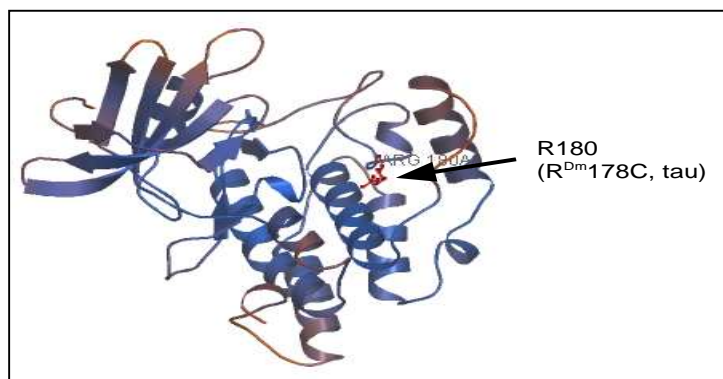


Figure: 1.1.6.1.1: Model of *S.pombe* Hhp1. The model is based on the crystal structure of *S.pombe* Cki1 (PDB ID: [1CSN](http://www.rcsb.org/pdb/explore/show?id=1CSN)). The model was produced with the Swiss Model tool (<http://swissmodel.expasy.org/interactive>; accessed 03 April 2015). The protein sequence identity is 57.39% and the covered sequence ranges from 5aa-294aa. The position of the arginine residue R180 in SpHhp1 is shown. This residue is equivalent to the tau mutation R178C in the Syrian hamster CKI protein.

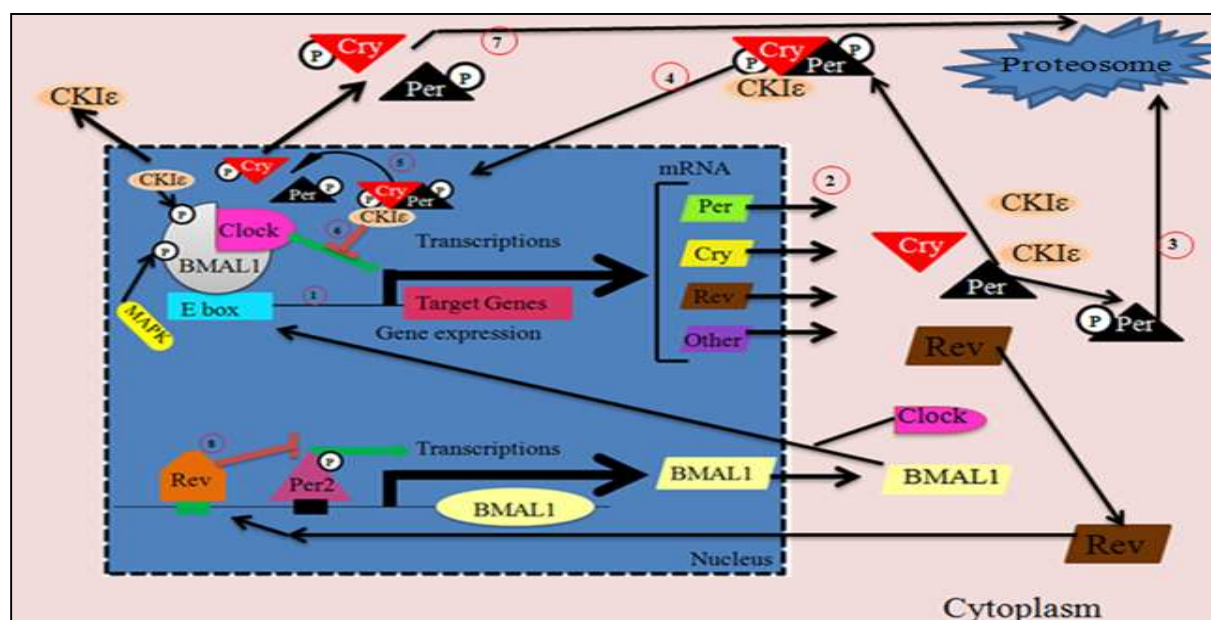


Figure: 1.1.6.1.2: A model of the clock in *Drosophila*: CKIs regulates the circadian clock. BMAL1-CLOCK binds to the *E-box* sequence upstream of the genes *PER1-3*, *CRY1* and *CRY2*, and *REV-ERBa*. In the cytoplasm CKI phosphorylate *PER* and forms the CK1-Per-Cry complex which translate to the nucleus to block the heterodimeric transcription factor *CLOCK-BMAL1* (1).

Another interesting mutation *CKI ϵ .S408N* is linked with familiar advanced sleep phase syndrome (FASPS). It is characterized by a long circadian rhythm caused by a polymorphism of in the *CKI ϵ* gene that removes the auto-phosphorylation site serine 408 by mutating it to asparagine (S408N). This mutation increases the activity of *CKI ϵ* (57). Another mutation linked to FASPS in mice is a permutation of serine-662 to glycine in the *Per2* protein which interferes with the binding of *mPer2* to *mCKI ϵ* decreasing *CKI ϵ* mediated phosphorylation of *mPER* (68). The polymorphism of V647G in human *Per3* also influences *CKI δ/ϵ* binding and causes delayed sleep phase syndrome (DSPS) (72).

1.1.6.2. Circadian rhythm and the cell cycle

The circadian clock is involved in cell cycle regulation in humans and mice (43). One major target of the circadian clock is Wee1 kinase, a negative regulator of cell cycle progression (Figure: 1.1.6.2.1). Wee1 regulation is known as Serial Coupling, also called the two-process model. In this, expression of Wee1 kinase (cell cycle gene) and of the transcription factor c-Myc (cell growth regulation gene) is regulated by the clock in a circadian manner to modulate onset of mitosis and DNA replication, respectively. In other words, the idea here is that proteins in one cycle regulate gene expression in another cycle. By controlling expression of Wee1 and c-Myc (Figure: 1.1.6.2.1), the clock changes the threshold for mitotic onset (Wee1) and DNA replication (c-myc) (101).

A more direct involvement of the clock is known as direct coupling, also called parallel coupling. Here, the circadian protein Timeless (Tim) (which is homologous to Swi1 in fission yeast) is necessary for both, the circadian clock and cell cycle regulation in the response to DNA replication problems (Figure: 1.1.6.2.2). In other words, Tim is a direct element of both cycles: the circadian cycle and the cell cycle. Thus, elimination of Tim would impair both cycles. However, the circadian cycle can operate in the absence of the cell cycle, as illustrated by the muscular circadian cycle, the liver circadian cycle and the neural circadian cycle. A direct role of clock proteins in cell cycle regulation is limited to some circadian factors like Tim, Period and Cryptochrome (101).

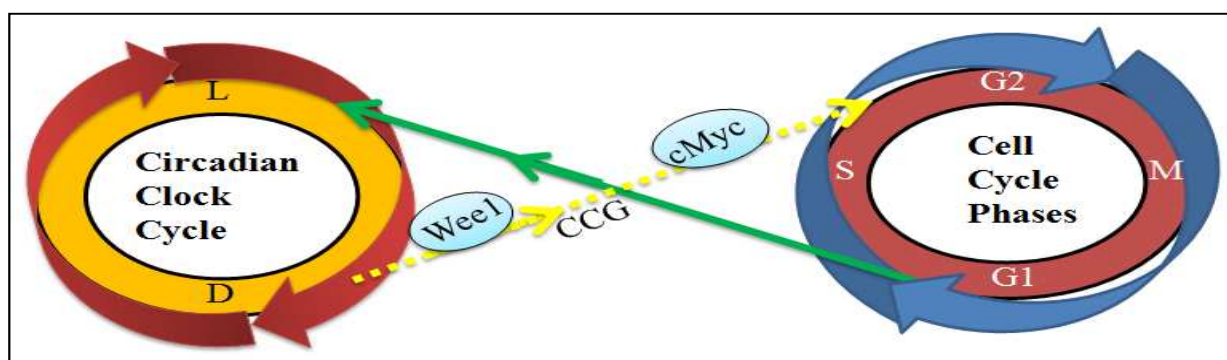


Figure: 1.1.6.2.1: Serial Coupling between circadian rhythm and the cell cycle: Synchronization of cell cycle and circadian clock may happen via co-operation among Wee1 and c-Myc. Reactions and expression of proteins in one cycle may control genes expression in another cycle. In other words, the circadian clock may control the cell cycle steps by regulating Wee1 kinase and the transcription factor c-myc. The effects of both cycles, the cell cycle and the circadian clock on each other are depended on the strength of the coupling between both cycles (101).

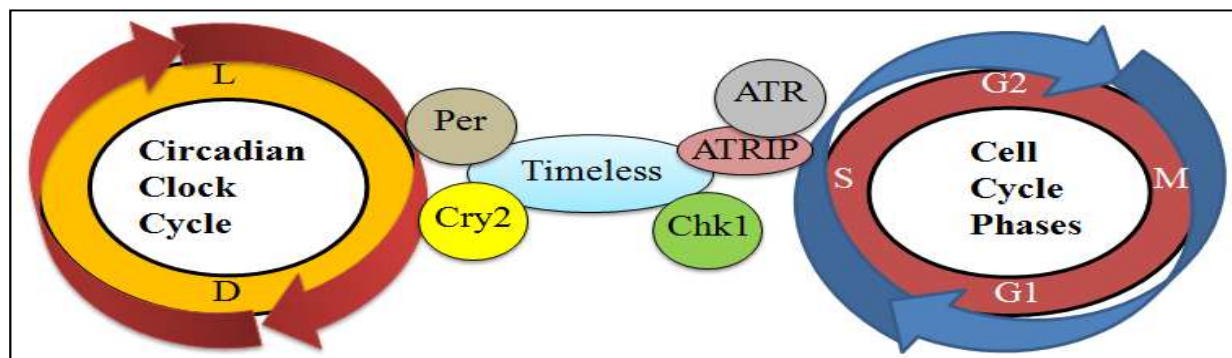


Figure: 1.1.6.2.2: Direct Coupling between the circadian rhythm and the cell cycle. Timeless (Tim) contributes to both cycles at the same time by forming two discrete protein complexes with Per and Cry2, and ATR-ATRIP and Chk1, respectively. ATR kinase binds to single-stranded DNA via its subunit ATRIP where ATR phosphorylates Chk1 kinase to mediate repair and to arrest cell cycle progression in the context of the DNA damage checkpoint (101).

1.1.6.3. Role of Circadian Proteins in the Direct Regulation of the Cell Cycle

Cell cycle controls response to DNA damage in all organisms, in a process known as DNA damage checkpoints. DNA damage stimulates these cellular stress response pathways and can cause cell cycle arrest, apoptosis and DNA repair. DNA damage is detected by ATR and ATM kinases, and the inhibitory cell cycle signal is conveyed by Chk1 and Chk2 kinase, respectively. While ATM binds to broken chromosomes, ATR detects single-stranded DNA (43, 66, 132, 499).

Interestingly, some circadian proteins such as Per1, Timeless and Per2 influence this DNA damage response. Per1 works as a tumour suppressor and regulates ATM kinase (Figure: 1.1.6.3.1) directly in the context of the detection of broken chromosomes (132). Overexpression of Per1 in cancer increases apoptosis caused by ionizing radiation which induces DNA double-strand breaks. Inhibition of Per1 has the opposite effect reducing apoptosis. Ionizing radiation may result in nuclear translocation of Per1 coinciding with the induction of c-Myc expression and repression of the CDK inhibitor p21 (Waf1/Cip1) (132). In summary, Per1 performs a second important role as regulator of ATM kinase in the context of apoptosis induced by broken chromosomes, a key function to suppress tumour formation (66).

In a similar way, Timeless interacts with ATR kinase (Figure: 1.1.6.3.1) to modulate the response to DNA replication problems (43). Like Per1, Per2 acts as tumour suppressor as indicated by the observation that *Per2* mutant mice develop γ -radiation induced lymphomas in a high ratio compared with wild type controls resulting from a partial impairment of p53-mediated apoptosis. Disruption of the circadian clock itself does not impact on DNA repair and DNA

damage checkpoints highlighting the fact that only some selected circadian proteins adopted this function (66).

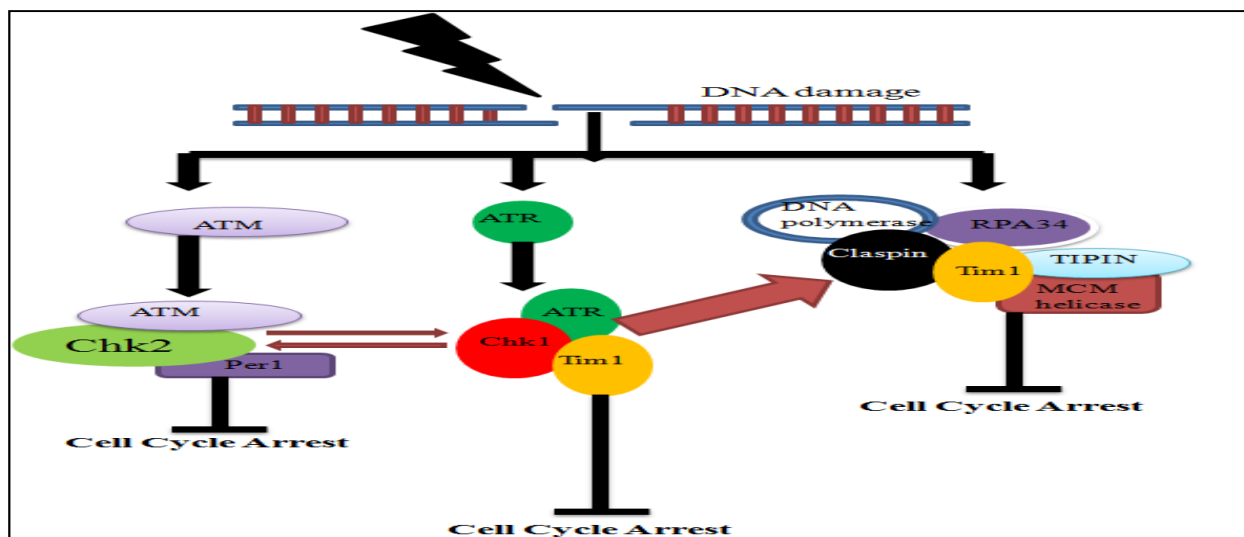


Figure: 1.1.6.3.1: ATM/Chk2/Per1 and ATR/Chk1/Tim1 activities. An interaction between circadian clock and cell cycle proteins. Per1 and Tim1 are both required to activate the DNA damage checkpoint kinases ATR-Chk1 in S phase and ATM-Chk2 in G2 (499).

1.1.6.4. Clock Genes and Aberrant Expression in Cancer

The circadian clock genes *CKI ϵ* , *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Clock*, and *Bmal1* are all involved in cancer development. Deregulation of *clock* gene expression is found in various cancer types (133, 134). Many breast cancer cells expressed *Per* genes abnormally due to changes in the methylation patterns at the *Per* gene promoters. This resulted in the use of the *Per3* gene as a biomarker for diagnosis of breast cancer in pre-menopausal women (66). Expression of *Per1* and *Per2* may be decreased in sporadic breast tumors compared to normal breast tissue (105), demonstrating the tumor suppressive nature of *Per2*. *Per2* activity can be promoted in the presence of its normal clock partner *Cry2*. Additionally, *Per2* expression in cancer cell lines is associated with a significant decrease in the expression of Cyclin D1 and an up-regulation of the tumor-suppressor p53 (103).

Proliferation in ovarian cancer cells has been found to follow a pattern of peaks and troughs that is out of phase with the circadian rhythm compared to normal tissue (93). Expression levels of *Per1*, *Per2*, *Cry2*, *Clock*, and *CKI ϵ* in ovarian cancers are lower than expression levels in normal ovaries. In normal tissues *Cry1* expression is higher than *Per3* and *Bmal1*, and also

expression levels of Per1 are lower than Cry1 in non-tumour tissues compared to cases of endometrial carcinoma (EC) (84). Per1 expression decreases in endometrial carcinoma (EC) possibly caused by DNA methylation of the promoter or other factors which lower Per1 gene expression thereby disrupting the circadian rhythm or the DNA damage response helping endometrial cancer cells to survive (134). Per1 is also down-regulated in human prostate cancer (136). The beneficial role of low Period protein levels is further illustrated by the observation that over-expression of Per1 in prostate cancer cells leads to growth inhibition and apoptosis (136), and by the finding that expression of Per1, Per2, Per3, Cry1, Cry2, and Bmal1 is strongly reduced in the chronic phase and blast crisis of chronic myeloid leukemia (CML) (135).

1.1.7. CKI, the Tumor-suppressor p53 and the Oncogene *mdm2*

CKI of the subtypes δ and ϵ are also involved in cancer formation. Both CKI δ and ϵ phosphorylate the important tumour suppressor p53 *in vitro* and *in vivo* (100). Since it is difficult to distinguish between both kinases many papers refer to CKI δ/ϵ when discussing the role of these kinases. CKI δ/ϵ phosphorylates murine p53 at serine 4, 6 and 9, and human p53 at serine 6, 9 and 15 and threonine 18 (96, 97, 98). Phosphorylation of the N-terminal serine 15 and threonine 18 by CKI may result in the inhibition of the interaction of p53 with MDM2, its partner protein required for the down-regulation and destruction of p53. Thus, CKI δ/ϵ phosphorylation is expected to stabilise the transcription factor p53 protecting organisms from damaged and potentially dangerous cells (97, 98, 99). Knippschild et al. (96) stated that CKI δ/ϵ phosphorylation of p53 is a feedback loop under stress situations. Consistent with this idea, CKI δ/ϵ phosphorylates MDM2 at serine 240, 242, 246 and 383 in the C-terminal domain (1). CKI is maybe suggested to play partly a role in aberrant proliferation of cells, because of dual roles of the oncogene *mdm2* and CKI-mediated modification of the tumor-suppressor p53 in the context of the role of CKI in Wnt signalling (i.e. inhibition of β -catenin). The tumor-suppressor activities of CKI are linked with the induction of apoptosis and with the stabilisation of the microtubule network and centrosome functions. CKI γ was also implicated in the development of kidney cancer (1). While, Frierson *et al.* (76) identified a role of CKI ϵ in adenoid cystic carcinomas (ACC) in the salivary gland, and Masuda *et al.* (77) recognized an impact of CKI activity on Acute Myelogenous Leukemia (AML). In mice, immunoreactivity for CKI δ was confirmed in cells of hyperplastic B follicles and advanced B-cell lymphomas in p53-deficient cells (75), and CKI δ activity was detected in choriocarcinomas (1, 560). CKI δ and ϵ

immunoreactively were found in breast carcinomas in many nuclei and the cytoplasm of ductal and acinar epithelial cells in the malignant tissue (78). Moreover, *CKI ϵ* has high immunoreactivity in invasive tumors of the breast tissue (78).

1.1.8. The role of CKI in Cell Physiology

Cki isoforms have a role in many cell processes such as the cell cycle and mitosis in eukaryotes. For example, Hrr25 (*CKI δ/ϵ*) in *S.cerevisiae* directly regulates mitosis and meiosis (86). The other two CKI isoforms in *S.cerevisiae*, *ScCKI* and *ScCKII*, have roles in bud formation (cell division) and cytokinesis (82, 83). Mammalian *CKI α* is involved in spindle dynamics and chromosome segregation (84, 85), while *CKI δ* is active at the spindle apparatus and the mitotic centrosomes (79). *CKI δ* phosphorylates tubulin, and the microtubule-associated proteins MAP4, MAP1A, Tau, and Stathmin in response to genotoxic stress that could result in genomic instability (79). Human *CKI δ/ϵ* may regulate some centrosome functions, because it interacts with the centrosomal scaffold protein AKAP450 (87). Taken together, *CKI δ/ϵ* are important to prevent uneven chromosome distribution in mitosis which could result in aneuploidy frequently observed in cancer cells. The latter may also explain why *CKI δ/ϵ* upregulates the tumour suppressor p53 which can remove such cells by inducing apoptosis (80, 81). Interestingly, SpHhp1 contains a potential microtubule binding domain (HIPYR) between histidine-167 (H167) and arginine-171 (R171) located in a loop above the ATP binding site (Figure 1.1.4.1; Figure 1.8.1).

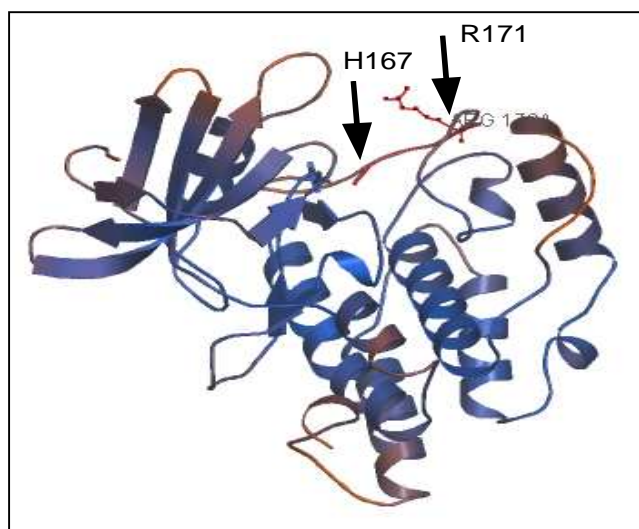


Figure: 1.1.8.1: Model of *S.pombe* Hhp1. The model is based on the crystal structure of *S.pombe* Cki1 (PDB ID: 1CSN). The model was produced with the Swiss Model tool (<http://swissmodel.expasy.org/interactive>; accessed 03 April 2015). The protein sequence identity is 57.39% to the model template *CKI δ* and the covered sequence ranges from 5aa-294aa. The position of microtubule binding domain 167-HIPYR-171 in SpHhp1 is shown. Arginine R171 is highlighted.

1.1.9. Reaction and Action of CKI in Apoptosis

Beyaert *et al.* (70) stated that CKI isoforms retard apoptosis by several pathways which includes phosphorylation of receptor for p75 tumor necrosis factor alpha (TNF α). In this context, CKI, especially CKI α , may prevent cell death by interfering with tumor necrosis-factor-related apoptosis including ligand (TRAIL) induced apoptosis. CKI mediated phosphorylation of the DISC complex, which forms at the cytoplasmic side of the TNF α receptor, may block activation of the caspase cells death response (41). CKI α , δ , and ϵ play also a role in the regulation of Fas-mediated apoptosis. Fas-mediated apoptosis is caused by caspase-8 activation (1). The induction of apoptosis through this pathway depends on caspase-8 mediated cleavage of the proapoptotic Bcl2 family member Bid at aspartate-59, which is negatively influenced by CKI dependent phosphorylation in direct neighbourhood to the cleavage site (48). In the case of mouse Bid, modification of serine-61 by a priming kinase stimulates phosphorylation of serine-64 and serine-66 by CKI thereby blocking cleavage of mBid (1, 39).

CKI α interferes with cell death mediated by the RXR class of retinoic acid receptors. The nuclear retinoic acid (RXR) receptors are able to build heterodimers with NGF1-B, insulin-like growth factor binding protein 3 (IGFBP) and β -catenin, which all play an important role in regulating cell survival. Upon an apoptotic stimuli through 9-cis retinoic acid and its derivatives, NGF1-B is released from the complex which then relocates to mitochondria where it induces the release of cytochrome C and apoptosis (36, 37, 42, 44, 47, 73). CKI α represses apoptosis by phosphorylating RXR without affecting RXR regulated gene transcription. Both proteins RXR and NGF1-B were discovered in interchromatin granule clusters, which led to the idea that the phosphorylated RXR–CKI α complex might be responsible for the relocalisation to the chromatin which would block apoptosis as RXR cannot move to the mitochondria (38, 45).

1.1.10. CKI and the Wnt Pathway

In both vertebrates and invertebrates the wingless (Wnt) signalling pathway performs important roles in development, for example, in forming the dorsal axis, the growth of tissues and the proliferation and differentiation of cells (e.g. neural cells, mammary cells and embryonic stem cells) (7, 17, 18). In fact, mutations in the Wnt-signaling components contribute to cancer in patients suffering from skin, liver, breast, brain, and colon tumours (1,7, 19).

The Wnt signaling pathway begins when the secreted wnt protein ligand binds to the frizzled receptors at the plasma membrane (Figure: 1.1.10.1) (22). This signal activates the frizzled

receptor and causes phosphorylation of the protein dishevelled (Dvl) (18, 22). At the heart of this signalling pathway is the Axin protein complex. Axin is a scaffold protein to which the kinases CK1 and Glycogen Synthase Kinase-3 β (GSK-3 β) bind to phosphorylate the transcription factor β -catenin (1, 20). Another member of this complex is the protein Adenomatous Polyposis Coli (APC) which is lost in approximately 90% of colon cancer cases (1, 18, 22). Phosphorylation of β -catenin by CK1 and GSK3 β reveals a recognition motif of β -transducin repeats which are a target of ubiquitin ligases that trigger the destruction of the phosphorylated β -catenin thereby preventing Wnt signalling (1, 20). These events take place in the absence of the wnt ligand to ensure that cells do not divide. Once the wnt ligand engages with the frizzled receptor, phosphorylation of β -catenin by GSK-3 β and CK1 stops which stabilises β -catenin and activates a nuclear transcriptional response (Figure: 1.1.10.1) (23).

CKI is predicted to phosphorylate β -catenin at serine 45 (24). Knippschild *et al.* (1) reviewed that CKI is a negative regulator of Wnt signaling, because it may phosphorylate β -catenin at serine 45, and this action could initiate GSK3 β -mediated phosphorylation of β -catenin at serine 33, serine 37 and threonine 41 (20). Loss of CKI activity would therefore prevent degradation of β -catenin in the absence of wnt signalling, a key event in cancer formation (1).

Although CKI is known as a negative regulator of wnt signalling, recent research has revealed that CKI family members have also positive roles in this pathway (7, 25). Axin, Dvl and APC may also be a CKI substrate resulting in a positive effect on Wnt signaling (7, 26). The precise functions of Dvl are however still unknown (7, 27). CKI ϵ -mediated phosphorylation of the Dvl causes disintegration of the Dvl, axin, GSK3 β complex by recruiting Frat to the complex (Figure: 1.1.10.1) (28). The phosphatase PP2A might activate CKI ϵ when Wnt signalling decreases by reducing phosphorylation of the C-terminal inhibitory autophosphorylation sites of CKI ϵ (30).

Another mechanism by which CKI ϵ could act as a positive regulator of Wnt signalling is through the phosphorylation of Tcf3. CKI ϵ and Tcf3 act synergistically, and phosphorylated Tcf3 binds to β -catenin thereby inhibiting its phosphorylation and degradation. In addition, CKI ϵ could act as molecular switch between two pathways: the canonical Wnt-signaling pathway and the planar cell polarity/JNK pathway. The latter regulates, the organization of the cytoskeleton and Dvl is involved in both pathways. Interestingly, over-expression of Dvl and inhibition of CKI ϵ may stimulate the JNK pathway (1).

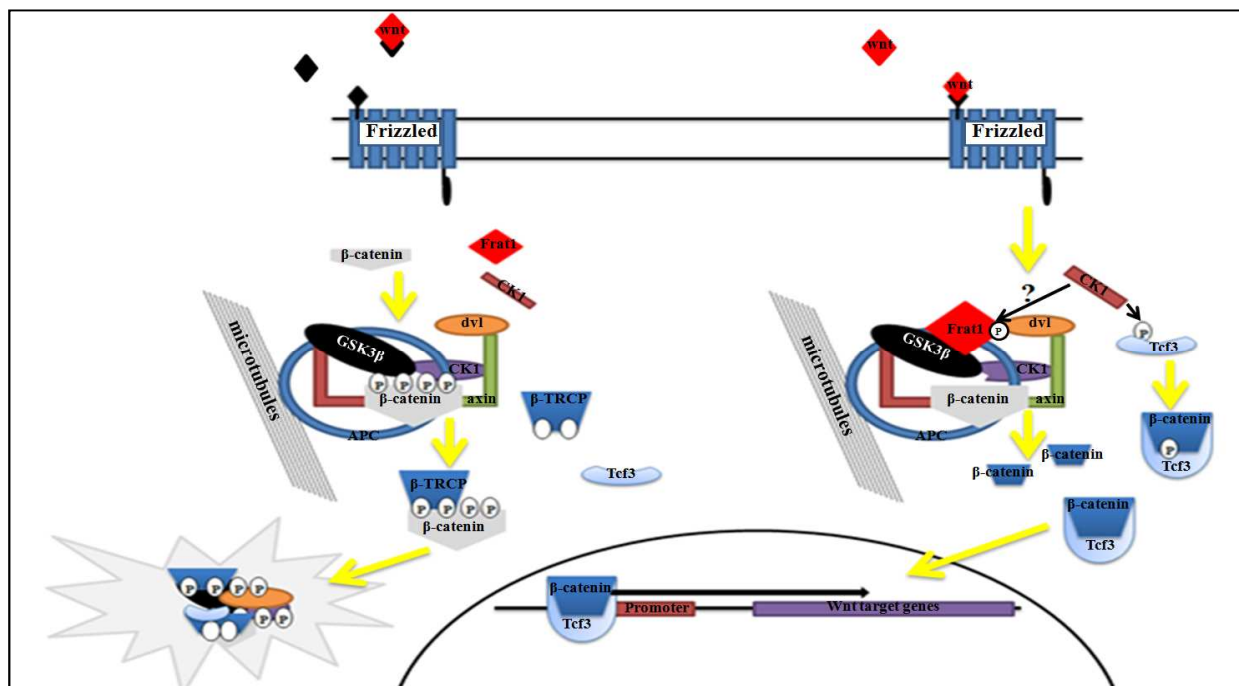


Figure: 1.1.10.1: Mechanism of CKI in the wingless (wnt) pathway. In the absence of Wnt-signalling, CKI and GSK3 β (glycogen synthase kinase 3 β) both phosphorylate β -catenin at its N-terminus. This reveals the β -TRCP (β -transducin repeat containing protein) domain of β -catenin. This allows the binding of β -TRCP which leads to the ubiquitination and destruction of β -catenin. In the presence of the Wnt ligand, which binds to the Fzl receptor (Frizzled receptors), the Dvl (dishevelled) protein is phosphorylated. The phosphorylation of Dvl enables it to bind to axin and to block the phosphorylation of β -catenin by GSK3 β and CKI ϵ (1).

1.1.11: CKI and the Response to DNA damage

As mentioned earlier in Chapter 5 CKI enzymes may regulate the DNA damage response via their role in the circadian clock. Especially in the case of ScHrr25, SpHhp1 and SpHhp2 there is evidence that CKI enzymes play a direct role in the DNA damage response (169, 171, 172). As shown in Figure 1.1.11.1, *S.pombe* cells contain the paralogue kinases of ATM (Tel1), and ATR (Rad3), but in contrast to mammalian cells, Tel1 (ATM) plays only a minor role in the DNA damage response. The reason for this is the rapid conversion of double-stranded DNA breaks into single-stranded DNA which activates Rad3 (ATR) kinase (179). In S-phase, Rad3 activates Cds1 (Chk2) kinase to signal replication stress, whereas in G2-phase Rad3 activates Chk1 kinase (Figure 1.1.11.1) (169, 171, 172, 179).

In G2, Chk1 phosphorylation at serine 345 by Rad3 kinase requires the loading of the Rad9-Rad1-Hus1 ring onto the ssDNA-dsDNA junction by the Rad17-RFC complex. In the presence of the scaffold proteins Rad4 (TopBP1) and Crb2 (53BP1), Rad3 phosphorylates Chk1 at S345

which results in a G2-M delay as Chk1 enforces the inhibitory phosphorylation of Cdc2 kinase at tyrosine 15 by stimulating Wee1 kinase and by removing Cdc25 phosphatase from the nucleus (169, 171, 172, 224, 395, 483, 484).

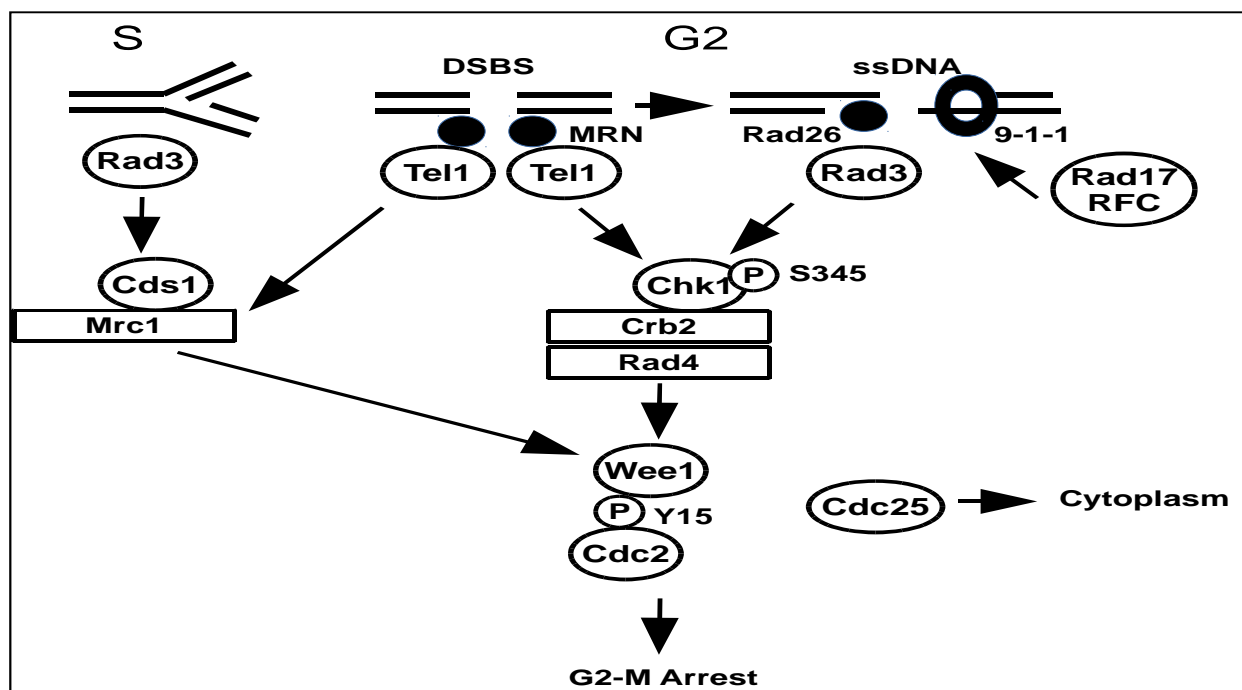


Figure: 1.1.11.1: The DNA Damage Response in *S.pombe*. Tel1 (ATM) kinase is recruited to DNA double-strand breaks (DSBS) by the Rad50-Mre11-NBS1 (MRN) complex to activate Chk1 kinase which associates with the scaffold protein Crb2 (53BP1). Since DSBS are rapidly converted into single-stranded DNA (ssDNA), which activates Rad3 (ATR) kinase, Tel1 plays only a minor role in fission yeast. Rad3 is recruited to ssDNA by Rad26 (ATRIP) where it phosphorylates Cds1 (Chk2) kinase in S and Chk1 kinase in G2. Cds1 binds to stalled DNA replication forks via the scaffold protein Mrc1. Tel1 may modulate activation of Cds1 by phosphorylating Mrc1. Activation of Cds1 and Chk1 prolongs the inhibitory phosphorylation on Cdc2 at tyrosine 15 (Y15) which blocks entry into mitosis. Chk1 activates Wee1 kinase and removes Cdc25 phosphatase from the nucleus. This maintains Y15 phosphorylation. Activation of Chk1 requires the presence of the Rad9-Rad1-Hus1 (9-1-1) ring which is loaded by Rad17-RFC, and the presence of a second scaffold protein Rad4 (TopBP1) (169, 171, 172).

In S phase, Rad3 activates Cds1 (Chk2) to stabilise and protect DNA replication forks, and to block cell cycle progression. Cds1 is recruited to stalled replication forks by the scaffold protein MRC1 (237). Zhou and Elledge (169) stated that Cds1 kinase has about 70% similarity to ScRad53, and that Cds1 kinase is required for cell survival caused by introducing the ribonucleotide reductase inhibitor hydroxyurea (HU). When replication forks break, Rad3 switches from Cds1 to Chk1 to signal DNA damage (236, 272). As activation of Chk1, phosphorylation of Cds1 requires the presence of the 9-1-1 ring at stalled forks. Unlike Ck2 which is involved in the response to DNA damage (502), there is so far only limited evidence of

a role of CKI enzymes. In *S.cerevisiae* and in *S.pombe*, Hrr25 and Hhp1 are required for the repair of DNA breaks (102, 169, 171). Similar evidence is not yet available in higher eukaryotic cells. The role of Hhp1 in DNA break repair could be linked with its association with the Rad50 protein (497).

The MRE11–RAD50–NBS1 (MRN) complex plays important roles at a DNA double-stranded break as it recruits via its Nbs1 subunit Tel1 (ATM) kinase (173, 332, 502) and helps to convert the break into ssDNA to activate Rad3 (ATR) kinase via its Mre11 exonuclease subunit. Hence the association between Hhp1 and Rad50 could affect these important DNA damage activities of the MRN complex (162). Given that the MRN complex acts in homologous recombination (HR) and Non-Homologous End Joining (NHEJ) it is possible that Hhp1 influences the choice of the repair pathways to mend broken chromosomes. Since the Cdc2-cyclin B kinase complex promotes HR and blocks NHEJ in G2 (272, 501), and because Hhp1 associates with Cdc2 and cyclin B (Cdc13) (272, 497), CKI could also affect the repair response indirectly via the main cell cycle regulator Cdc2-cyclin B.

1.2. Aims of this Study

Currently the study of CK1 enzymes in mammalian cells is complicated by the large number of isoforms and by the limited availability of specific reagents. The overarching aim of this work is to utilize the model eukaryote *S.pombe* to understand the consequences of mutations in conserved amino acids or domains of CK1 ϵ (Hhp1) on DNA repair, mitosis and cell cycle regulation.

Previous work in the group revealed a novel function of Hhp1 kinase in the response to DNA replication stress caused by replication of methylated templates (methylmethanesulfonate, MMS) or when replication forks collide with Topoisomerase 1 proteins immobilized by the anti-cancer drug camptothecin (CPT). Genetic data suggest that Hhp1 acts downstream of the DNA replication protection complex consisting of Swi1 (Timeless) that travels with the DNA replication fork (Figure: 1.2.3), and the scaffold protein MRC1 (Clasplin) which activates a DNA damage response specifically in S-phase (Figure: 1.1.11.1; Figure: 1.2.2) (Caspari, unpublished). Given the close link between the circadian clock, the DNA damage checkpoint and cell cycle regulation (Chapter 5), regeneration of the circadian *tau* mutation in Hhp1 (R180C) which results in a changed auto-phosphorylation pattern (69), was also included.

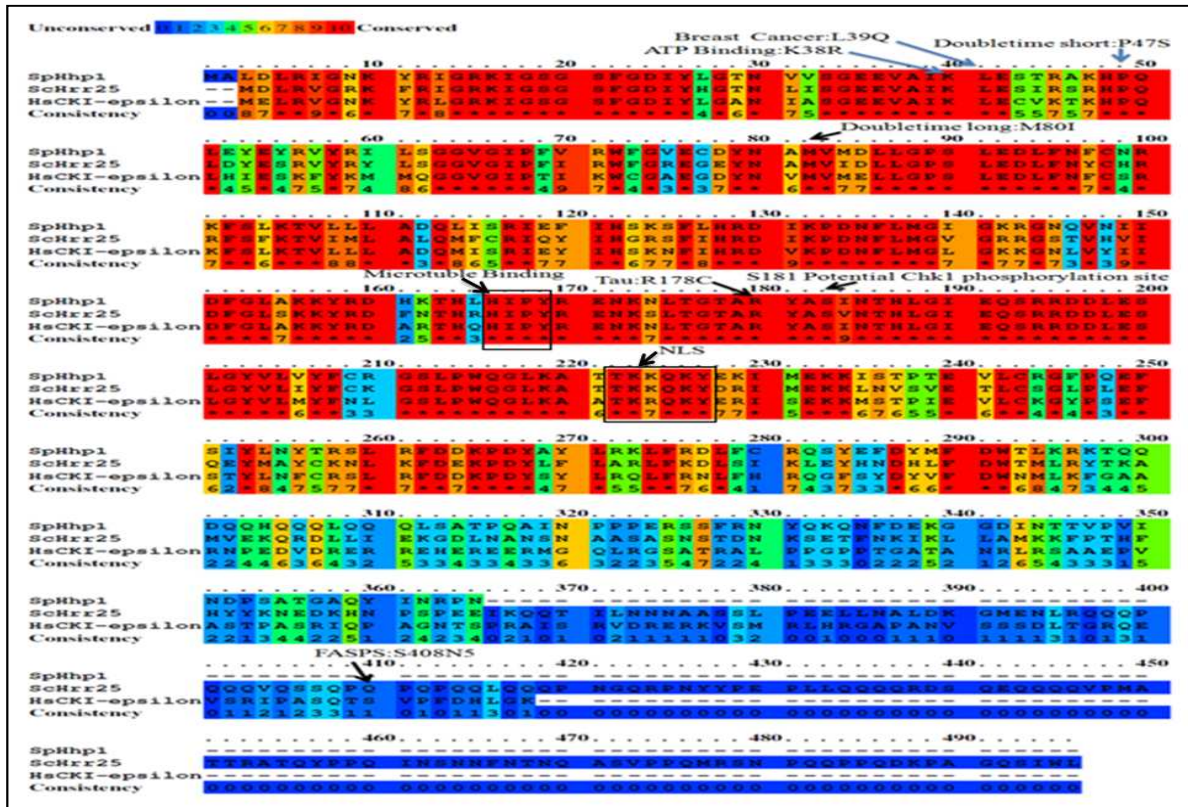


Figure: 1.2.1: Alignment of *S.pombe* Hhp1, *S.cerevisiae* Hrr25 and human CKI ϵ . The conserved residues involved in kinase activity, circadian clock functions and disease development are indicated (numbers refer to the human protein). (source: PRALINE sequence alignment tool; available at: <http://zeus.few.vu.nl/jobs/ad211873028694a24fd126b6d9ae7064/>; accessed 13 October 2015). Human CKI ϵ : NP_689407.1, SPHhp1: CAA20311.1, and SCHrr25: CAA97918.1.

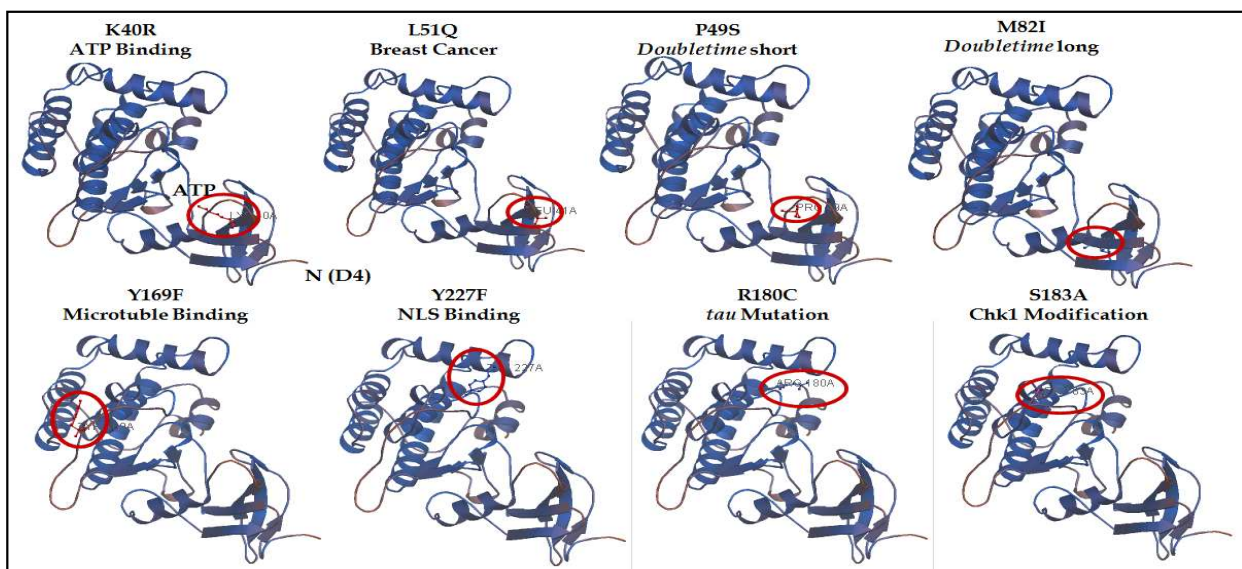


Figure: 1.2.2: Model of *S.pombe* Hhp1 with the mutation sites indicated. The model is based on CKI (PDB ID: 3SVO) (500) with 70.43% identity ranging from 4aa-297aa which covers the complete kinase domain but only the beginning of the C-terminal domain. The model was generated with Swiss Model (<http://swissmodel.expasy.org/interactive>; accessed 04 April 2015). The numbers refer to the amino acid position in *S.pombe* Hhp1 kinase.

Unpublished work revealed that *S.pombe* cells expressing the *tau* mutation are partly sensitive to the topoisomerase 1 poison CPT and have an extended G2 arrest in response to this type of DNA damage (Williams and Caspari, unpublished).

In addition to the previously generated mutation in the ATP binding site (K40R) and the circadian clock mutation *tau* (R180C) (Figure: 1.2.2), the following mutations will be re-created in Hhp1 (Figure: 1.2.2). A replacement of leucine-38 by glutamine in human CKI is frequently found in aggressive breast cancers (~12% of 42 screened breast cancer patients) (141, 142), but the biological consequences of this mutation are currently unknown (Hhp1: L41Q). The *Drosophila* mutations *doubletime short* (P47S; Hhp1.P49S) and *doubletime long* (M80I; Hhp1.M82I) lengthen or shorten the circadian clock in the fruit fly, respectively. Both decrease the kinase activity *in vitro*. Interestingly P49 in Hhp1 is replaced by a serine residue in Hhp1 (Figure: 1.2.2). Serine 183 is a potential phosphorylation site of the DNA damage response kinase Chk1 which was identified during this study (Sayed and Caspari, unpublished). In addition to these point mutations, the highly conserved microtubule and nuclear localisation (TKKQKY-227) domains (Figure: 1.2.2) will be deleted in frame, and the conserved tyrosine residues within both domains, which may act as phosphorylation sites (Hhp1: Y169F; Y227F), will be replaced by a phenylalanine residue. The resulting mutant strains will be tested in (i) DNA damage sensitivity assays, (ii) cell cycle assays and (iii) their phosphorylation status will be tested using isoelectric focusing.

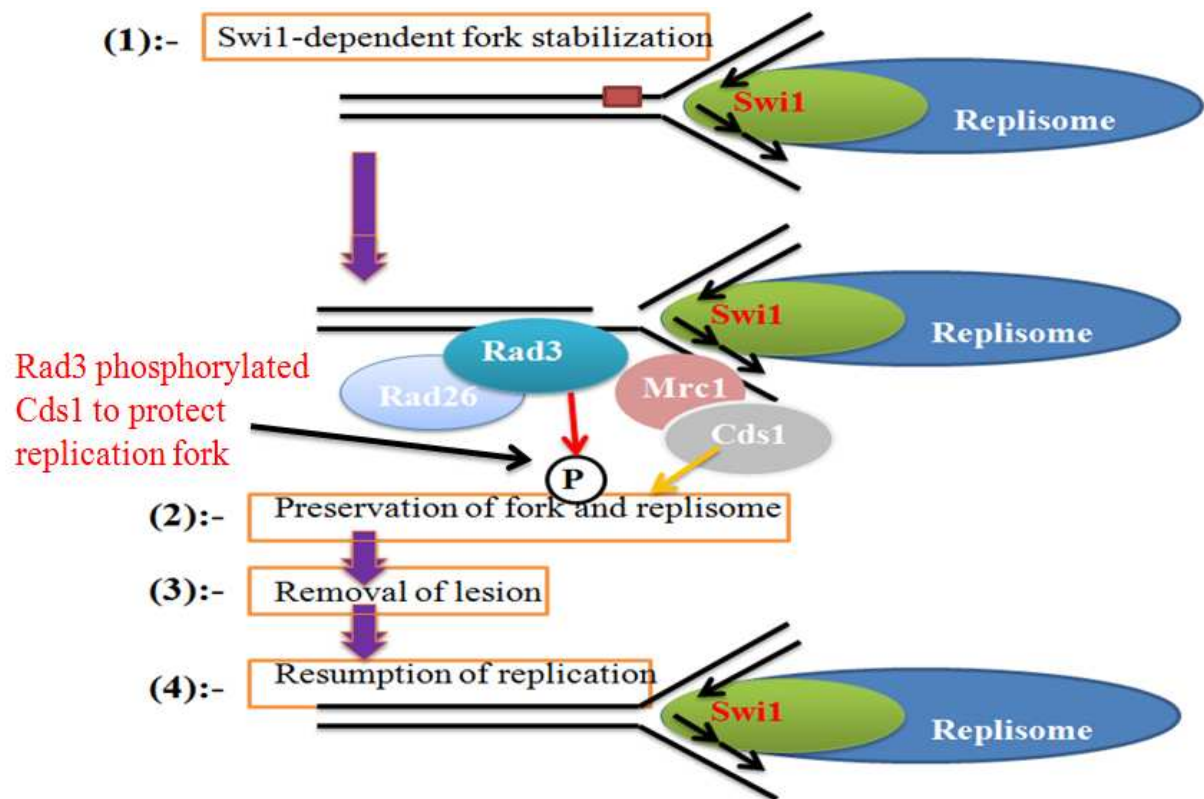


Figure: 1.2.3: Implication of Swi1 in response to replication fork arrest. Swi1 is implicated in the response to fork arrest during S-phase (118). Recruitment of Swi1 that travels with the replication fork is important to activate Cds1 kinase in response to DNA replication lesions. This activation requires also the scaffold protein Mrc1 (Clasplin). Swi1 and Mrc1 act jointly with the DNA damage checkpoint kinase a Rad3-Rad26 and the PCNA-like ring complex Rad9-Rad1-Hus1 (49, 118).

Chapter 2: Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Media

2.1.1.1. *S.pombe* media:

2.1.1.1.1. YEA (Yeast extract Agar)

0.5% w/v yeast extract

3.0% w/v glucose

0.01% w/v adenine

H₂O

2% w/v agar

2.1.1.1.2. YEA broth medium

0.5% w/v yeast extract

3.0% w/v glucose

0.01% w/v adenine

H₂O

2.1.1.1.3. YEA+ 5-FOA

0.5% w/v yeast extract

3% w/v glucose

0.01% w/v adenine phosphate

2% w/v Agar

dH₂O

after autoclaving add in amount of 1mg/ml 5-fluororotic acid

2.1.1.1.4. G418 ((Geneticin) aminoglycoside antibiotic)

0.5% w/v yeast extract

3% w/v glucose

0.01% w/v adenine phosphate

2% w/v agar

dH₂O

after autoclaving add in amount of 75-100 μ g/ml G418

2.1.1.1.5. YEA+CPT (camptothecin)

0.5% w/v yeast extract

3% w/v glucose

0.01% w/v adenine phosphate

2% w/v agar

dH₂O

after autoclaving add camptothecin (CPT) from a 10mM stock solution in DMSO to the required final concentration

2.1.1.1.6. YEA+MMS (methyl methanesulfonate)

0.5% w/v yeast extract

3% w/v glucose

0.01% w/v adenine phosphate

2% w/v agar

dH₂O

after autoclaving add MMS from a 99% stock solution (SIGMA 129925) to the required final solutions of 0.005% or 0.01% MMS

2.1.1.1.7. Minimal Media minus Leucine or Adenine or Uracil (MM-L or A or U)

3% w/v glucose

2% w/v agar

dH₂O (1litre)

Yeast nitrogen base 6.7g (dissolve into 40 ml dH₂O, spin at 3000rpm for three minutes and then filter sterilize onto the recipe)

Adenine 7.5mg/ml 30x 33.2 ml/L

Uracil 3.5mg/ml 15x 66.6 ml/L

Leucine 7.5mg/ml 30x 33.2 ml/L

2.1.1.1.8. EMM (Edinburgh minimal medium)

3.0% w/v glucose

0.67% w/v yeast nitrogen base

0.5% w/v NH₄Cl

225mg/L of supplement: adenine / histidine /uracil / leucine

pH = 5.5 adjusted with KOH

H₂O

2% w/v agar

2.1.1.1.9. ME (Malt extract)

3% w/v Bacto-malt extract

225mg/L of supplement: adenine / histidine /uracil / leucine

dH₂O

pH 5.5 adjusted with NaOH.

2% w/v agar

2.1.1.2. *E. coli* media**2.1.1.2.1. LB Medium (Luria-Bertani broth)**

1% w/v tryptone

0.5% w/v yeast extract

1% w/v NaCl

dH₂O (to 1Liter)

2.1.1.2.2. LB Agar Medium

10g/L tryptone

5g /L yeast extract

10g/L NaCl

20g/L agar

dH₂O (to 1Liter)

2.1.1.2.3. LB Agar Medium+ ampicillin

10g/L tryptone

5g/L yeast extract

10g/L NaCl

20g/L agar

dH₂O (to 1Liter), after autoclaving add ampicillin (100 mg/ml)

2.1.2. Buffers and others

2.1.2.1. 10X DNA loading dye

0.025g xylene cyanol

0.025 g bromophenol blue

1.25ml of 10% SDS

12.5ml glycerol

6.25ml dH₂O

2.1.2.2. 4% Paraformaldehyde (10ml volume)

0.4g Paraformaldehyde

2μl 10M NaOH

dH₂O

2.1.2.3. 10X PBS buffer (phosphate- buffered saline) 4 litre volume (pH 7.2)

360g NaCl

43.6g Na₂HPO₄

12.8g NaH₂PO₄·7H₂O

dH₂O

2.1.2.4. 10X SDS buffer (4 litre volume)

576g Glycine

40g SDS

121g Tris-HCl

dH₂O

2.1.2.5. 10X Transfer buffer (4 litre volume)

124g Tris-base

56g Glycine

dH₂O

2.1.2.6. 50X TAE buffer (Tris-Acetate-EDTA) (1 litre volume)

242g Tris base

47.1ml glacial acetic acid

37.2g Na₂EDTA. 2H₂O

dH₂O

2.1.2.7. 10 X TBE buffer (for agarose gel electrophoresis) (1 litre volume)

108g Tris base (890mM)

55g Boric acid (890mM)

40ml EDTA pH8 (20mM)

dH₂O

2.1.2.8. 1X TE buffer (Tris-EDTA)

10mM Tris-HCl pH7.5

1mM EDTA pH8

2.1.2.9. 100% TCA (Trichloroacetic Acid)

500g TCA

350ml dH₂O

2.1.2.10. 10mM dNTPs

10ul dATP 100mM stock solution

10ul dCTP 100mM stock solution

10ul dGTP 100mM stock solution

10ul dTTP 100mM stock solution

60ul dH₂O

2.1.2.11. Lithiumacetat buffer

100mM lithiumacetate

10mM Tris pH6.5

1mM EDTA pH8

2.1.2.13. 30X Adenine (7.5 mg/ml) (250 ml volume)

1.9g adenine

dH₂O

2.1.2.14. 15X Uracil (3.5 mg/ml) (250 ml volume)

0.9g uracil

dH₂O

2.1.2.15. 30X Leucine (7.5 mg/ml) (250 ml volume)

1.9g leucine

dH₂O

2.1.2.16. DNA extraction buffer (genomic DNA)

2% w/v triton 100x

1% w/v SDS

100mM NaCl

10mM Tris-HCl pH8

1mM EDTA

2.1.2.17. 1X SDS running buffer (1 Litre volume)

50 ml 10x SDS buffer (dissolve 30g of Tris base, 144g of glycine, and 10g of SDS in 1L of water. The pH of the buffer should be 8.3)

dH₂O (to 1 Liter)

2.1.2.18. 1X transfer buffer (5 Litre volume)

100ml 10x Transfer buffer

750ml Methanol

dH₂O

2.1.2.19. 1X PBS + 0.05 % Tween 20

200ml 10x PBS pH7.2 (80g NaCl, 2g KCl, 14.4g Na₂ HPO₄, 2.4g KH₂PO₄ in 800ml water, adjust pH to 7.2, adjust volume to 1L with additional distilled water)

1mL Tween 20

dH₂O (to 2000ml)

2.1.2.20. 40% Polyethylglycol (PEG) (100ml volume)

40g PEG 4000

100ml Yeast Transformation Buffer

2.1.2.21. 1M Lithium acetate (250ml volume)

25.505g Lithium acetate

dH₂O

2.1.2.22. 0.5M EDTA pH 8 (1 Litre volume)

186.1g Na₂EDTA.2H₂O

dH₂O

Note: use 10M NaOH to adjust the pH

2.1.2.23. 1M Tris HCl pH 6.8/8.8

1M Tris Base 121.1g [adjust pH with concentrated hydrochloric acid](#)

add H₂O up to 1000 ml

2.1.2.24. Buffer H

50mM HEPES, pH = 8.0

150mM NaCl

0.1% w/v NP40

10% w/v glycerol

5mM EDTA

60mM β-glycerophosphate

2.1.2.25. Protease inhibitors for soluble protein extract

50mM NaF

1mM Na₃VO₄

5mM N-ethylmethylanine

1mM PMSF

1 Protease Inhibitor Cocktail Tablet (Roche, cat no 11836 153 001) per 10ml of buffer H

2.1.2.26. Transfer buffer for Western blotting

25mM Tris base

190mM glycine

15% methanol

pH around 8.3

Note: For proteins larger than 80kD, it was recommended that SDS was included at a final concentration of 0.1% and concentration of methanol was reduced to 10%.

2.1.2.27. Blocking membrane buffer (milk buffer)

3 - 5% milk powder or BSA in PBST buffer.

1x PBS

0.05% Tween 20

2.1.2.28. Running buffer for SDS-PAGE (Tris-glycine buffer)

25mM Tris base

190mM glycine

0.1% w/v SDS

pH around 8.3

2.1.2.29. 1X Soc medium (1Litre volume)

20g yrypton

5g yeast extract

0.5g NaCl

dH₂O

Note:- aliquot in 50ml and autoclave

2.1.2.30. 5M NaCl (500ml volume)

146g NaCl

dH₂O

2.1.2.31. 20% SDS

40g SDS

dH₂O (in 500 ml)

2.1.2.32. Yeast Transformation buffer pH 6.8

100mM lithium acetate

10mM Tris-HCl pH6.8

1mM EDTA pH8

Note: Acetic Acid can be used to adjust the pH

2.1.2.33. 40% Polyethyleneglycol

100mM lithium acetate

10mM Tris-HCl pH6.8

1mM EDTA pH8

2.1.2.34. 1M HEPES (1Litre volume)

238.3g HEPES

dH₂O

2.1.2.35. 4X SDS sample buffer

40% w/v glycerol

240 mM Tris-HCl pH6.8

8% w/v SDS

0.04% w/v bromophenol blue

5% w/v β-mercaptoethanol

2.1.2.36. Ponceau Stain

0.1% w/v ponceau

5% w/v acetic acid

2.1.3. Agarose gel

1% w/v agarose

1x TAE buffer/or 1x TBE buffer

heat in microwave until agarose is completely dissolved

0.5µg/ml ethidium bromide

2.1.4. SDS polyacrylamide gel**2.1.4.1. Resolving gel/bottom gel (total of 10 ml)**

Reagent	Gel percentage			
	8%	10%	12%	15%
H ₂ O	8.5ml	7.5ml	6.5ml	5ml
1M TrisHCL pH8.8	7.5ml	7.5ml	7.5ml	7.5ml
20% SDS	150µl	150µl	150µl	150µl
40% Acrylamide/Bis Solutin, 37.5:1	4ml	5ml	6ml	7.5ml
10% APS	100µl	100µl	100µl	100µl
TEMED	20µl	20µl	20µl	20µl

Table: 2.1.4.1.1: Recipe for the resolving acrylamide gels

2.1.4.2. Stacking gel/top gel

Components	Gel percentage 4%
H ₂ O	7.5ml
1M TrisHCL pH6.8	1.5ml
20% SDS	50µl
40% Acrylamide/Bis Solutin, 37.5:1	1ml
10% APS	100µl
TEMED	10µl

Table: 2.1.4.2.1: Recipe for the stacking acrylamide gel

2.1.5. *E.coli* strain used in this study

The *E.coli* strain used to amplify plasmids was TOP10. Genotype: *F-mcrAD(mrr-hsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D(ara-leu)7697 galK rpsL (Str^R) endA1 nupG*.

2.1.6. Plasmids used

Plasmids names	References
pAW8 Plasmid	Watson Adam, Garcia Valerie, Bone Neil, Carr Antony and Armstrong John. (2008). Gene tagging and gene replacement using recombinase-mediated cassette exchange in <i>Schizosaccharomyces pombe</i> . <i>Gene</i> , (407): 63–74.
Plasmid pREP41- <i>Leu2+</i>	Prudden John, Evans Joanne, Hussey Sharon, Deans Bryan, O'Neill Peter, Thacker John and Humphrey Tim. (2003). Pathway utilization in response to a site-specific DNA double-strand break in fission yeast. <i>The EMBO Journal</i> , (22): 1419-1430.

Table: 2.1.6.1: Plasmids used in this study

2.1.7. List of antibodies used in this study

Antibody	Company	Catalog Number	Description	Concentration
MYC tag	Covance	MMS-150R	Mouse monoclonal	1:1000
MYC tag	Santa Cruz	SC40	Mouse monoclonal	1:1000
HA tag	Covance	MMS-101R	Mouse monoclonal	1:1000
HA tag	Santa Cruz	SC7392	Mouse monoclonal	1:1000
HA tag	Santa Cruz Biotechnology	sc-7392	Mouse monoclonal	1:1000
Cdc2	Abcam	AB70860 & ab5467	Mouse monoclonal	1:1000
Anti-Rabbit IgG	Sigma	A 6154	Horseradish Peroxidase-conjugated Affinity Pure Goat	1:5000
Anti-Rabbit IgG	DacoCytomation	P 0399	Horseradish Peroxidase-conjugated Swine polyclonal	1:5000

Anti-Mouse IgG	DacoCytomation	P 0161	Horseradish Peroxidase-conjugated polyclonal Rabbit	1:5000
Anti-Mouse IgG, Light Chain Specific	Jackson ImmunoResearch	115-035-174	Horseradish Peroxidase-conjugated Affinity Pure Goat	1:5000
Anti-mouse secondary AB-HRP anti-light chain	Jackson ImmunoResearch	115-035-174	Horseradish Peroxidase-conjugated	1:10,000
STREP Tag	Source Bioscience	ABE3837	rabbit	1:1000
Anti-HA Tag	Covance	MMS 101R	Mouse	1:1000
Anti-HA Tag (best for Chk1-HA)	ABCAM	AB9110	Rabbit	1:1000
Anti-mouse HRP secondary AB	Sigma	A4416	Goat	1:10,000
Anti-rabbit HRP secondary AB	Sigma	A6154	Goat	1:10,000

Table: 2.1.7.1: List of Antibodies used in this study.

2.1.8. *S. pombe* strains used in this study

Name	Genotype
<i>hhp1-HA-wt</i> (1388)	<i>h- ade6-M216 leu1-32 ura-D18 hhp1::hhp1-HA3-kanMX4</i>
<i>hhp1 base strain</i> (1495)	<i>h- loxP-hhp1--loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.R180C-C-terminal.deletion</i> (2007)	<i>h- hhp1::loxP-hhp1-Δ298-356-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M82I</i>	<i>h- hhp1::loxP-hhp1-M82I-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.L51Q</i>	<i>h- hhp1::loxP-hhp1-L51Q-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.S183A</i>	<i>h- hhp1::loxP-hhp1-S183A-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.R180C.K40R</i> (1986)	<i>h- hhp1::loxP-hhp1-R180C K40R-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M84G</i> (2008)	<i>h- hhp1::loxP-hhp1-M84G-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M84G.R180C</i>	<i>h- hhp1::loxP-hhp1-M84G R180C-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.P49S</i>	<i>h- hhp1::loxP-hhp1-P49S-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.NLS.deletion.M84G</i>	<i>h- hhp1::loxP-hhp1-Y227F Δ298-356-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.Y227F.M84G</i>	<i>h- hhp1::loxP-hhp1-Y227 M84G-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.Y169F.M84G</i>	<i>h- hhp1::loxP-hhp1-Y169F M84G-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>chk1.HA</i> (1682)	<i>chk1.HA ura4.D18 ade6.M210leu1.32</i>
<i>Δcds1</i> (1043)	<i>h- ade6-M210 cds1::ura4+ ura4-D18</i>
<i>cdc2.1w Δsrs2</i>	<i>cdc2.1w srs2::kanMX4 ade6-M216 leu1-32 ura4-D18</i>
<i>Δsrs2</i> (1405)	<i>ade6-M216 srs2::kanMX4 leu1-32 ura4-D18</i>

<i>cdc2.1w</i>	<i>cdc2.1w ade6-M216 leu1-32 ura4-D18</i>
wild type 804	<i>h- ade6-M216 leu1-32 ura4-D18</i>
Δ <i>srs2</i> Δ <i>chk1</i> Δ <i>hhp1</i>	<i>srs2::kanMX4 chk1::ura4+ hhp1::hphMX6 leu1-32 ura4-D18 ade6-M210</i>
Δ <i>hhp1</i> Δ <i>chk1</i> (1251)	<i>chk1::ura4+ hhp1::hphMX6 leu1-32 ura4-D18 ade6-M210</i>
Δ <i>chk1.hhp1.S183A</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-S183A-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
Δ <i>chk1.hhp1.M82I</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-M82I-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
Δ <i>chk1.hhp1.M84G.R180C</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-M84G R180C-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>crb2-T215A</i>	<i>crb2::ura4+ ade6-M216 leu1-32 ura4-D18</i>
Δ <i>rqh1</i> (881)	<i>rqh1::kanMX4 leu1-32 ura4-D18 ade6-M210</i>
Δ <i>rqh1</i> Δ <i>hhp1</i> (1048)	<i>hhp1::hphMX6 rqh1::kanMX4 leu1-32 ura4-D18 ade6-M210</i>
Δ <i>tel1</i>	<i>tel1::leu2 leu1-32 ura4-D18 ade6-M210</i>
Δ <i>tel1::L</i> Δ <i>hhp1</i>	<i>hhp1::hphMX6 tel1::leu2 leu1-32 ura4-D18 ade6-M210</i>
Δ <i>mus81</i> Δ <i>hhp1</i> (1109)	<i>hhp1::ura4+ mus81::kanMX4 leu1-32 ura4-D18 ade6-M210</i>
<i>Hhp1.HA</i> Δ <i>chk1</i> (2068)	<i>chk1::ura4+ ade6-M216 leu1-32 ura4-D18 hhp1::hhp1-HA3-kanMX</i>
<i>hsk-1312</i> (1188)	<i>h hsk1-1312 ura4-D18 leu1-32 ade6-M210</i>
<i>hsk-1312</i> Δ <i>hhp1</i> (1123)	<i>hsk1-1312 hhp1::ura4+ leu1-32 ura4-D18 ade6-M210</i>
Δ <i>hhp1</i> Δ <i>rad3</i> (2222)	<i>hhp1::hphMX6 rad3::ade6+ leu1-32 ura4-D18 ade6-M210</i>
<i>hhp1.K40R</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-K40R-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M82I</i> Δ <i>wee1</i>	<i>wee1::ura4+ hhp1::loxP-hhp1-M82I-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.L51Q</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-L51Q-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>Hhp1.K40R-R180C</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-R180C K40R-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M84G</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-M84G-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M84G.R180C</i> Δ <i>Chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-M84G R180C-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.M82I</i> Δ <i>Chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-M82I S183A-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.R180C.C-terminal.deletion</i>	<i>hhp1::loxP-hhp1-R180C Δ298-356-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
Δ <i>wee1</i> (868)	<i>h- wee1::ura4+ ade6-M216 leu1-32 ura4-D18</i>
Δ <i>wee1</i> (869)	<i>h+ wee1::ura4+ ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.R180C.C-terminal.deletion</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-Δ298-356-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.Y169F</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-Y169F M84G-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.K40R</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-K40R-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>hhp1.K40R-R180C</i> Δ <i>chk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-K40R R180C-HA-loxM ade6-M216 leu1-32 ura4-D18</i>

<i>crb2.YFP</i> (820)	<i>h⁻ leu1-32 ura4-D18 crb2yep2HA6::ura4+</i>
<i>chk1.Myc.His</i> (2256)	<i>h⁻ leu1-32 ura4-D18 chk1-9myc2HA6His::ura4+</i>
<i>hhp1.Y169F.M84G-Δchk1</i>	<i>chk1::ura4+ hhp1::loxP-hhp1-M84G Y169F M84G-HA-loxM ade6-M216 leu1-32 ura4-D18</i>
<i>Δhhp1.ura4+-Δmus7::KAH</i> (1157)	<i>mus7::kanMX4 hhp1::ura4+ leu1-32 ura4-D18 ade6-M210</i>
<i>Δmus7::KAH</i> (1128)	<i>mus7::kanMX4 leu1-32 ura4-D18 ade6-M210</i>
<i>cdc2.1w</i>	<i>cdc2.1w ade6-M210 leu1-32 ura4-D18 ade6-L469-ura4+- ade6-M375</i>
<i>cdc2.1w Δsrs2</i>	<i>h⁻ cdc2.1w srs2::kanMX4 ade6-M210 leu1-32 ura4-D18 ade6-L469-ura4+- ade6-M375</i>
wild-type 804 (804)	<i>h⁻ ade6-M210 leu1-32 ura4-D18</i>
<i>Δchk1:u</i> (1174)	<i>h⁻ ade6-M210 chk1::ura4+ leu1-32 ura4-D18</i>
<i>cdc2.1w</i>	<i>h⁻ ade6-M210 leu1-32 ura4-D18 cdc2.1w</i>
<i>cdc25.22</i> (833)	<i>h cdc25.22 ade6-M210 leu1-32 ura4-D18</i>
<i>Δhhp1:H</i> (1824)	<i>h⁻ ade6-M210 hhp1::hphMX6 leu1-32 ura4-D18</i>
<i>Δwee1</i>	<i>h⁻ ade6-M210 wee1::ura4+ leu1-32 ura4-D18</i>
<i>hhp1-HA</i>	<i>h⁻ ade6-M210 leu1-32 ura4-D18 hhp1::hhp1-HA3-kanMX4</i>
<i>ku70-GFP-HA</i>	<i>h90 ade6-M210 leu1-32 lys1-131 ku70::ku70-GFPHA3-kanMX4</i>
<i>ku80-HA</i>	<i>h90 ade6-M210 leu1-32 ura4-D18 ku80::ku80-HA3-ura4+</i>
<i>srs2-Myc</i>	<i>h⁻ ade6-216 leu1-32 ura4-D18 srs2::srs2-HA3-kanMX4</i>
<i>cdc13-HA</i>	<i>h⁻ ade6-216 leu1-32 ura4-D18 cdc13::cdc13-HA3-ura4+</i>
<i>hhp1-GFP</i> (2262)	<i>h90 hhp1-GFP-kanMX4 ade6-216 leu1-32 ura4-D18</i>
<i>Δchk1</i>	<i>h90 ade6-M210 chk1::kanMX4 leu1-32 ura4-D18</i>
<i>Δrad3</i>	<i>h90 ade6-M210 rad3::ade6+ leu1-32 ura4-D18</i>
<i>Δcrb2:u</i>	<i>h90 ade6-M210 crb2::ura4+ leu1-32 ura4-D18</i>
<i>Δswi1:Leu</i> (1692)	<i>h⁻ swi1::LEU2 ura4-D18 leu1-32 ade6-M216</i>
<i>Δmrc1:G418R</i> (1499)	<i>h⁻ MRC1::KANMX ura4-D18 leu1-32 ade6-M216</i>
<i>Δhhp1:uΔswi1:L</i>	<i>h⁻ hhp1::hphMX4 swi1::LEU2 ura4-D18 leu1-32 ade6-M216</i>
<i>Δhhp1:u Δmrc1:KR</i>	<i>h⁻ hhp1::hphMX4mrc1::kanMX ura4-D18 leu1-32 ade6-M216</i>
<i>Δhhp1 Δku80</i>	<i>h⁻ hhp1::hphMX4ku80::ura4+ ura4-D18 leu1-32 ade6-M216</i>
<i>Δhhp1Δwee1.50</i> (1527)	<i>h⁻ hhp1::ura4+ wee1.50 ura4-D18 leu1-32 ade6-M216</i>
<i>Δhhp1Δcdc2.1w</i>	<i>h⁻ hhp1::ura4+ cdc2.1w ura4-D18 leu1-32 ade6-M216</i>
<i>hhp1.R180C</i> (1885)	<i>h⁻ hhp1-R180C ura4-D18 leu1-32 ade6-M216</i>
<i>hhp1.K40R</i> (1886)	<i>h⁻ hhp1-K40R ura4-D18 leu1-32 ade6-M216</i>
<i>rad4.116</i>	<i>h+ rad4-116, ura4-D18, leu1-32, ade6-M210</i>
<i>rad9.HA</i>	<i>h⁻ rad9::ura4+, ura4-D18, leu1-32, ade6-704</i>
<i>hus1.Myc</i>	<i>hus1::13myc, ura4-D18, leu1-32, ade6-704</i>
<i>rad3.KD</i> (918)	<i>rad3-KD, ura4-D18, leu1-32, ade6-704</i>
<i>cds1</i>	<i>cds1::ura4+, ura4-D18, leu1-32</i>

<i>rad9.Myc</i>	<i>rad9::13myc ura4-D18, leu1-32, ade6-704</i>
<i>cdc25.22</i>	<i>h- ura4.D18leu1.32</i>
<i>cdc25.22cdc2.1w</i>	<i>cdc25.22cdc2.1w ura4.D18 ade6.M210leu1.32</i>
<i>cdc2.1w</i> (834)	<i>h- ura4-D18 ade6.M210leu1.32</i>
<i>cdc2.1w</i>	<i>h+ ura4-D18 ade6.M210leu1.32</i>
Δ <i>chk1:u</i>	<i>Chk1::ura4.D18 ade6.M210leu1.32</i>
Δ <i>chk1 Cdc2.1w</i>	<i>Chk1::ura4 cdc2.1w D18 ade6.M210leu1.32</i>
Δ <i>ku70</i> (978)	<i>h- ku70::kan MX6 ura4.D18 ade6.M210leu1.32</i>
Δ <i>ku70</i>	<i>h+ ku70::kan MX6 ura4.D18 ade6.M210leu1.32</i>
Δ <i>mus81</i> (1053)	<i>h+ mus81::kan MX6 ura4.D18leu1.32</i>

Table: 2.1.8.1: List of *S. pombe* strains used in this study.

2.1.9. Oligonucleotides used in this study

Name	Sequence 5' to 3'
Hhp1-B3.1	CGAAGTTATGCATGCATCGCATGCTAACTTGCTCTACTTTTTTCGAACCCC
Rad9.S8.1	GCTATCACACTAGTTAGACTAGTCAGATCTATATTACCCTGTTATCCC
Hhp1-forward	ATCTTACAGGTAAGTCTGCTATGCTAGCATCAATAC
Hhp1-reverse	GTATTGATGCTAGCATAGCAAGCAGTACCTGTAAGAT
Hhp1-Microtubule.Del.forward	GTATCGTGATCACAAAACCTCCCTGGCTGCACCTCCCAAGGACAACAAGAATCTTACAG G
Hhp1-Microtubule.Del.reverse	CAGTACCTGTAAGATTCTTGTCTCCTTCGCAGGTCAGCCAGGTGAGTTTTGTGATCA C
Hhp1-Del.NLS.forward	CTGCCTTGGCAGGGATTGAAGGCTACCGAAAAGATTATGGAGAAGAAGATCTCTACGC
Hhp1-Del.NLS.reverse	GCGTAGAGATCTTCTTCTCCATAATCTTTTCGGTAGCCTTCAATCCCTGCCAAGGCAG
Hhp1-L41Q.forward	GGTGAAGAGGTCGCTATCAAGCAAGAATCAACTCGTGCTAAACAC
Hhp1-L41Q.reverse	GTGTTTAGCACGAGTTGATTCTTGTGATAGCGACCTCTTCACC
Hhp1-P49S.forward	TCAACTCGTGCTAAACACTCTCAATTGGAGTATGAATAC
Hhp1-P49S.reverse	GTATTCATACTCCAATTGAGAGTGTTTAGCACGAGTTGA
Hhp1-M82I.forward	TGTGATTACAACGCTATTGTGATGGATTTATTG
Hhp1-M82I.reverse	CAATAAATCCATCACAATAGCGTTGTAATCACA
Hhp1-Y277F.forward	CCACGAAAAAGCAAAAAGTTTGAAGAAGATTATGGAG
Hhp1-Y277F.reverse	CTCCATAATCTTTTCAAACCTTTTGCTTTTTTCGTGG
Hhp1-Y169F.forward	CACCTGCACATTCCTTTTCGCGAGAACAAGAATC
Hhp1-Y169F.reverse	GATTCTTGTCTCGCGAAAAGGAATGTGCAGGTG
Hhp1-L51Q.forward	GCTAAACACCCTCAACAAGAGTATGAATAC
Hhp1-L51Q.reverse	TCTGTATTCATACTCTTGTGAGGGTGTTC
Hhp1-S103R.forward	CAATCGAAAGTTTAGATTGAAAACAGTTC
Hhp1-S103R.reverse	CAAAAATTAACAAGTCTTCC

Hhp1-S183A.forward	CTTACAGGTAAGTGCACGCTATGCTGCTATC
Hhp1-S183A.reverse	AATACCTAAATGAGTATTGATAGCATAGCGTGCGTACCTGTAAG
Hhp1-M84G.forward	GATTACAACGCTATGGTGGGAGATTTATTG
Hhp1-M84G.reverse	GAAGGACCCAATAAATCTCCCACCATAGCG
Hhp1-K40R.forward	CTGGTGAAGAGGTCGCTATCCGTCTAGAATCAACTCGTGC
Hhp1-K40R.reverse	GCACGAGTTGATTCTAGACGGATAGCGACCTCTTCACCAG
Hhp1-R180C.forward	ATCTTAACAGGTAAGTGCCTGCTATGCTAGCATCAATAC
Hhp1-R180C.reverse	GTATTGATGCTAGCATAGCAAGCAGTACCTGTAAGAT
Hhp1-C-terminal.deletion.forward	CTATATGTTTGATTGGACCTTGAAGAGAAAGGGAGCTCAATATATCAACACACCTAAT
Hhp1-C-terminal.deletion.reverse	ATTAGGTCTGTTGATATATTGAGCTCCCTTTCTTCAAGGTCCAATCAAACATATAG

Table: 2.1.9.1: List of oligonucleotides f used in this study.

Note:- Some of *S.pombe* mutants were made but DNA sequencing had confirmed they are not right. They are: Hhp1. S103A; Hhp1. L41Q (those two are cancer mutants); Hhp1.MBD. deletion; Hhp1.NLS. deletion; Hhp1.Y169F; Hhp1.Y227F; and Hhp1.C-terminal.deletion.

2.2. Methods

2.2.1. Molecular biological and biochemical methods

2.2.1.1. Preparation of genomic DNA from *S. pombe* cells

YEA cultures *Hhp1.HA.wt* cells were grown overnight at 30 °C. Yeast cells were harvested at 3000 rpm in a Sorvall RT Legend benchtop centrifuge for 3-5 minutes. The supernatant was discarded and the pellet was resuspended in 20ml of dH₂O. The cells were spun again at 3000 rpm for 3-5 minutes and the pellet was resuspended in 1ml dH₂O, and then transferred to a 2ml screw-lid tube. Cells were pelleted at 12000rpm for one minute. 200µl DNA extraction buffer, 200µl Phenol:Chloroform:Isoamylalcohol (25:24:1), and 6-7 micro-spoons of silica or glass beads were added to the cell pellet. The mixture was shaken for 10-12 minutes on a cell disrupter vortex at maximum speed. The tube was spun at 12000rpm for five minutes, the aqueous phase was moved to an eppendorf tube and the genomic DNA was precipitated with 1ml ethanol 99% by spinning the mixture for five minutes at 12000rpm. The supernatant was discarded and the DNA pellet was resuspend in 400µl TE buffer containing 5µl RnaseA. The RNA was degraded for a period of 20-30 min at 37 °C. 1ml 99% ethanol and 40µl 4M ammonium acetate was added to precipitate the DNA. The tube was spun for five minutes at 12000rpm. The supernatant was

discarded and the tube was placed upside down on a paper tissue for several minutes. The dried pellet was resuspended in 100-500 μ l TE buffer and an aliquot was analysed on a 1% agarose gel (Figure: 2.2.1.1.1).

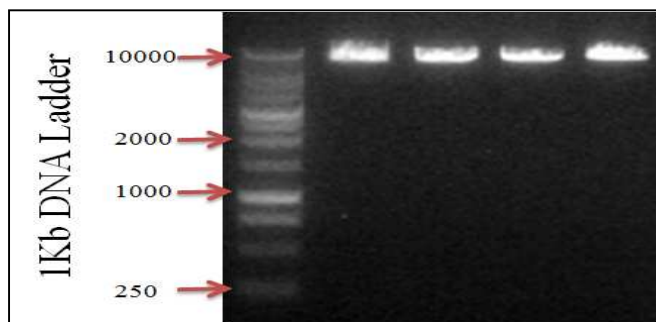


Figure: 2.2.1.1.1: Image of Hhp1 genomic DNA.

2.2.1.2. Polymerase chain reaction (PCR)

The reaction mixtures and cycles for the amplification of different fragments and to generate fusion PCR products were prepared on ice.

2.2.1.2.1. PCR to generate the *hhp1* mutants

One PCR reaction contains:

- 2 μ l genomic DNA
- 10 μ l 5X HF buffer (Finnzymes)
- 5 μ l 2mM dNTPs
- 0.5 μ l PhusionTM DNA polymerase (Finnzymes)
- 28 μ l dH₂O

A master mix was made and divided into several tubes and then the following pairs of primers were added to make fragment A and fragment B as following:

Tube A:

- 2.5 μ l Rad9.S8.1 and 2.5 μ l Hhp1-(specific nucleotide mutation)-Forward

Tube B:

- 2.5 μ l Hhp1.B3.1 and 2.5 μ l Hhp1-(specific nucleotide mutation)-Reverse

The PCR was run for 30 cycles.

Note:

1):- stock concentration 10 μ M for Rad9.S8.1 and Hhp1.B3.1 primers.

2):- heated lid of PCR machine is 98 $^{\circ}$ C, 30 cycles: 98 $^{\circ}$ C for ten seconds, 55 $^{\circ}$ C for thirty seconds, 72 $^{\circ}$ C for one and half minute, and hold at 10 $^{\circ}$ C.

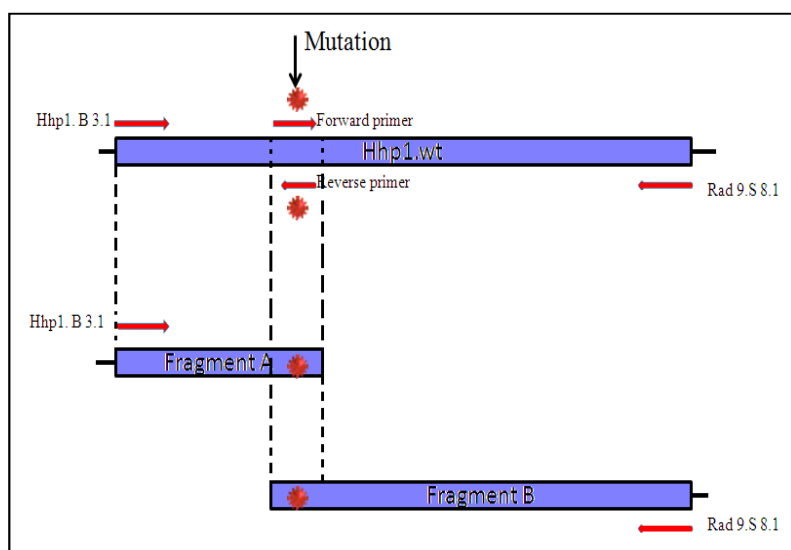


Figure: 2.2.1.2.1.1. Fusion PCR. The first PCR reaction amplifies two *hhp1* fragments which overlap by 25-25bp in the region of the desired mutation. The mutation is encoded in the internal primers. The two flanking primers contain a *Sph*I (forward) and *Spe*I (reverse) restriction site. The two fragments were purified and mixed with only the flanking primers present. This results in the full-length *hhp1* fragment which can be clone using the *Sph*I and *Spe*I sites.

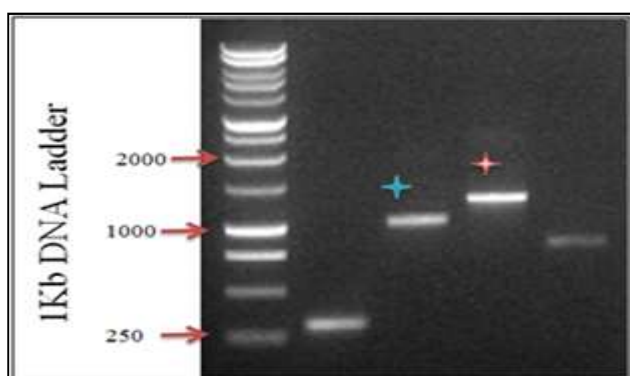


Figure: 2.2.1.2.1.2: Images of DNA fragments after purification of DNA, one fragment has size of about 1000-1200 Kb and related fragment has size of 1000-1200 Kb, so when they join together they suppose to give DNA size in about 1500Kb which is the estimated size for *Hhp1* with its HA tag.

2.2.1.2.2. Fusion PCR for such *Hhp1*-specific Mutant

Mix fragment A and fragment B to achieve creation of certain mutant as following:

5 μ l fragment A

5 μ l fragment B

10 μ l 5X GC buffer
 5 μ l 2mM dNTPs
 0.5 μ l PhusionTM DNA polymerase (Finnzymes)
 20 μ l dH₂O

The mix was placed in a PCR machine under these conditions: heated lid is 98 °C, 15 cycles: 98 °C for thirty seconds, 55 °C for thirty seconds, 72 °C for one and half minute, and hold at 10 °C.

After the PCR had finished , the following materials were added to each tube:

15 μ l 5X GC buffer
 10 μ l 2mM dNTPs
 1 μ l PhusionTM DN polymerase (Finnzymes)
 15 μ l dH₂O
 5 μ l Rad9.S8.1
 5 μ l Hhp1.B3.1 (PCR was run again for 35 cycles).

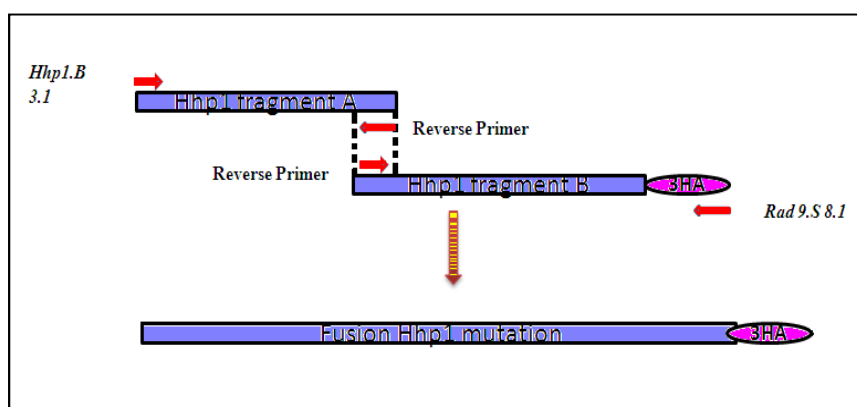


Figure: 2.2.1.2.2.1: Addition of the HA tag. The primer Rad9.S8.1 binds down-stream of the haemagglutinin tag sequence which is common to all integrated HA tags (653). Since the genomic DNA was prepared from a *hhp1-HA* strain, the PCR fragment contains the three HA epitopes.

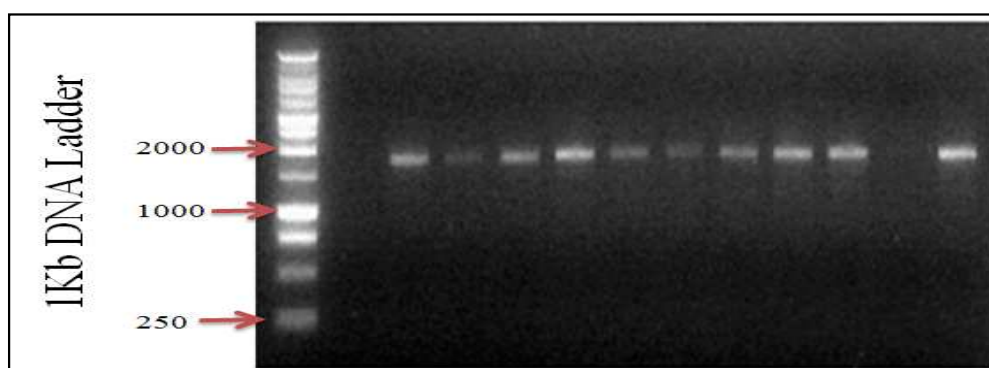


Figure: 2.2.1.2.2.2. An example image of purified *hhp1*-HA fusion PCR fragments

Note: after each PCR step DNA products were purified by using 1% agarose gel in 1x TAE buffer and GenElute™ Gel Extraction Kit (SIGMA Product Code NA1111) (Figure: 2.2.1.2.2.3).

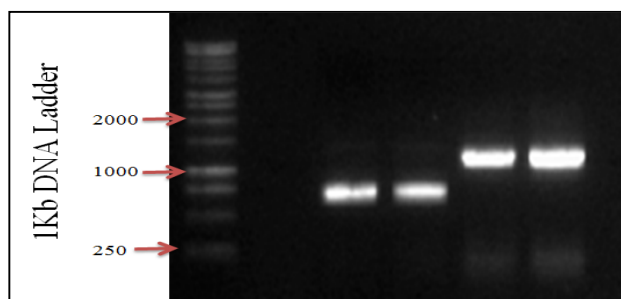


Figure: 2.2.1.2.2.3. Size of the PCR products after gel purification. 5 μ l of each PCR product was mixed with 2 μ l of DNA loading dye and loaded onto a 1% agarose gel. The two small fragments were fused to the larger size.

2.2.1.3. Elution of DNA fragments from agarose gels

DND fragments (fusion PCR products) were run on a 1% agarose gel with 0.5 μ g/ml ethidiumbromide. The DNA bands were cut out under blue light, and extracted from the gel slice using the GenElute® Gel Extraction Kit. Gel extractions were processed according to the manufacturers' instructions. The DNA eluted from the columns was stored at -20 °C (Figure: 2.2.1.2.2.3).

2.2.1.4. Restriction digest

Restriction enzymes and buffers were from New England Biolabs (NEB); reaction conditions were processed according to the manufacturer's recommendations.

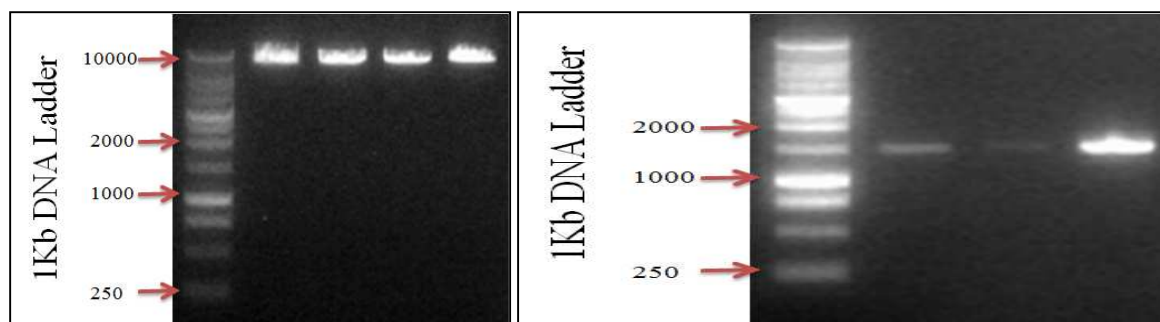


Figure: 2.2.1.4.1: Examples of digested pAW8 fragments (left panel) and purified fusion PCR products (i.e. *hhp1.M84G.Y227F*, *hhp1.M84G.NLS.deletion*, and *hhp1.M84G.Y169F* (right panel)

Note: the digest of the PCR products and the vector pAW8 were done according to New England BioLabs (NEB) (<https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions>).

2.2.1.4.1. Restriction Digest for the PCR Products (certain Hhp1 mutant)

30 µl of DNA (fusion PCR product)

1 µl Sph I

1 µl Spe I

5 µl NEB 2 (10x) buffer

15 µl ddH₂O

The reaction was incubated for 1-2 hours at 37 °C.

Note: Digest protocol with restriction enzymes is in this website (NEB): <https://www.neb.com/protocols/2014/05/07/double-digest-protocol-with-standard-restriction-enzymes>

2.2.1.4.2. Restriction Digest for the Vector pAW8

10 µl of DNA (fusion PCR product)

0.5 µl Sph I

0.5 µl Spe

5 µl NEB2 (10x) buffer

14 µl ddH₂O

The digested reaction was incubated for 1-2 hours at 37 °C incubator.

After the digest all DNA fragments were loaded onto a 1% agarose gel and purified in order to check for the right size of the products and to deactivate the restriction enzymes, which was also after the digest for 20 min at 70°C.

5µl of each sample was tested on a 1% agarose Gel in 1X TAE buffer.

Note: Restriction Digest Enzyme SpeI DNA sequence is provided from the NEB website (<https://www.neb.com/products/r0133-spei>) as below:

5' A CTAG T 3'
3' T GATC A 5'

Restriction Digest Enzyme SphI DNA sequence is provided from the NEB website (<https://www.neb.com/products/r0182-sphI>) as below:

5' G CATG C 3'
3' C GTAC G 5'

2.2.1.5. Ligation

Digested inserts and vector (pAW8 plasmid) were incubated with 10X DNA ligation buffer, one unit of T4 DNA ligase in a final volume of 20µl in a 16 °C water bath overnight:

- 5µl pAW8 plasmid (digested and purified)
- 5µl PCR product (digested and purified)
- 2µl (10x) DNA ligase buffer
- 1µl 10U/µl ligase enzyme
- 7µl ddH₂O

The rationale behind the Cre-Lox integration system is explained in Watson et. al. (258). The digested *hhp1* fragments were cloned into the unique SphI and SpeI sites of pAW8 (Figure: 2.2.1.5.1).

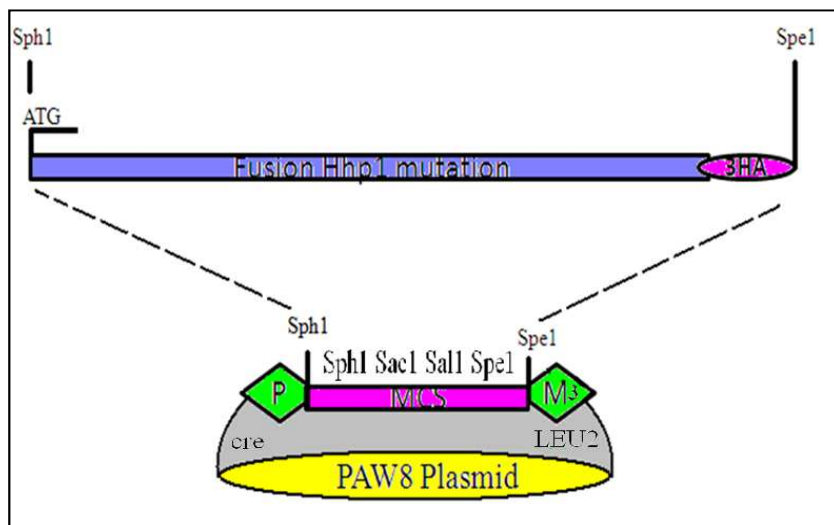


Figure: 2.2.1.5.1: The pAW8 Plasmid. Insertion of the mutated *hhp1* between the *loxP* (P) and *loxM3* (M3) recognition sequences for the Cre recombinase. The Cre/*lox* site-specific recombination enzyme is encoded by the *cre* gene on the plasmid. It will recognize the corresponding sites at the modified *hhp1* locus in the base strain and insert the fragment from the plasmid thereby removing the *ura4+* marker gene which resides between *loxP* and *loxM3* at the *hhp1* locus in the base strain 1495.

The *Cre/lox* site-specific recombination system is based on the sequence specific recombination between the identical *loxP* and *loxM3* sequences in the base strain and in the pAW8 plasmid. The Cre enzyme catalyses the exchange between the identical sequences thereby exchanging the *ura4* marker gene in the base strain for the mutated DNA fragment in pAW8 (Figure: 2.2.1.5.2). Loss of the active *ura4+* gene can be selected on 5-FOA plates as cells containing the active *ura4* gene convert the prodrug 5-Fluororotic (5-FOA) into a toxic drug (e.g. cells which underwent the exchange are *ura4* negative and 5-FOA resistant).

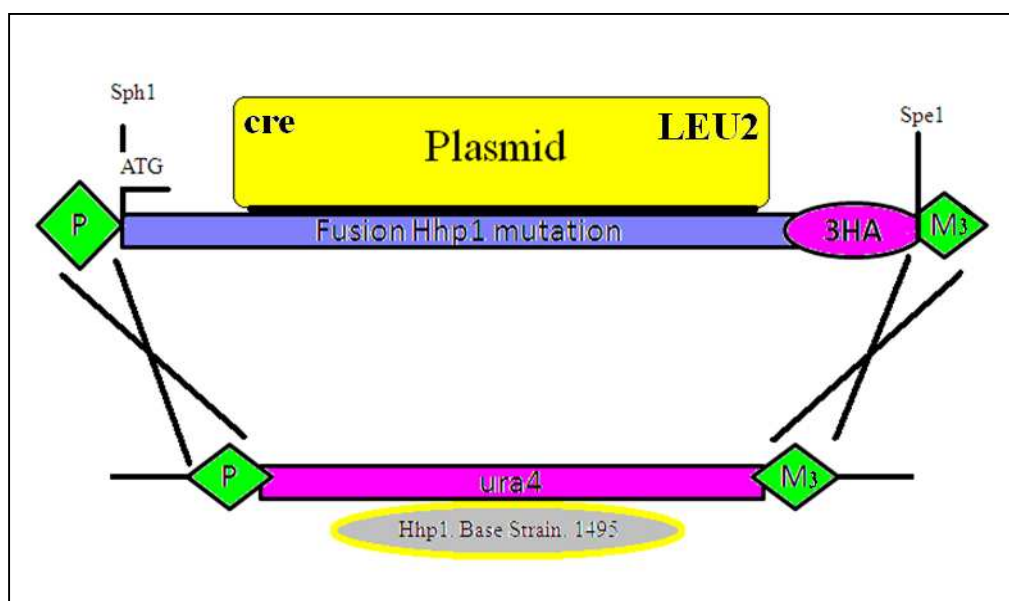


Figure: 2.2.1.5.2: The Cre-Lox cassette exchange system. See text for details (258).

2.2.1.6. Transformation of *E. coli* Top10 Competent Cells

50 μ l competent cells were used for one transformation. Cells were purchased from Bioline. Cells were thawed on ice and mixed by flicking the tube. 5 μ l of the ligation mixture was added to the cells, which were then incubated on ice for 30 minutes. After the heat shock at 42 °C for 45 seconds, cells were incubated for one minute on ice before 1ml of LB broth was added. Cells were then incubated for one hour in a 37 °C shaker at 200rpm. Cells were harvested by a 1 min spin at 12,000rpm and the cell pellet was plated on a LB+ampicillin plate. Plates were incubated at 37 °C overnight.

Note: Transformation is done by following steps in NEB website: <https://www.neb.com/protocols/1/01/01/transformation-protocol-c2528>

2.2.1.7. Preparation of plasmid DNA from *E. coli*

Plasmid DNA for cloning was prepared using the GenElute[®] Plasmid Miniprep Kit according to the manufacturer's instructions. Samples of 5 μ l from the final product were digested again with *Spe*I and *Sph*I to test the plasmid (Figure: 2.2.1.7.1).

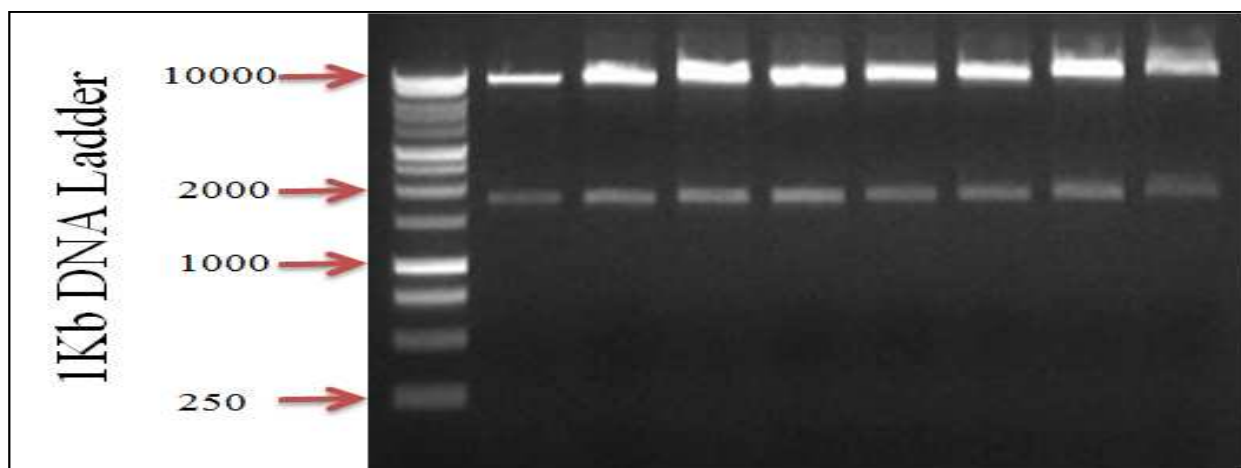


Figure: 2.2.1.7.1: Examples of pAW8 plasmids (about 8000kb) containing *hhp1* (approximately 1,500bp) inserts.

2.2.1.8. Transformation of *S. pombe* Cells

Cells (*Hhp1* base strain 1495) were grown in minimal medium overnight as this increases the transformation efficiency. Cells were harvested by centrifugation at 3000 rpm for three minutes. The supernatant was discarded and the pellets were washed with 20ml sterile water ddH₂O) and

centrifuged again. This step was repeated with transformation buffer. The cell pellet that was then suspended in 1ml transformation buffer and cells were harvested. 100µl aliquots of cells were transferred to an eppendorf tube and the plasmid DNA plus 2µl carrier DNA (10µg/ml sonicated & heated [5 min 95°C] salmon sperm DNA) were added. The samples were incubated for ten minutes at room temperature before adding 260 µl 40% PEG. Incubation was continued for one hour at 30 °C before 43µl DMSO were added. After five minute heat shock at 40-42 °C, cells were washed in 1ml of sterile water and plated on selective agar plates (minimal medium minus leucine, plus uracil and adenine). In case of counter selection for *ura* negative colonies, the transformed cells (*leu2+*) were replica plated onto a YEA plate with 5-FOA.

2.2.1.9. Total Cell Extract with Trichloroacetic acid (TCA)

For 1 sample 5×10^8 cells were harvested, washed in dH₂O, wash in 1ml 20% (w/v) trichloroacetic acid (TCA) and re-suspended in 200 µl of 20% TCA with 6-8 micro-spoon of silica or glass beads, and proteins were extracted on the fastprep machine.

The protein extract was moved to an eppendorf tube and proteins were pelleted for five minutes at 12,000rpm. The protein pellet was re-suspended in 150µl of 4x SDS sample buffer and 150 µl Tris-HCl pH8.8. Finally, the sample was heated at 95°C for 5 minutes and either stored at -20°C or used directly for further analysis (230).

2.2.1.10. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

SDS-PAGE gels were used to analyze/value protein extracts. Proteins were run through acrylamide gel and stopped at certain location which is indicated its size.

In this experiment two types of gels are used in top of each other, the stacking gel or focus gel with sample wells and the separation or resolving gel. The stacking Gel contains normally 4% acrylamide (Table: 2.1.10.1). The resolving gel (Table: 2.1.10.2) separates proteins according to their molecular weight (Table: 2.1.10.3). Large proteins required a small gel percentage (Table: 2.1.10.3).

Stacking Gel

Components	Gel percentage 4%
H ₂ O	7.5ml
1M TrisHCL pH6.8	1.5ml
20% SDS	50μl
40% Acrylamide	1ml
10% APS	100μl
TEMED	10μl

Table: 2.1.10.1: Recipe of stacking acrylamide gel for focusing stained protein samples.

Separation Gel

Reagent	Gel percentage			
	8%	10%	12%	15%
H ₂ O	8.5ml	7.5ml	6.5ml	5ml
1M TrisHCL pH8.8	7.5ml	7.5ml	7.5ml	7.5ml
20% SDS	150μl	150μl	150μl	150μl
40% Acrylamide	4ml	5ml	6ml	7.5ml
10% APS	100μl	100μl	100μl	100μl
TEMED	20μl	20μl	20μl	20μl

Table: 2.1.10.2: Recipe of resolving acrylamide gels in 10 ml volume for protein separations.

Protein Size (kDa)	Gel %
4 to 40	20%
12 to 45	15%
10 to 80	12%
15 to 100	10%
25 to 20	8%

Table: 2.1.10.3: Protein size range and required acrylamide concentrations.

Gels were run at 100V for 2-2:30 hours. The protein size standard from ThermoScientific (PageRuler Pre-stained Protein Ladder, cat.no.26616) was used.

After the run, proteins were transferred in 1x Transfer buffer onto nitrocellulose blotting membrane (Amersham HybondTM-Ecl) for 2hr at 65V or overnight at 10V for 12 hours. Free membrane space was blocked with Blocking buffer (3% Milk buffer: 3g Milk powder (fat-free),

10ml of 10x PBS, 0.05% Tween-20 and dH₂O up to 100ml) for at least 30-45 minutes at room temperature.

Incubation with the primary antibody was performed in blocking buffer at 4 °C on a rocking platform overnight. The membranes were sealed with 2ml antibody solution in a plastic bag.

Before incubation with the secondary antibody, membranes were washed three times with 1x washing buffer for 10 minutes. The secondary antibody was applied for 1-1:30 hours at room temperature in blocking buffer.

Finally, the membranes were washed three times with 1x washing buffer as previously, incubated in the Western Lightning® Plus-Enhanced Chemiluminescence substrate solution and exposed to an X-ray film (Exposure film) in a light tight developing cassette. The membrane was placed between two plastic sheets and the film was placed on top of the plastic. Films were developed using the X-ray film processor (MI-5, JENCONS-PLS).

Note: All Western blot steps are explained at this website:
<http://www.piercenet.com/method/overview-western-blotting>

2.2.1.11. Soluble Protein Extract

For one sample 5×10^8 cells were harvested, washed in dH₂O, and re-suspended in 300 μl of buffer H with inhibitors and with 6-8 micro-spoons of silica or glass beads. Proteins were extracted on the fastprep machine for 10 min. The protein extract was moved to an eppendorf tube and the insoluble material was pelleted for five minutes at 12,000 rpm. The protein supernatant was moved to a new tube and used for further analysis.

2.2.1.12. Isoelectric Focusing and 2D-PAGE (Two-dimensional electrophoresis)

Between 10-30μl of soluble protein extract were mixed to a final volume of 125μl of in Destreak™ rehydration solution with 0.2% of the corresponding IPG buffer (pH 3-10). Samples were loaded onto linear Immobiline™ DryStrip gels pH3-10 7cm (GE Healthcare) and run according to the manufacturer's specifications (468).

Running conditions: Biorad PROTEAN IEF cell; rehydration at 50V for 12 hours, followed by the rapid focusing program at 10,000Vh.

After the run, the strips were placed in a IPG tray and wash with 2 ml equilibration buffer I (6M urea 0.375M Tris-HCl (pH8.8), 2% SDS, 20% glycerol, 2% (w/v) DTT) for 10 minutes at room

temperature on a rocking platform, and then washed again for 10 minutes in equilibration buffer II (6M urea, 0.375M Tris-HCl (pH8.8), 2% SDS, 20% glycerol, 2.5% (w/v) Iodoacetamide). Strips were then placed on the top of a 10% resolution acrylamide gel and run under normal conditions (120V/2h) (229).

2.2.1.13. Taking Cell Images

Cells were grown in YEA broth overnight to logarithmic phase at 30 °C. 5×10^8 cells per sample were harvested and treated as required (e.g 40 μ M CPT at 30 °C for four hours). Cells were harvested and fixed in 300 μ l 99% methanol at room temperature. Cells can be stored at room temperature over a long period of time in methanol.

Cells were pelleted and washed three times with the staining solution (DAPI:Calcofluor) as described in (229).

10 μ l of cells were applied to a poly-L-lysine coated cover slip (64mm long) and covered with a second cover slip. Images were taken with a Nikon ECLIPSE TE2000-U fluorescence microscope (60x objective with oil) (262).

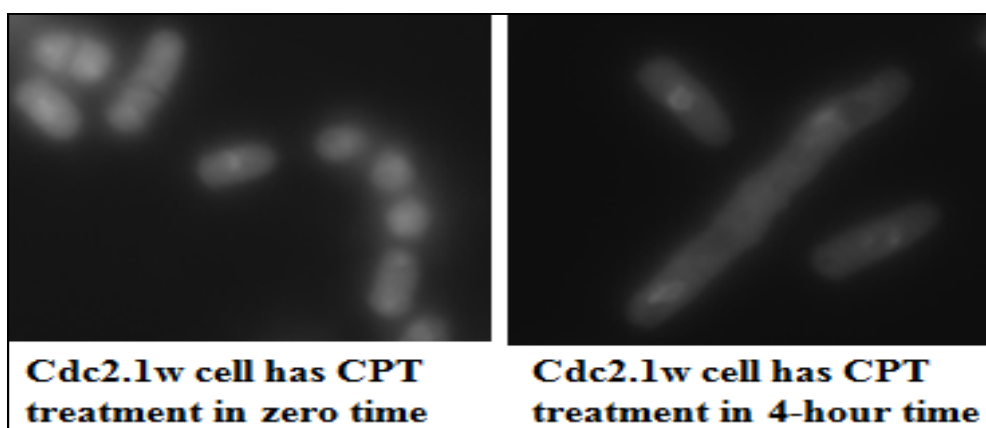


Figure: 2.2.1.13.1: Cell Images. Example images of untreated (left panel) and CPT treated *cdc2.1w* cells (right panel) The camptothecin (CPT) concentration was 40 μ M.

2.2.1.14. Lactose Gradient Centrifugation

Lactose gradients were performed as described previously (259) with the following changes. Cells were grown at 30°C in rich medium to a low cell number 10^6 – 10^7 cells/ml, and 5×10^8 cells were harvested from these cultures. Lactose gradients were centrifuged at 750 rpm for 7 min in a Sorvall RT Legend bench top centrifuge and small G2 cells were taken from the top of the cell

cloud. G2 cells were washed in rich medium and resuspended in 1 ml YEA medium. For the heat-induced G2 arrest, one sample (500 μ l) was mixed with 500 μ l YEA (30°C) and incubated at 30°C, whereas the second sample was mixed with 500 μ l YEA (40°C) and incubated at 40°C. For the CPT-induced G2 arrest, one sample (500 μ l) was mixed with 500 μ l YEA, whereas the second sample was mixed with 500 μ l YEA containing 80 μ M CPT. All samples were incubated at 30°C. 40 μ l aliquots were withdrawn in 20 min intervals and added to 200 μ l methanol. Cells were pelleted and stained with 30 μ l of a Hoechst (1:1000)-calcofluor (1:100) solution (stocks: calcofluor 1 mg/ml in 50 mM sodium citrate, 100 mM sodium phosphate pH 6.0; Hoechst 10 mg/ml in water) prior to scoring under a fluorescence microscope. 5 μ l of fixed and stained cells were placed on a microscope slide and examined under a fluorescent microscope. For each sample, 100 cells were counted and the percentage of the G1/S phase (separated cells without sign of separation) was scored (229, 230).

Number of tube	7% Lactose	30% Lactose
1	10	0
2	8.75	1.25
3	7.5	2.5
4	6.25	3.75
5	5	5
6	3.75	6.25
7	2.5	7.5
8	1.25	8.75
9	0	10

Table: 2.2.1.14.1: Preparation of the different lactose concentrations from a 7% (w/v) and 30% (w/v) stock solutions in YEA medium. 1ml from each mixture was overlaid (blue tip with narrow end cut off) with 1ml from the next highest concentration starting with 1ml 30% and finishing with 1ml 7% lactose.

2.2.2. Genetic Methods for *S.pombe*

2.2.2.1. The Acute DNA Sensitivity Assay

Cultures were grown in YEA broth at 30 °C overnight. The cell concentration was determined and cells were diluted in 10ml of YEA medium to 5x10⁴ cells/ml. From this dilution 500 μ l of cells were mixed with 500 μ l of 80 μ M CPT or 0.01% MMS. An initial aliquot of 75 μ l was taken and plated on one YEA plate (zero hour or start time (T=0)). All samples were then incubated in a 30 °C shaker at 200 rpm and further 75 μ l aliquots were taken at the indicated time points and plated on YEA plates. After 3 to 4 days the surviving colonies were counted (229, 230). If different MMS doses were tested, six eppendorf tubes were set up and 0, 5, 10, 15, 20, or 25 μ l of

1% MMS was added to 500µl cells. Cells were incubated for the indicated time and the MMS was inactivated by the addition of 500µl 2% sodium thiosulfate. 75µl aliquots were plated from each sample on one YEA plate.

2.2.2.2. The Chronic DNA Sensitivity Assay and Heat Test

Cultures were grown in YEA broth at 30 °C overnight. The cell number was determined and cells were diluted in 1ml of YEA medium to a final concentration of 1×10^7 /ml. A 10-times serial dilution was then prepared by diluting 100µl to 900µl YEA broth. From each dilution, 5µl of cells were dropped on YEA plates containing the indicated drug concentrations or no drug. Plates were incubated for 3 to 4 days and images were taken. To test for temperature-sensitive growth, YEA plates were incubated at 37°C instead of 30°C.

2.2.2.3. Strain Construction

To construction new strains by mating, two strains of the opposite mating type were mixed in a drop of sterile water (dH₂O) on a mating plate (Malt Extract Plate). The plate was then incubated at 25 °C for 2–4 days (Figure: 2.2.2.6.1). Successful mating was indicated by the formation of asci, which was confirmed by microscopic examination of samples taken from the mating plate. Spores were isolated from vegetative cells by incubating a small amount of cell material with asci in 500µl 30% ethanol for 20 to 30 minutes. 10-20µl of this solution was than plated on one YEA plate. After 3-4 days, the colonies formed by the surviving spores were further analysed to select the desired double mutant.

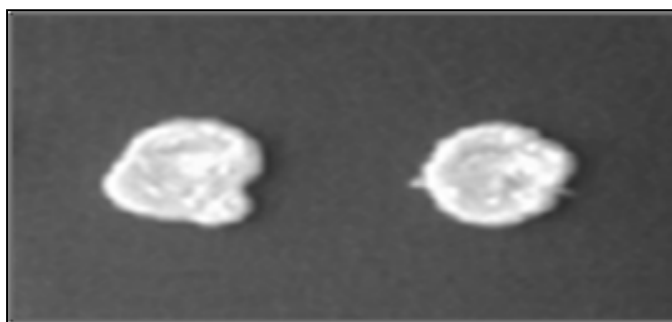


Figure: 2.2.2.3.1: Example of mating cell patches on a malt extract plate after 4 days at 25 °C.

Chapter 3: The Role of Hhp1 Kinase in the Repair of broken DNA Replication Forks

Chapter summary

The role of the dual-specific kinase Hhp1 (CK1) in the response to DNA lesions is not well understood. While early work in 1994 suggested a function for the kinase when chromosomes break in the presence of methyl-methanesulfonate (MMS), the cellular details remained so far unexplained. This chapter summarises genetic and cell cycle experiments which place Hhp1 kinase firmly in the regulatory network which controls the activity of the structure-specific endonuclease Mus81-Eme1. When DNA replication forks stall, Cds1 kinase is recruited to the inactive fork by the scaffold protein Mrc1 where the kinase inactivates the Mus81-Eme1 endonuclease to protect the fork. When replication forks break in the presence of the topoisomerase 1 inhibitor camptothecin (CPT), this inhibition needs to be reversed as Mus81 is important for the repair. The results of this chapter suggest that the Mrc1-Cds1 pathway primes Mus81-Eme1 for its phosphorylation by Hhp1 kinase. Hhp1 is predicted to phosphorylate Eme1 and that this modification is important for the activation of the endonuclease. The deletion of the *hhp1* gene is epistatic with loss of *cds1*, *mrc1*, *swi1* (Swi1 binds also to the Mrc1-Cds1 complex), *hsk1-1312* (Hsk1 kinase regulates the Mrc1-Cds1 complex), *mus81*, *mus 7* (Mus7 acts in the Mus81 repair pathway) and *chk1* (Chk1 phosphorylates Eme1) in the presence of CPT. The Hhp1 kinase may be important for the switch from the Cds1-dependent protective mode to the Chk1-dependent repair mode when cells exit S phase with a broken DNA replication fork. The repair activities of the Hhp1-Mus81 pathway are independent of the recombination protein Rad51, the DNA end binding protein Ku70 and the nuclease Ctp1.

The chapter also reveals a role for Hhp1 in cell cycle regulation. Cells without Hhp1 delay exit from a CPT-induced G2 arrest by 100 min to 120 min compared to the short delay (20 min-40 min) of wild type cells. The cell cycle activities of Hhp1 may be distinct from its DNA repair functions. Cells without Hhp1 are highly CPT and MMS sensitive.

3. Introduction

Previous work revealed sensitivities of *S.pombe* cells deleted for *hhp1* to the alkylating agent methyl-methanesulfonate (MMS), ionising radiation (IR) and chronic exposure to the replication inhibitor hydroxyurea (HU), but not to UV light (15). This led to the conclusion that Casein kinase 1 (Hhp1) is required for the repair of broken chromosomes. It transpired however later that MMS is unlikely to cause DNA double-strand breaks (DSBs) directly as the initial DNA lesion, methylated adenine and guanine, is excised by base excision repair (503) and that especially methylated adenine causes DNA replication stress (504). Since UV photoproducts interfere also with DNA replication (505), it is unlikely that Δ *hhp1* cells are defective in bypassing modified bases or in translesion synthesis during S phase. The sensitivity to chronic HU exposure, which breaks DNA replication forks (506), and to IR points more towards a role of Hhp1 kinase when DNA replication forks break. Such a link between CKI and DNA replication may also indicate a link between the circadian clock and DNA replication.

For example, in human the risk of breast cancer is increased among females who work night shifts (185), which could be explained by an accumulation of DNA replication linked mutations. Such a link is supported by the regulation of DNA replication by the metabolic clock in *S.cerevisiae* where replication does not take place under high oxidative conditions (507). Interestingly, the intra-S DNA checkpoint kinase Rad53 (Cds1, Chk2) is essential for this regulation in budding yeast. Supporting evidence for a role of CKI in the response to DNA replication stress comes from work in *Xenopus* where CKI γ I phosphorylates a domain in the scaffold protein Claspin (Mrc1) which activates Chk1 kinase at damaged replication forks (267). In higher eukaryotic cells, Chk1 kinase protects stalled forks from breakage (508), a role which is covered by Cds1 kinase in *S.pombe* (509). Interestingly, Chk1 phosphorylates at CK1 δ at serine 328, 331, 370, and threonine 397 as well as the human CK1 δ variants 1 and 2 which results in a decrease in CKI kinase activity (268). Mammalian Chk1 acts as a priming kinase for CKI α to prepare Cdc25A phosphatase for degradation upon activation of the DNA damage checkpoint in G1/S (269).

3.1. *S.pombe* cells without Hhp1 are sensitive to DNA Replication Fork Damage caused by the Topoisomerase 1 Inhibitor Camptothecin

To test whether Hhp1 acts on broken DNA replication forks, wild type, $\Delta hhp1$ and $\Delta cds1$ cells were incubated with the topoisomerase 1 poison camptothecin (CPT) which breaks DNA replication forks (510) either on plates (Figure: 3.1.1) or of a period of 5 hours (Figure: 3.1.2).

While cells without Cds1, which only acts on intact but stalled forks, were CPT resistant, cells devoid of Hhp1 were highly sensitive. This supports the conclusion that CKI performs important functions when DNA replication forks break. This experiment revealed also a slow growth phenotype of $\Delta hhp1$ cells at 37 °C even in the absence of CPT.

Consistent with the work by Dhillon N, and Hoekstra M (15), $\Delta hhp1$ cells are sensitive to methyl-methanesulfonate (MMS) on plates (Figure: 3.1.3) and when exposed to 0.05% for five hours (Figure: 3.1.4). Interestingly, $\Delta cds1$ cells were not MMS sensitive at 0.01% MMS on plates (Figure: 3.1.3), but displayed a sensitive when exposed to 0.05% for 5 hours (Figure: 3.1.4) which is in line with the limited requirement of Cds1 kinase for the response to alkylated bases (511).

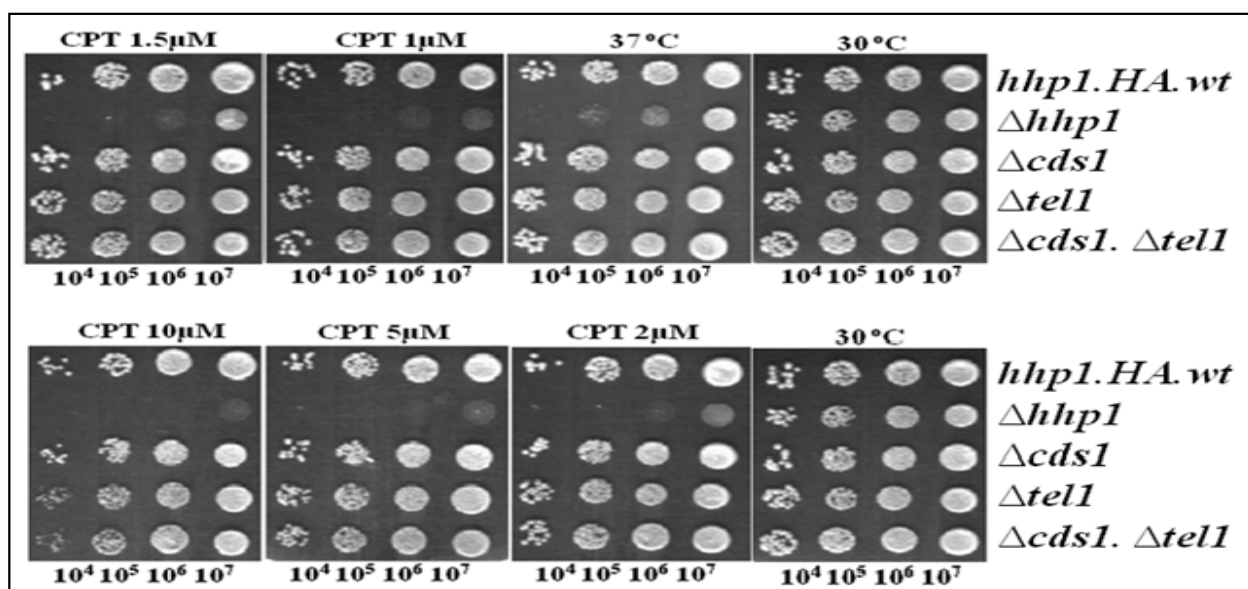


Figure: 3.1.1: Cells without Hhp1 kinase are highly camptothecin (CPT) sensitive. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. Cells lacking Tel1 (ATM) kinase or Tel1 and Cds1 kinase were also included.

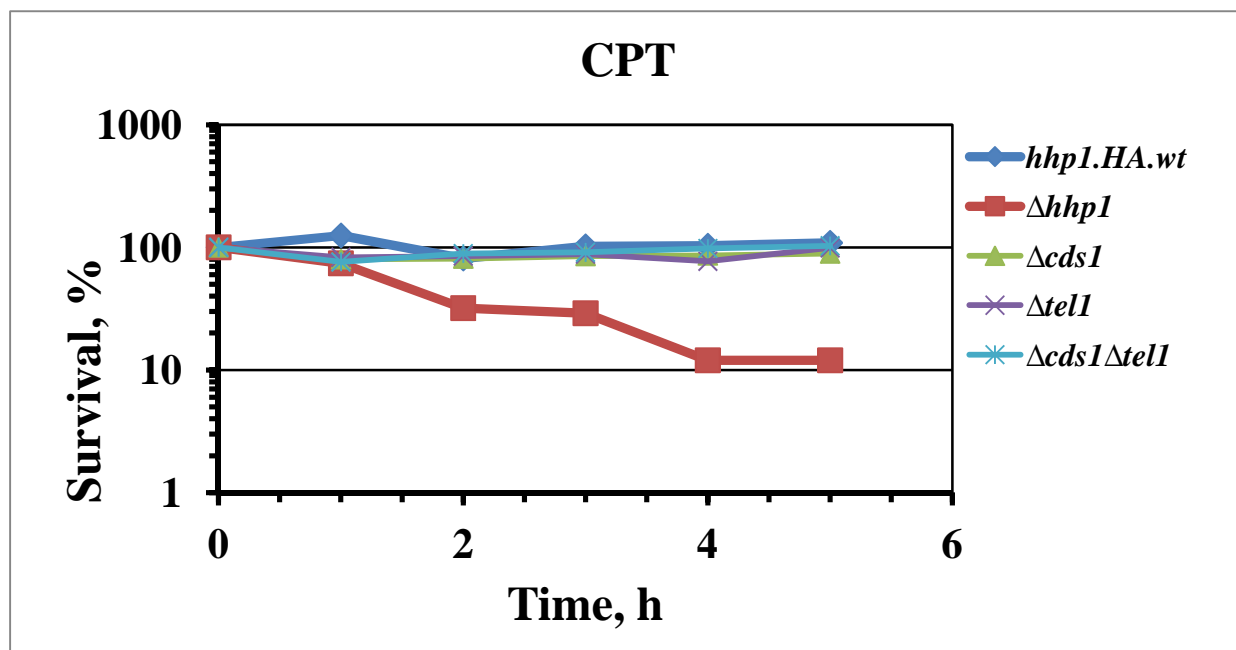


Figure: 3.1.2: Acute camptothecin (CPT) survival test. The indicated yeast strain cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40μM CPT and incubated for five hours at 30 °C. Aliquots of 75 μl were collected every hour and plated on one YEA plate.

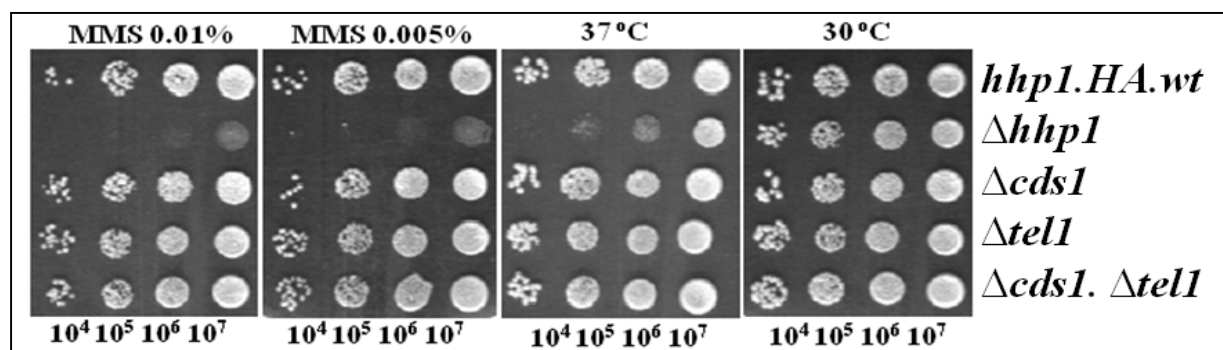


Figure: 3.1.3: Cells without Hhp1 kinase are highly methyl-methanesulfonate (MMS) sensitive. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. Cells lacking Tel1 (ATM) kinase or Tel1 and Cds1 kinase were also included.

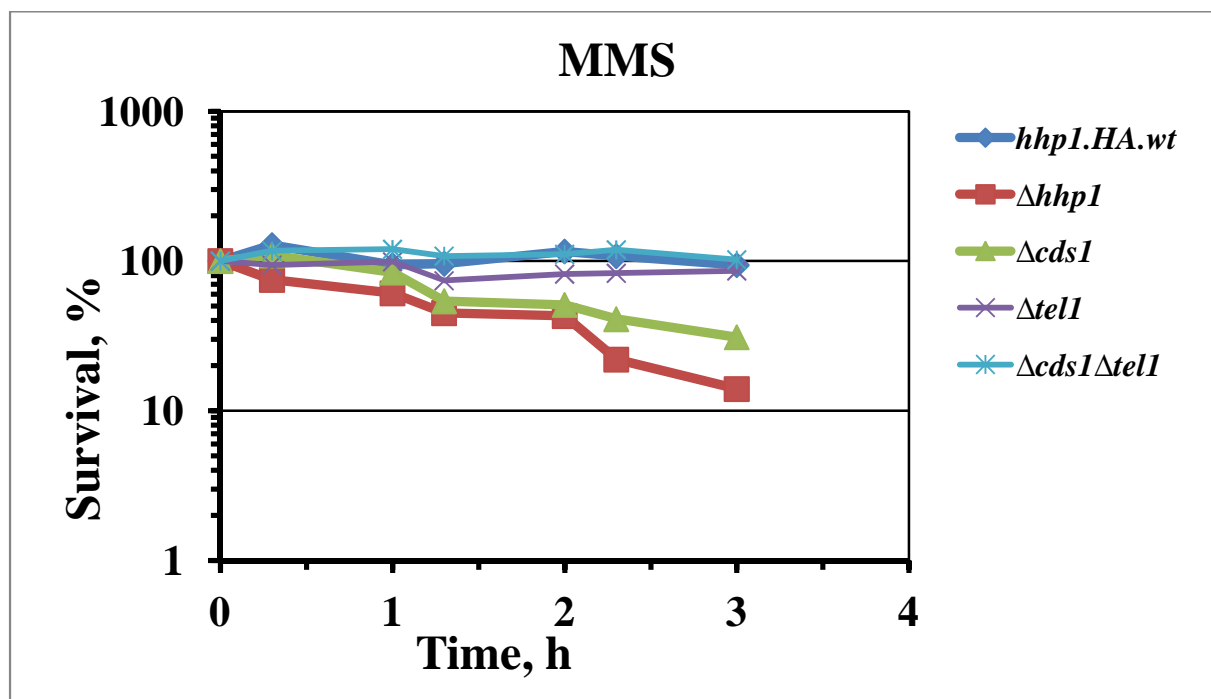


Figure: 3.1.4: Acute methyl-methanesulfonate (MMS) survival test. The indicated strains were exposed to 0.05% MMS for three hours. Aliquotes were withdrawn and plated on rich medium plates every 30 min. Plates were incubated for 4 days at 30°C and colonies were counted.

To test the effect of broken replication forks on cell cycle progression, wild type (*hhp1-HA*) cells expressing a C-terminally HA (haemagglutinin)-tagged Hhp1 kinase from its endogenous locus and $\Delta hhp1$ cells were synchronised in early G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT. As shown in [Figure: 3.1.5.A](#), *hhp1-HA* cells delay only for 20 min entry into the second cycle when replication forks break. A similar short delay has been reported in *S.cerevisiae* where CPT-induced breaks are repaired in G2 ([234](#)). In contrast, cells without Hhp1 delayed for 120 min in CPT medium ([Figure: 3.1.5.B](#)). The extended G2 delay of $\Delta hhp1$ cells could be caused by a repair defect when DNA replication forks collide with the immobilised topoisomerase 1 enzyme in the previous S phase (first peak of septation) or it could be caused by a delayed re-activation of the cell cycle machinery (i.e. Cdc2 kinase).

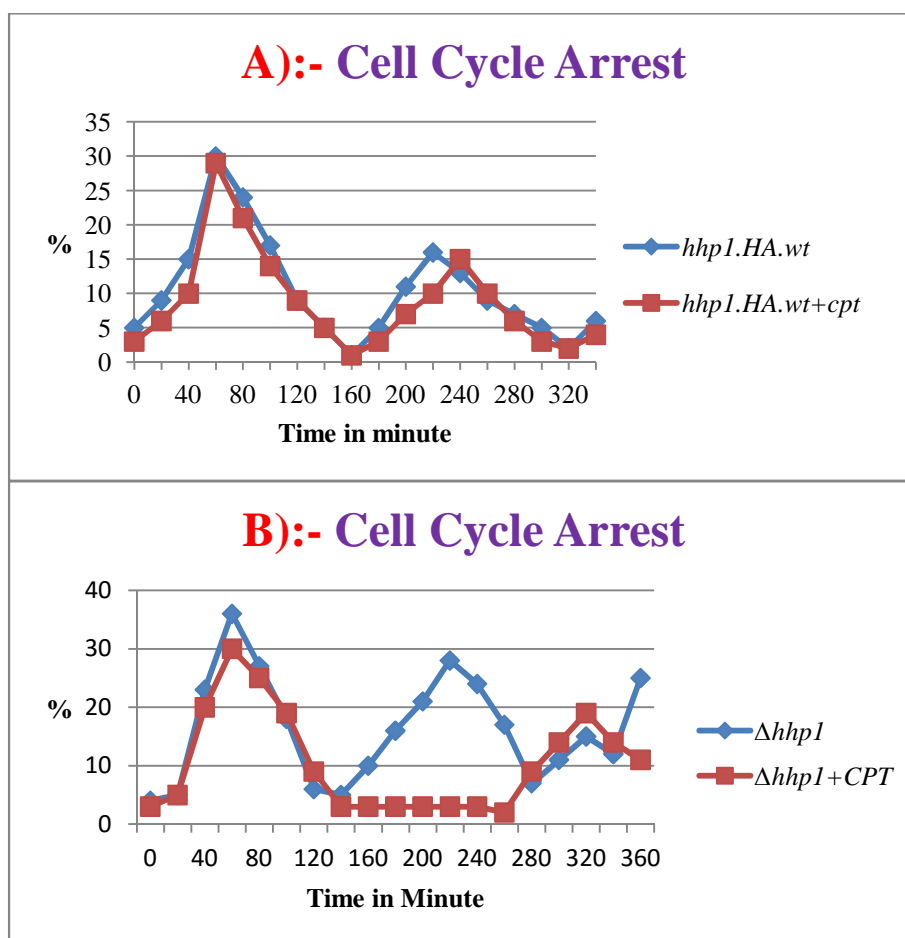


Figure: 3.1.5: Cell cycle for *S.pombe* strains. A: Hhp1-HA wild type cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). B: Cells without Hhp1 (Δ *hhp1*) were treated in the same way. Δ *hhp1* cells have an extended G2 delay compared to wild type *hhp1.HA.wt* cells.

3.2. Hhp1 Kinase and Tel1 (ATM) Kinase act in Parallel Pathways

Since Tel1 (ATM) kinase responds to unprocessed DNA double-stranded breaks after its recruitment by the Rad50-Mre11-Nbs1 (MRN) complex (512), the requirement of Tel1 for the response to CPT-induced DNA breaks was analysed in Δ *tel1* and Δ *tel1 Δ *hhp1* cells. As shown in Figure: 3.2.1, cells without Tel1 are only very mildly CPT sensitive suggesting a minor role of this checkpoint kinase when DNA replication forks break. The increased CPT sensitivity of the Δ *tel1 Δ *hhp1* double mutant compared to the Δ *hhp1* single mutant shows that both kinases act in two parallel repair pathways under these conditions. In line with a minor role of Tel1 in the response to broken replication forks, G2-synchronised Δ *tel1* cells arrest for approximately 40 min in CPT medium (Figure: 3.2.2), 20 min longer than wild type cells, but 100 min shorter than**

Δhhp1 cells. The *Δtel1Δhhp1* double mutant displayed an extended G2 arrest in CPT medium (80 min) that was somewhat shorter compared to the *Δhhp1* single mutant (120 min) (Figure: 3.2.3) which could suggest that Tel1 plays a role in the extended G2 arrest in the absence of Hhp1 kinase.

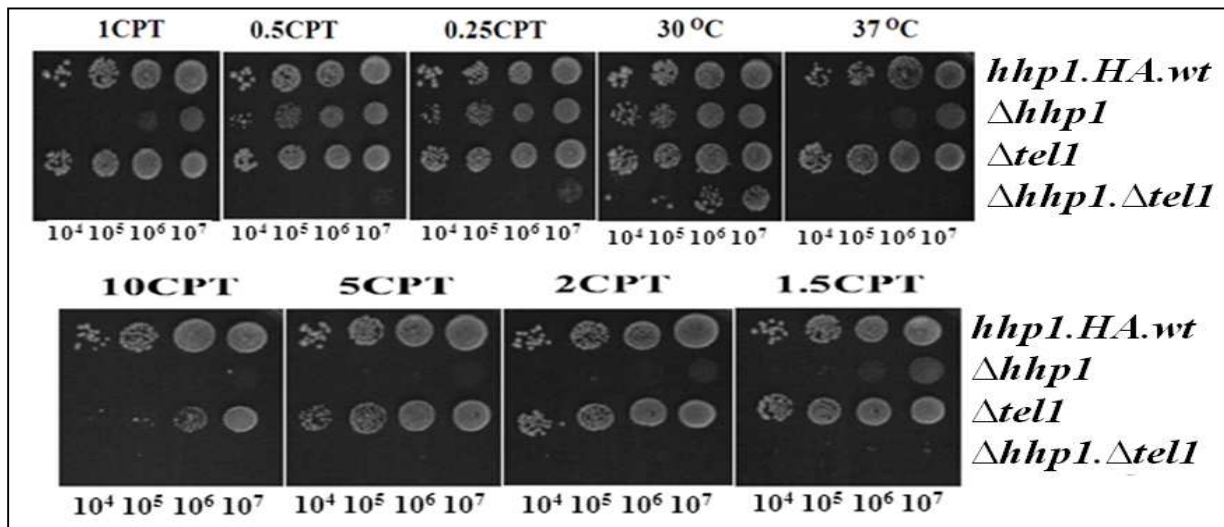


Figure: 3.2.1: Tel1 kinase and Hhp1 kinase act in two parallel pathways in the presence of camptothecin (CPT). Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. *S.pombe* cells without Hhp1 and Tel1 cannot grow in CPT medium.

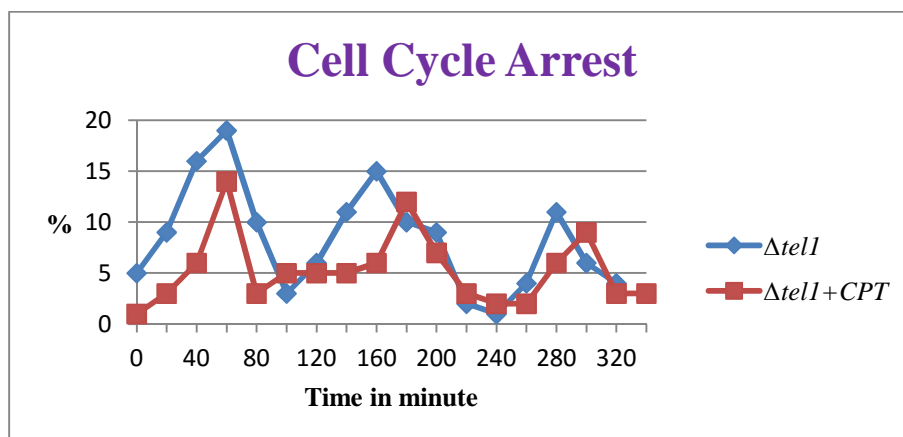


Figure: 3.2.2: *Δtel1* G2 arrest. Cells without Tel1 (*Δtel1*) were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40μM camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

The extended G2 arrest in the absence of Hhp1 kinase strongly implies a role of CK1 in the repair of broken DNA replication forks. The conclusion that Tel1 and Hhp1 act in two parallel repair pathways extended as well to MMS as indicated by the increased sensitivity of the $\Delta tel1 \Delta hhp1$ double mutant (Figure: 3.2.4). This parallel activity extended also to the slow growth at 37°C of $\Delta hhp1$ cells which was worsened by the deletion of *tel1*.

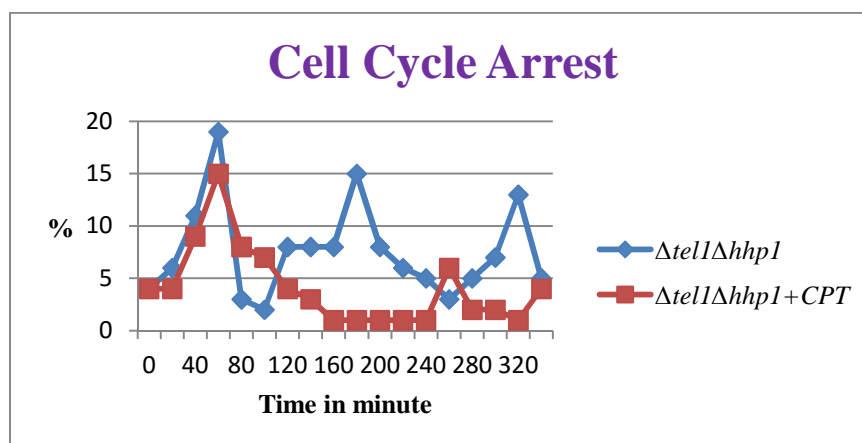


Figure: 3.2.3: $\Delta tel1 \Delta hhp1$ G2 arrest. Cells lacking both Tel1 and Hhp1 ($\Delta tel1 \Delta hhp1$) were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

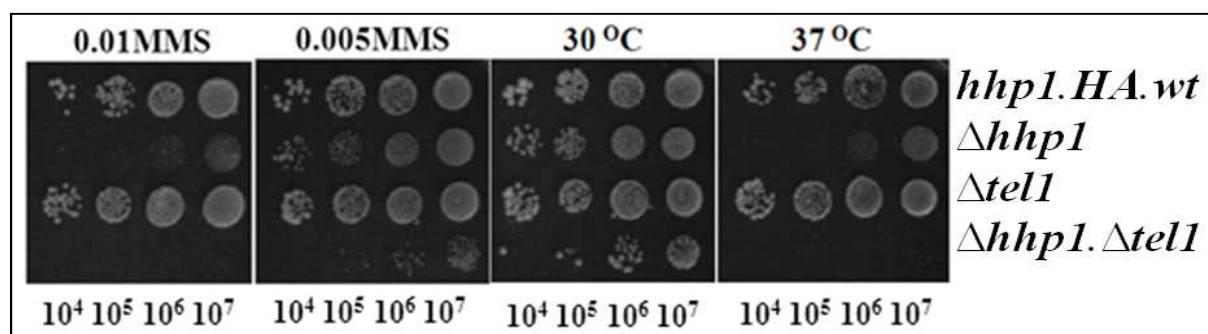


Figure: 3.2.4: testing Tel1 kinase and Hhp1 kinase. Tel1 kinase and Hhp1 kinase act in two parallel pathways in the presence of methyl-methanesulfonate (MMS). Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C.

3.3. Loss of Cds1 Kinase partly reduces the CPT and Heat Sensitivity, but has no Impact on the extended G2 Arrest in CPT Medium

Currently there is only indirect evidence linking Tel1 and Cds1 kinase in *S.pombe* since Tel1 phosphorylates the scaffold protein Mrc1 (Claspin) which recruits Cds1 to stalled DNA

replication forks (352). Cds1 was considered because its human paralogue Chk2 acts downstream of ATM kinase at broken chromosomes (513).

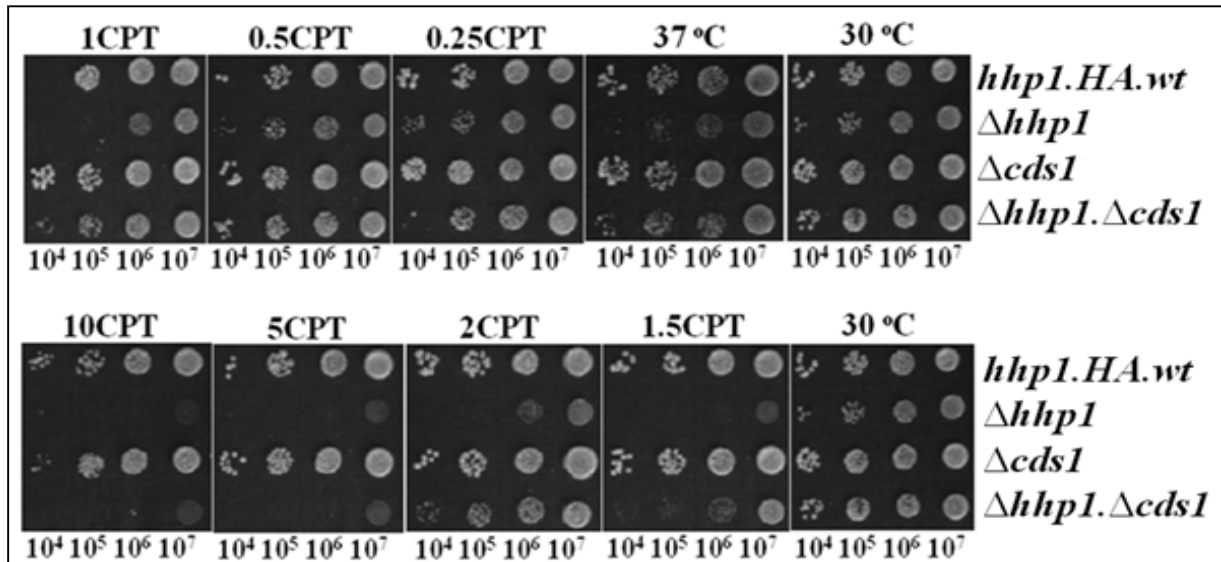


Figure: 3.3.1: Loss of Cds1 reduces the CPT (camptothecin) and heat sensitivity of cells without Hhp1 kinase. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. The data reveals epistatic genetic association between Hhp1 and Cds1.

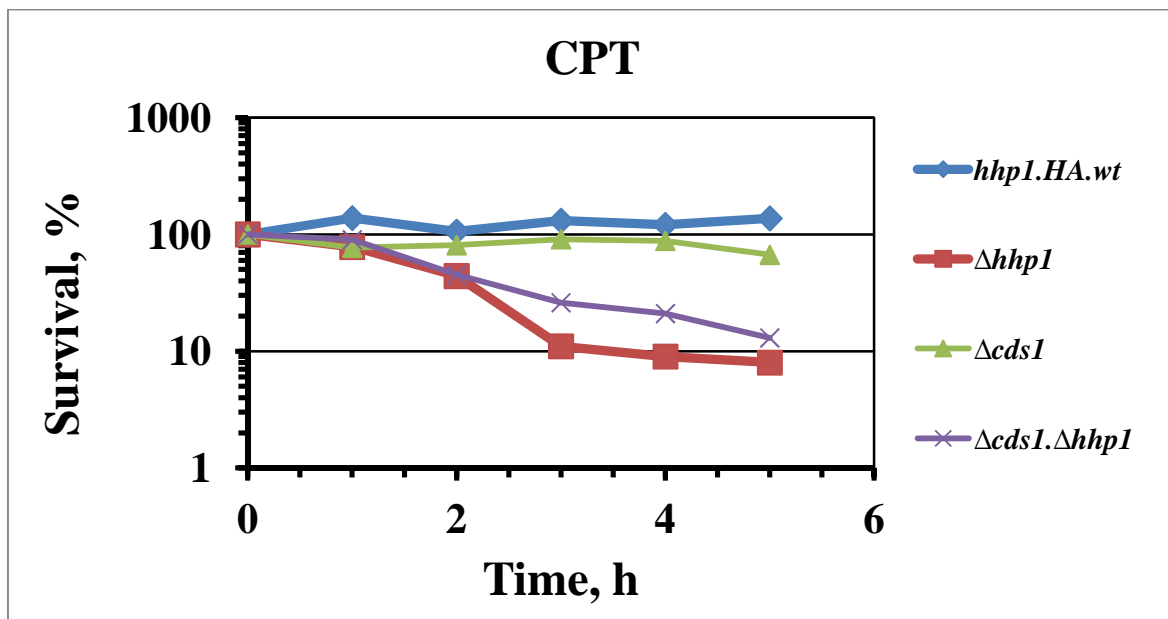


Figure:3.3.2: CPT (camptothecin) acute genetic survival test. Loss of Cds1 kinase in *S.pombe* cells renders cells less sensitive to CPT-induced DNA damage.

Unexpectedly, deletion of *cds1* in Δ *hhp1* cells partially reduced the temperature and CPT sensitivity (Figure: 3.3.4). The suppression of the CPT sensitivity was limited to concentrations of 2 μ M CPT or less. Loss of Cds1 on its own had no impact on cell survival as expected from its role at stalled, but not at broken forks (509). A similar weak reduction in CPT sensitivity was also evident when the same strains were exposed to 40 μ M CPT for 5 hours (Figure: 3.3.2). A similar suppression was not observed in the presence of the DNA alkylation agent MMS (Figure: 3.3.4, Figure: 3.3.5).

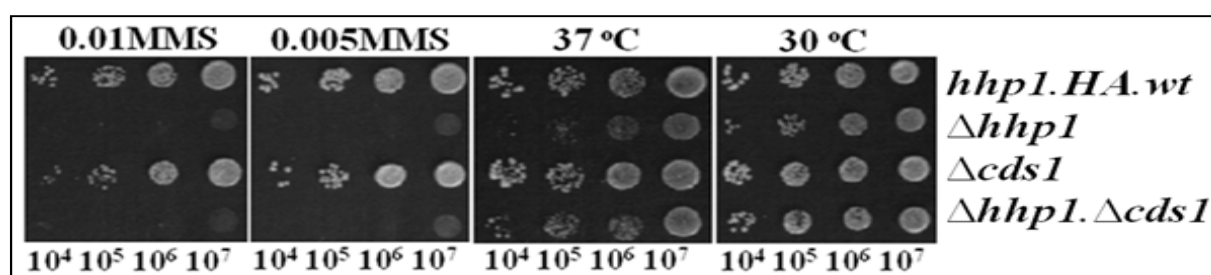


Figure: 3.3.4: Loss of Cds1 does not reduce the methyl-methanesulfonate (MMS) of cells without Hhp1 kinase. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C.

To test whether loss of *cds1* would also reduce the CPT-induced G2 arrest of Δ *hhp1* cells, the Δ *cds1.Δhhp1 double mutant was synchronised in G2 and released into rich medium with and without CPT. As shown in Figure: 3.3.6, inactivation of Cds1 had no real impact on the extended G2 arrest as the double mutant arrested for approximated 100 min (Δ *hhp1*: 120 min; Figure: 3.1.5).*

Taken together, these observations point towards a role of Cds1 kinase upstream of Hhp1 at low CPT concentrations and under heat stress conditions. Since heat effectively blocks S-phase progression without causing DNA breaks (514), it might be possible that DNA replication only slows down in the presence of low CPT concentrations. Cds1 kinase would then be required to stabilise the replication forks. When forks break at higher CPT concentrations (above 2 μ M CPT in plates), Cds1 would no longer be required since Chk1 would be activated (236). Since CKI kinase often require a priming kinase, Cds1 might act as such priming kinase on a yet unknown substrate which is later modified by Hhp1 when forks stall under heat stress conditions or at low CPT concentrations.

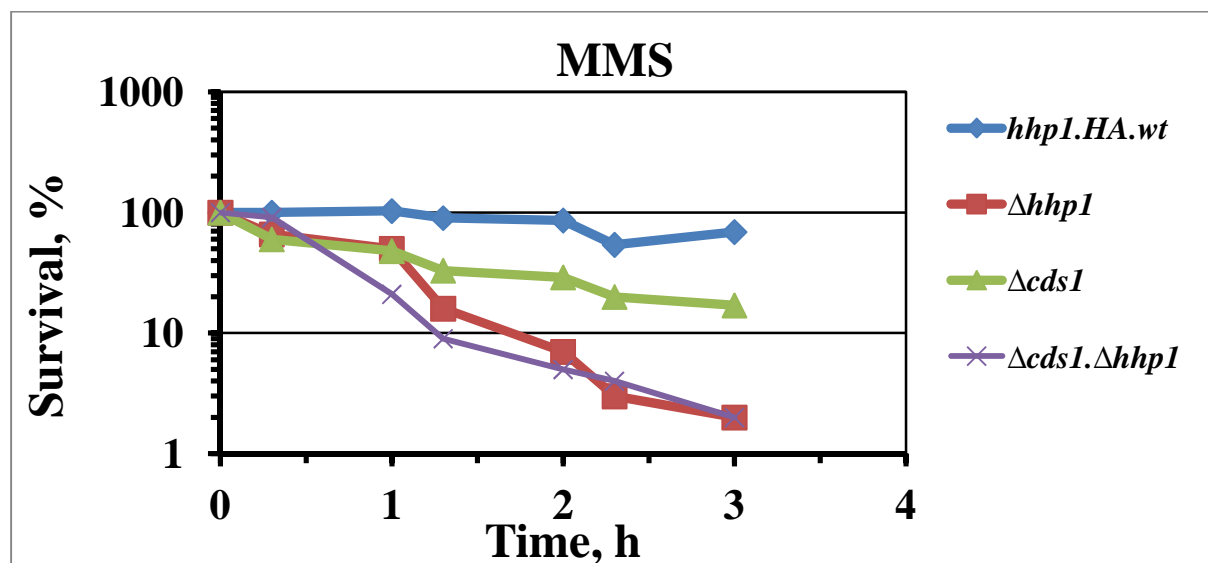


Figure 3.3.5: Acute methyl-methanesulfonate (MMS) survival tested $\Delta hhp1.\Delta cds1$. The indicated strains were exposed to 0.005% MMS at 30°C for the 3-hour. Aliquotes were withdrawn every 30 min and plated on rich medium plates. Plates were incubated for 4 days at 30°C and colonies were counted. As response to MMS-DSBs *hhp1* and *cds1* are acting in similar DNA repair pathway.

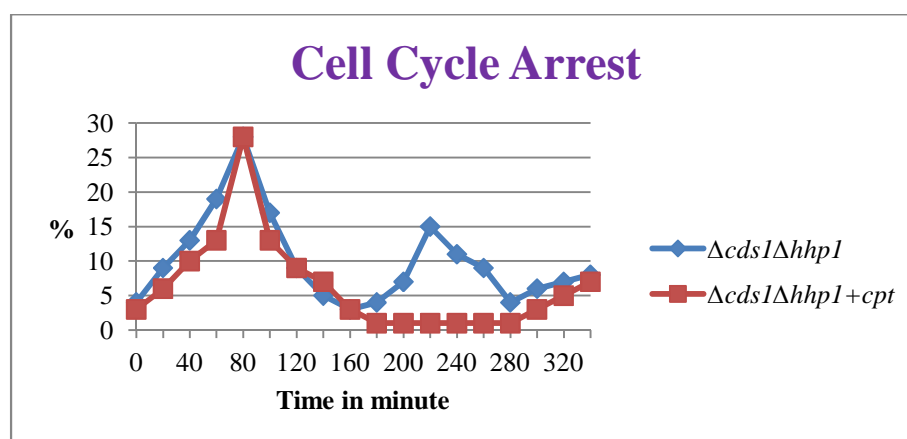


Figure 3.3.6: $\Delta cds1.\Delta hhp1$ cell cycle arrest. Loss of Cds1 does not affect the extended G2 arrest in the absence of Hhp1. Cells lacking both Cds1 and Hhp1 ($\Delta cds1.\Delta hhp1$) were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

3.3.1. Hhp1 Kinase acts jointly with Mus81 Endonuclease and the DNA Repair Protein Mus7

Given that Cds1 phosphorylates Mus81 to protect stalled DNA replication forks from cleavage (509), the endonuclease Mus81 could be a target of Hhp1 kinase (Figure: 3.3.2.5). To test this idea, the deletion of *hhp1* was combined with gene deletions of *mus81* and *mus7*.

Mus7 is a large protein (1888aa, 217.43 kDa) with unknown function which acts jointly with Mus81 when DNA is methylated by methyl-methanesulfonate (MMS) (257). As shown in [Figure: 3.3.1.1](#), deletion of *hhp1* in cells without Mus81 ($\Delta hhp1\Delta mus81$) did not further increase the high camptothecin (CPT) sensitivity of the $\Delta mus81$ single mutant. Consistent with the conclusion that Hhp1 kinase and Mus81 endonuclease act in the same CPT response pathway, deletion of *mus7* in the $\Delta hhp1\Delta mus81$ double mutant ($\Delta hhp1\Delta mus81\Delta mus7$) did not further increase the CPT sensitivity. This epigenetic relationship was confirmed in an acute survival test when cells were exposed to 40 μ M CPT for 5 hours ([Figure: 3.3.1.2](#)). The close functional link between the kinase and the endonuclease extended also to the DNA methylating drug MMS ([Figure: 3.3.1.3](#)). To confirm this important finding, the deletion of *hhp1* was combined with a deletion of *mus7* ($\Delta hhp1\Delta mus7$) and the tests were repeated. As in the case of the $\Delta hhp1\Delta mus81$ strain, the $\Delta hhp1\Delta mus7$ double mutant was as CPT sensitive as the $\Delta mus7$ single mutant ([Figure: 3.3.1.5](#), [Figure: 3.3.1.6](#)). In some experiments ([Figure: 3.3.1.1](#), [Figure: 3.3.1.5](#)), the $\Delta hhp1\Delta mus7\Delta mus81$ triple mutant was slightly more CPT sensitive especially at higher CPT concentrations. While this is not arguing against an epigenetic relationship between the three proteins, the triple mutant may however be defective in an additional repair response which becomes more important at higher CPT concentrations. Interestingly, Dehé P et al. (515) stated that 'Mus81-Eme1 activation prevents gross chromosomal rearrangements in cells lacking the BLM-related DNA helicase Rqh1'. However, according to [Figure: 3.3.1.7](#), and [Figure: 3.3.1.8](#) Hhp1 kinase and Rqh1 are more likely to act in different pathways in the presence of CPT since the $\Delta rqh1\Delta hhp1$ double mutant is more sensitive compared to the corresponding single mutants ([Figure: 3.3.1.8](#)).

In summary, these results place Hhp1 kinase in the Mus81-Mus7 DNA damage repair pathway when DNA replication forks break in the presence of the topoisomerase 1 inhibitor CPT or when replication forks encounter a methylated DNA template (MMS). Since Hhp1 and Cds1 are also epistatic ([Figure: 3.3.1](#)), it is possible that Cds1 acts as a priming kinase for Hhp1 to regulate Mus81 endonuclease ([Figure: 3.3.2.5](#)). When DNA replication stalls, Cds1 phosphorylates Mus81 to remove it from the nucleus (509). This inhibition of Mus81 could be reversed by the subsequent phosphorylation of Mus81 by Hhp1. Since active Mus81 forms a complex with Eme1 and because Eme1 undergoes regulatory phosphorylations by Rad3 and Cdc2 kinase (515), it could well be that Hhp1 modifies Eme1 instead of Mus81 ([Figure: 3.3.2.5](#)).

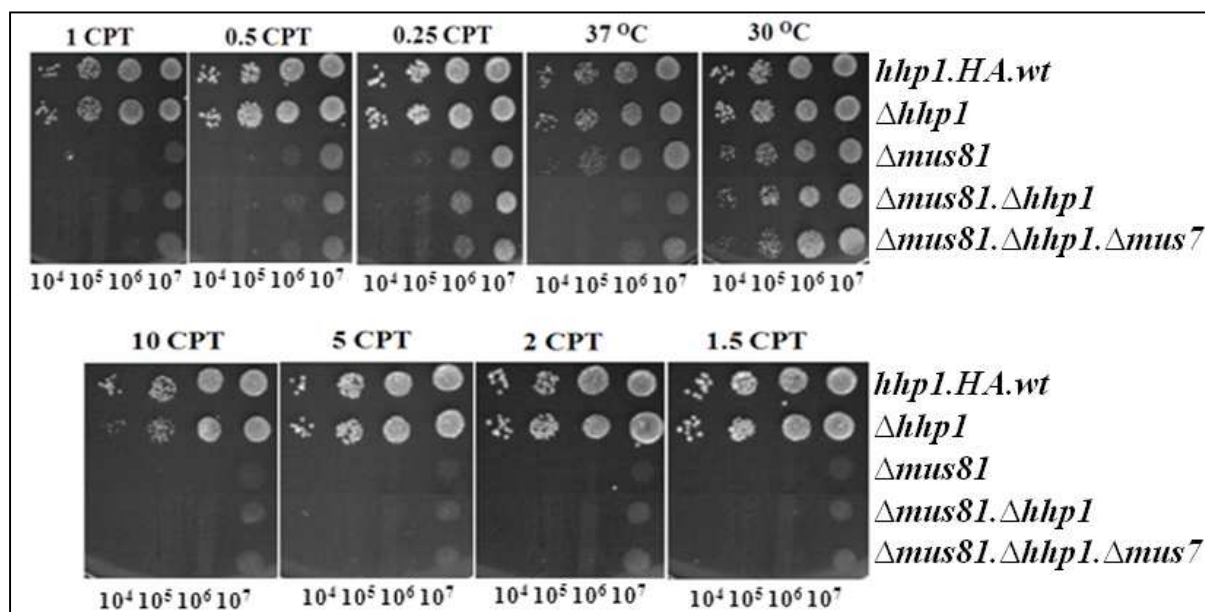


Figure: 3.3.1.1: Mus81, Mus7 and Hhp1 act in the same CPT response pathway. Drop test with the indicated strains on YEA plates containing between 0.25μM and 10μM camptothecin (CPT). All plates but one were incubated at 30°C for 4 days. One plate was incubated at 37°C to test for the temperature sensitivity of the *hhp1* deletion.

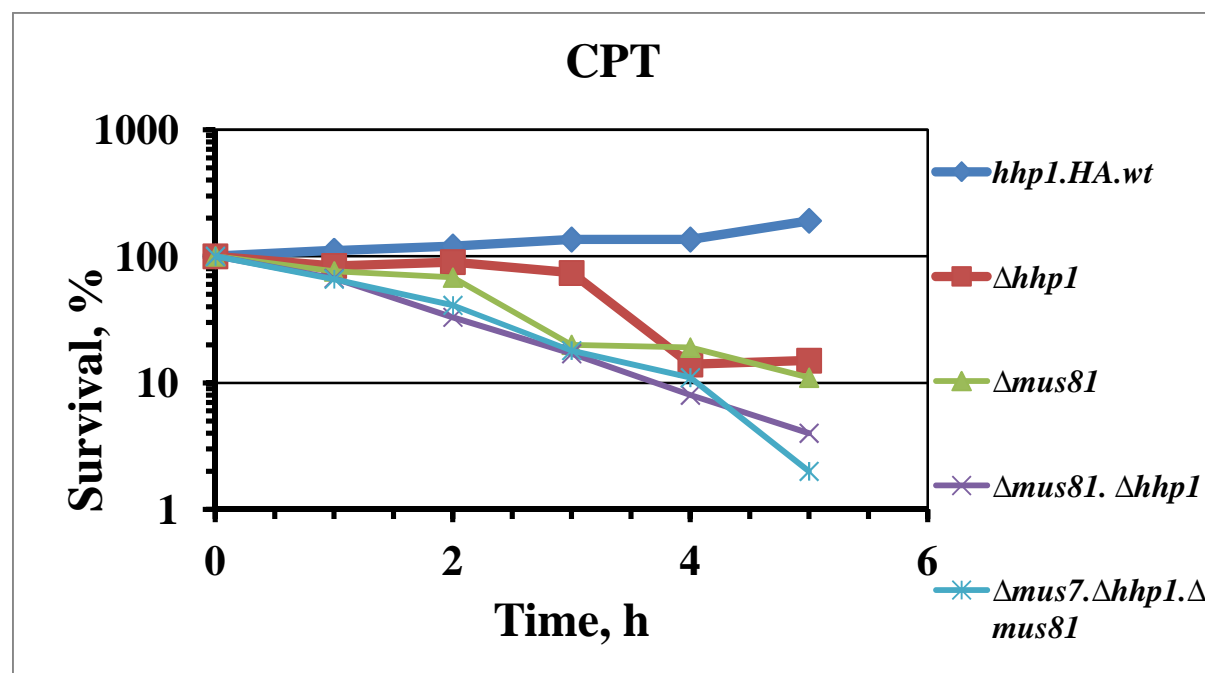


Figure: 3.3.1.2: Mus81, Mus7 and Hhp1 act in the same CPT (camptothecin) response pathway. Yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40μM CPT and incubated for five hours at 30° C. Aliquots of 75 μl were collected every hour and plated on one YEA plate.

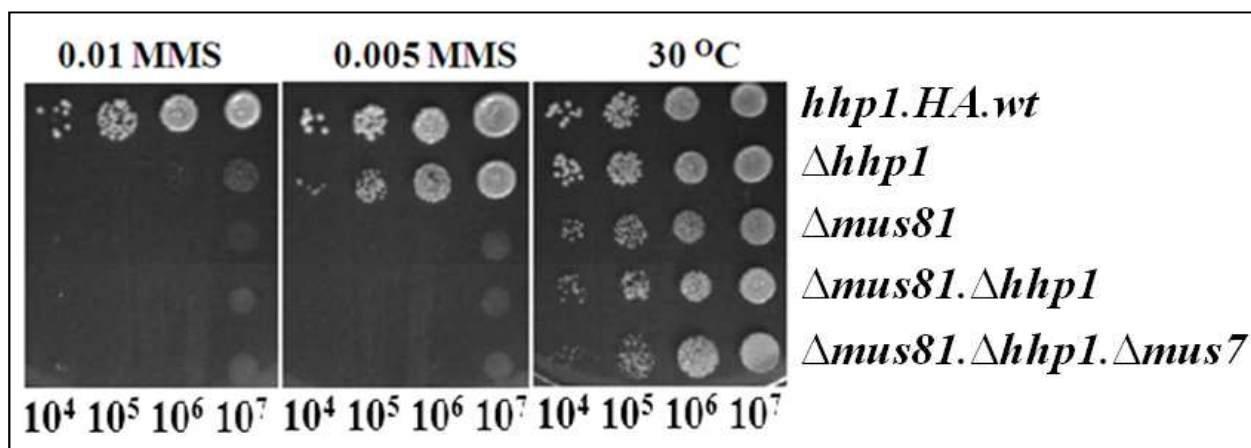


Figure: 3.3.1.3: MMS (methyl-methanesulfonate) spot test for the indicated strains. Mus81, Mus7 and Hhp1 act in the same MMS response pathway. Drop test with the indicated strains on YEA plates containing 0.005% or 0.01% methyl methanesulfonate (MMS). All plates were incubated at 30°C for 4 days. Cells without Hhp1 kinase are highly methyl-methanesulfonate (MMS) sensitive. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations were plated, $\Delta hhp1$ cells lacking Mus81 ($\Delta mus81$) or both Mus81 ($\Delta mus81$) and Mus7 ($\Delta mus7$) were also included.

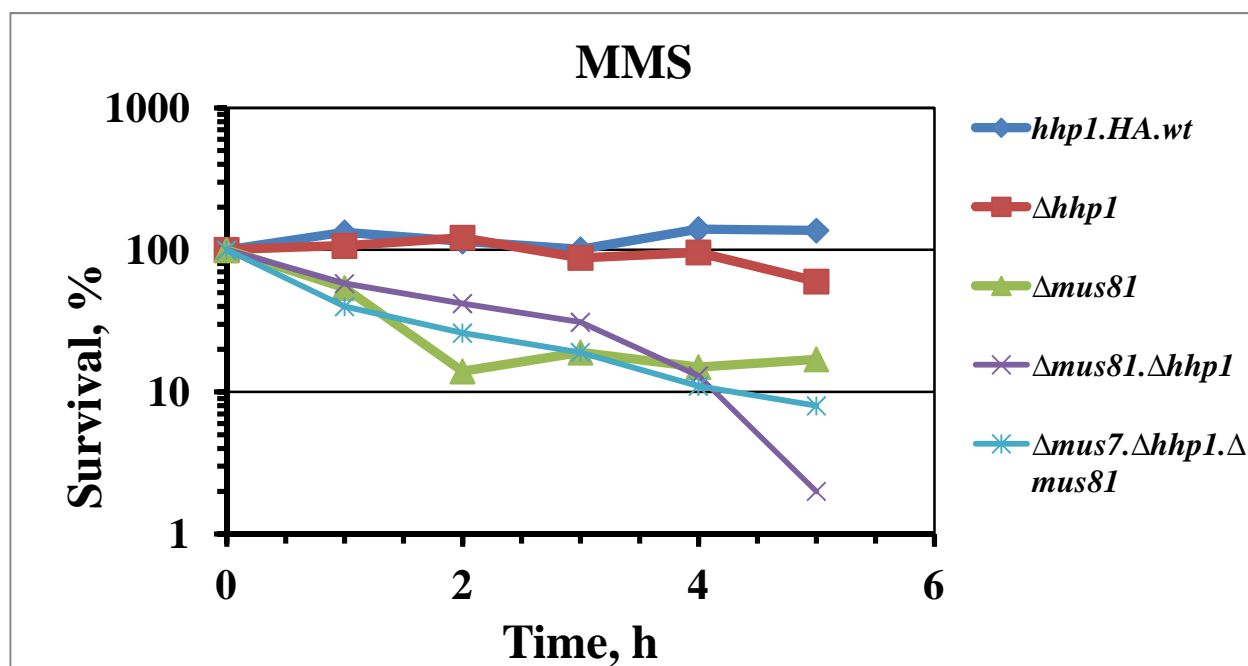


Figure: 3.3.1.4: Mus81 and Hhp1 act in the same MMS (methyl-methanesulfonate) response pathway. The indicated strains were exposed for 5 hours to 0.005% MMS concentrations at 30°C. Samples were withdrawn every one hour and plated on YEA plates. Colonies were scored after 4 days.

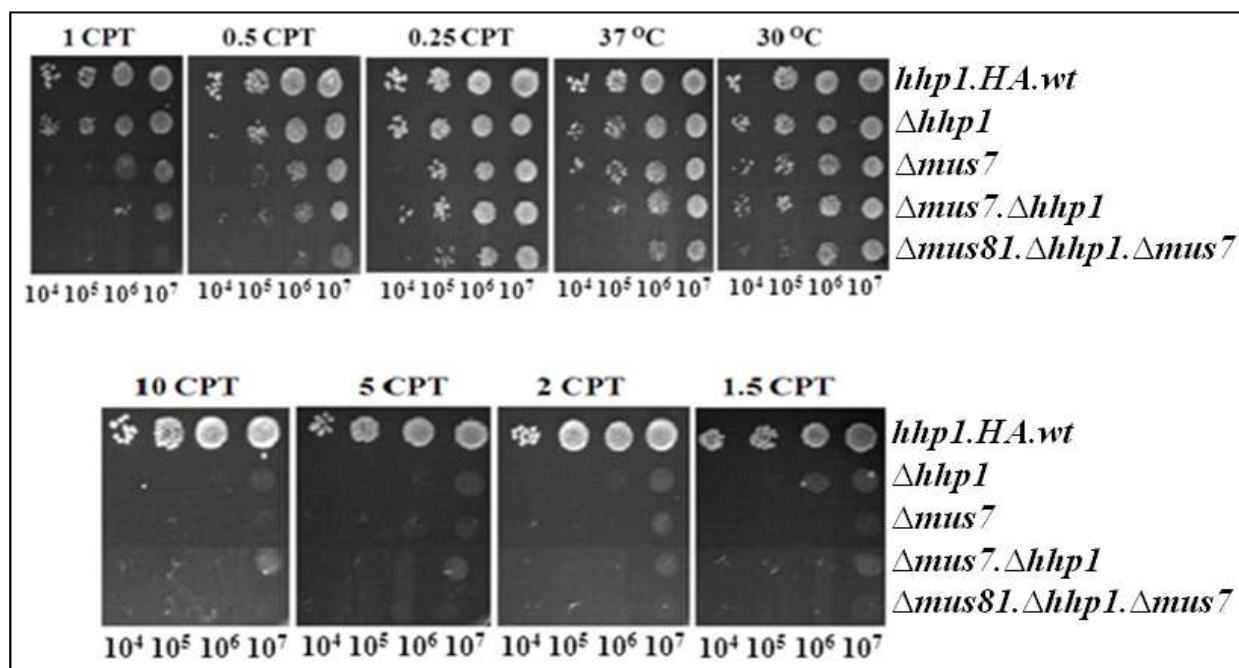


Figure: 3.3.1.5: CPT spot test for Mus7 and Hhp1. Mus7 and Hhp1 act in the same CPT response pathway. Drop test with the indicated strains on YEA plates containing between 0.25μM and 10μM camptothecin (CPT). All plates except one were incubated at 30°C for 4 days. One plate was incubated at 37°C to test for the temperature sensitivity of the *hhp1* deletion. The *Δmus81 Δhhp1 Δmus7* triple mutant is slightly more CPT and heat sensitive.

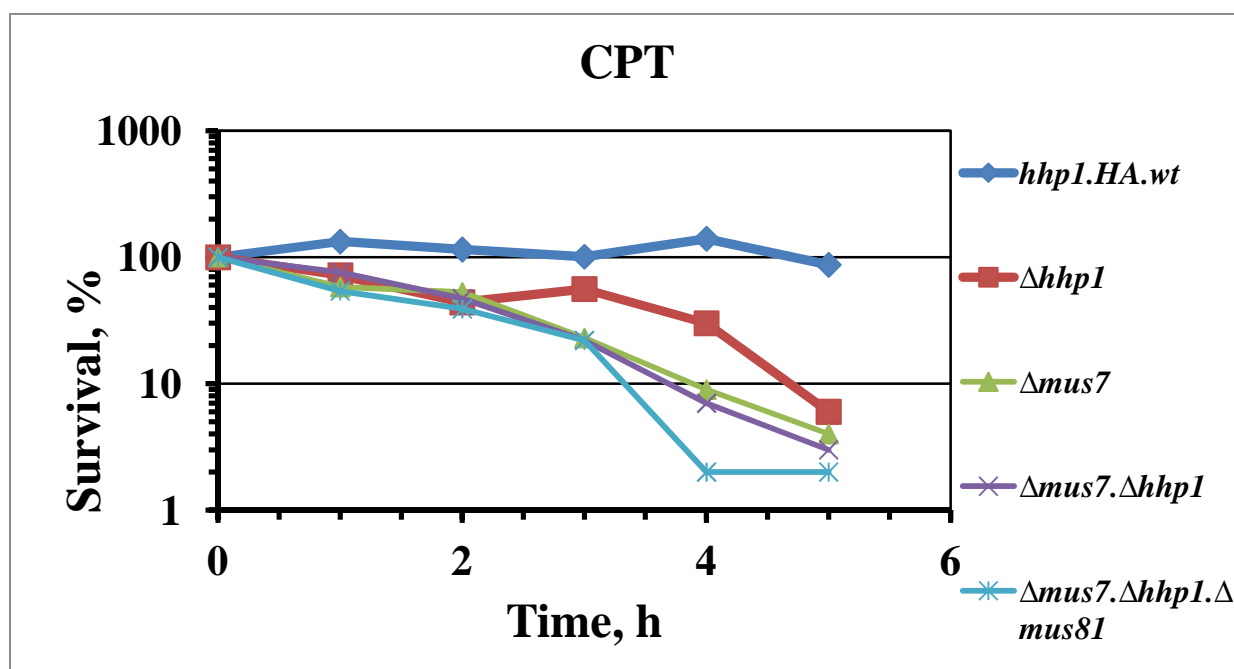


Figure: 3.3.1.6: Mus81, Mus7 and Hhp1 act in the same CPT (camptothecin) response pathway. Yeast strain cells were cultured into YEA medium overnight at 30°C. Cells were harvested and treated with 40μM CPT and incubated for five hours at 30°C. Aliquots of 75 μl were collected every hour and plated on one YEA plate.

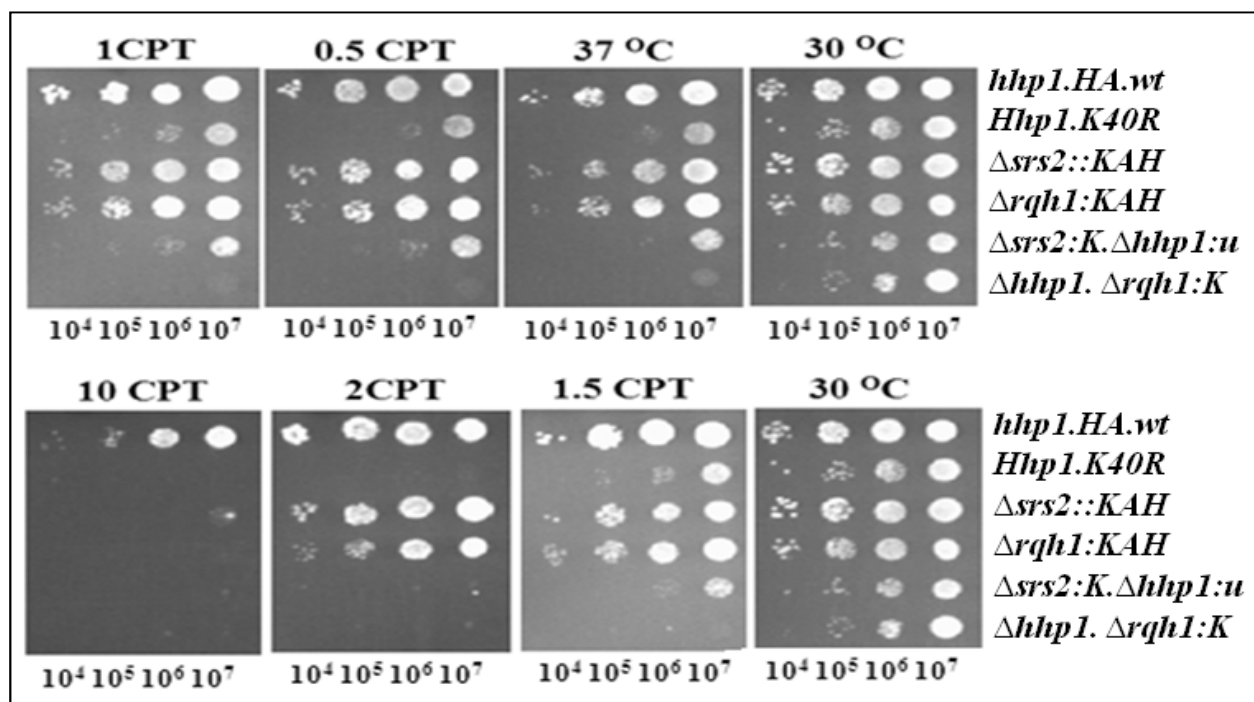


Figure: 3.3.1.7: Rqh1 DNA helicase acts in a parallel pathway to Hhp1. Serial dilutions of the indicated strains were applied to YEA plates with increasing concentrations of CPT (camptothecin). All plates, except one, were incubated at 30°C for 4 days. One plate was incubated at 37°C to test for the temperature sensitivity of the *hhp1* deletion. The Δ *rqh1Δhhp1* mutant is slightly more CPT and heat sensitive.

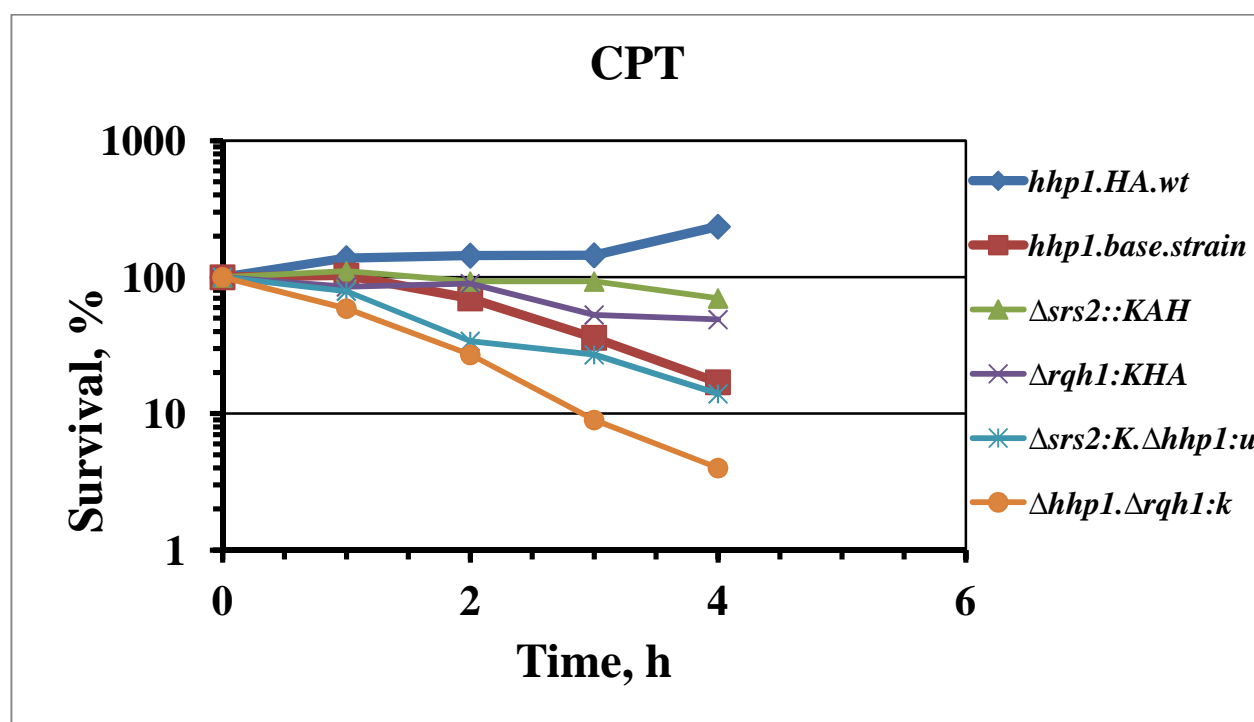


Figure: 3.3.1.8: Rqh1 and Hhp1 act in different CPT (camptothecin) response pathways. Yeast cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40μM CPT and incubated for five hours at 30 °C. Aliquots of 75 μl were collected every hour and plated on one YEA plate.

3.3.2. Hhp1 Kinase acts jointly with Mrc1

An alternative explanation for the role of Hhp1 kinase at damaged replication forks may be provided by the role of the scaffold protein Mrc1. Mrc1 binds directly to stalled forks or D-loop structures in the chromatin (516) and recruits Cds1 to stalled forks (237). May be Hhp1 kinase could impact on the Cds1-Mus81 pathway more indirectly by modulating the activity of Mrc1 rather than Mus81-Eme1 directly. This idea was tested by analysing the CPT and MMS sensitivity of a $\Delta mrc1\Delta hhp1$ double mutant. Interestingly loss of Mrc1 suppresses the CPT sensitivity of the $\Delta hhp1$ mutant at concentration of below 2 μ M CPT (Figure: 3.3.2.1) close resembling the suppression upon loss of *cds1* (Figure: 3.3.1). This suppression was limited to CPT and not evident when cells were treated with MMS. As shown in Figure: 3.3.2.2, and Figure: 3.3.2.3, the $\Delta mrc1\Delta hhp1$ double mutant was as MMS sensitive as the $\Delta hhp1$ strain placing both proteins in the same pathway. The suppression of the CPT sensitivity at low concentrations implies that Hhp1 kinase acts down-stream of Mrc1 and Cds1 as a genetic suppression often infers that the suppressing mutation abolishes a cellular event which later requires the activity of the down-stream protein, Hhp1 kinase in this case. A later function of Hhp1 after Mrc1 recruited Cds1 to stalled forks is therefore more consistent with a role of CK1 in the regulation of the Mus81-Eme1 endonuclease. This conclusion is further supported by an extension of the G2 arrest in CPT medium upon deletion of *mrc1* in $\Delta hhp1$ cell ($\Delta mrc1.\Delta hhp1$) (Figure: 3.3.2.4). The double mutant delays approximately 40 min longer than the $\Delta hhp1$ single mutant which implies a further DNA repair defect when replication forks break. Since Mus81-Eme1 is required for the resolution of recombination intermediates during DNA repair after replication fork collapse (515, 539), damaged forks may remain unrepaired for longer in the $\Delta mrc1 \Delta hhp1$ double mutant. This indicates that Hhp1 kinase has at least two repair execution points under these conditions, from which one is very likely the Mus81-Eme 1 endonuclease.

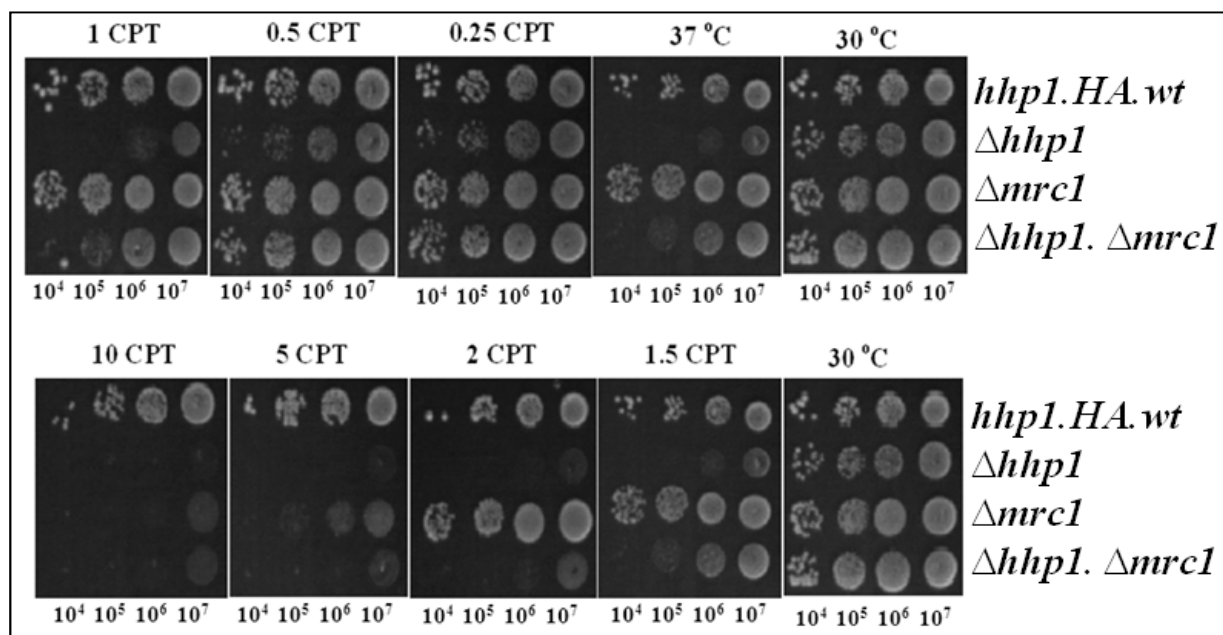


Figure: 3.3.2.1: Loss of *mrc1* partly suppresses the CPT (camptothecin) sensitivity of the Δ *hhp1* mutant. Serial dilutions of the indicated strains were spotted onto rich medium plates which indicated the CPT concentrations. Plates were incubated for 4 days at 30°C. A plate was incubated at 37°C to test the temperature sensitivity of the Δ *hhp1* strain. The data reveals that Hhp1 and Mrc1 are genetically connected.

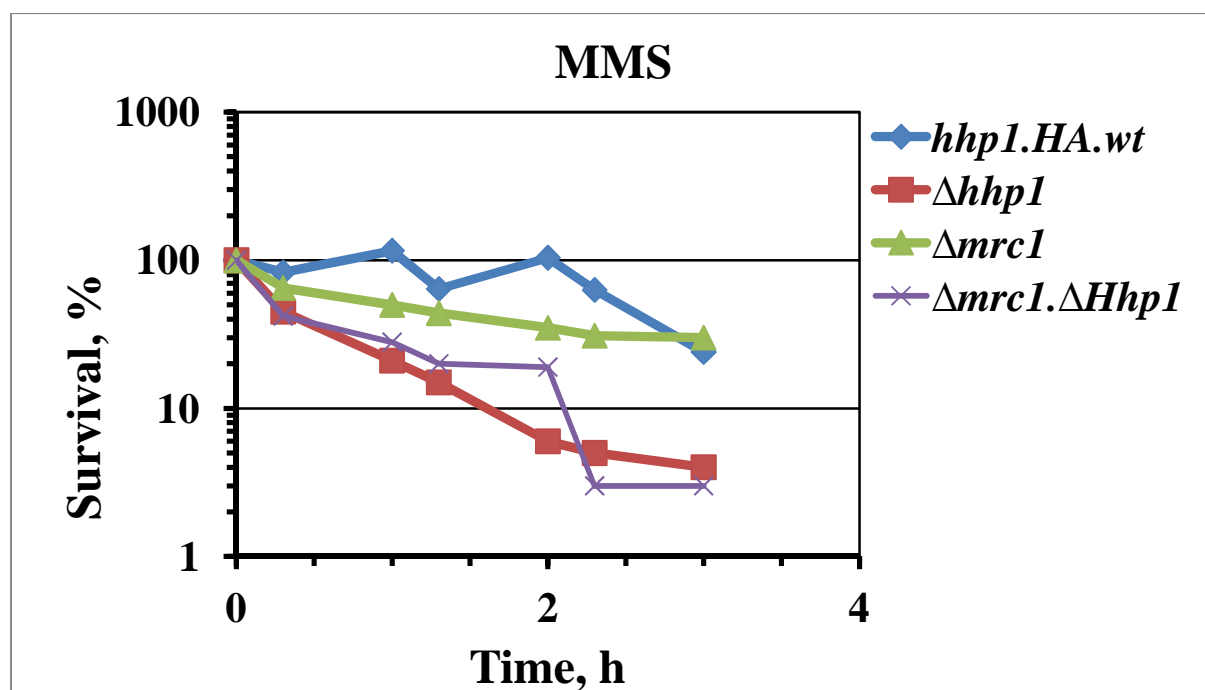


Figure: 3.3.2.2: Mrc1 and Hhp1 act in the same MMS (methyl-methanesulfonate) response pathway. The indicated strains were exposed for 3 hour to 0.05% MMS at 30°C. Samples (75µl) were withdrawn every 30 min and plated on YEA plates. Colonies were scored after 4 days.

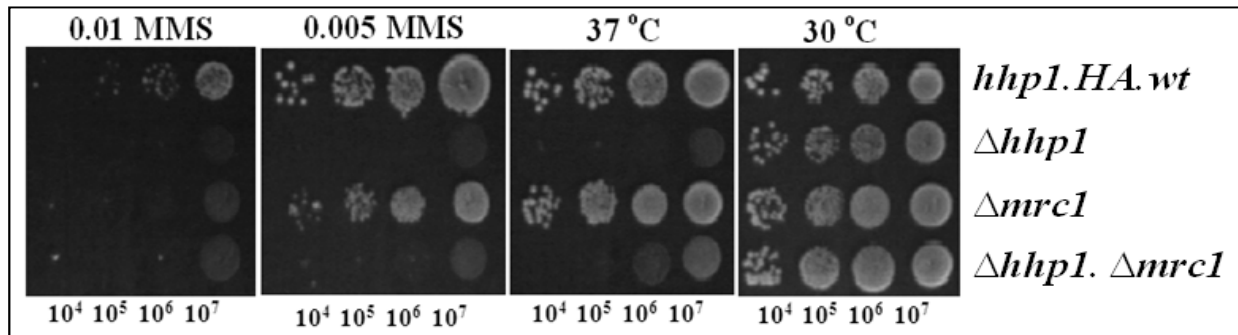


Figure: 3.3.2.3: Mrc1 and Hhp1 act in same pathway. Mrc1 and Hhp1 act in the same MMS (methyl-methanesulfonate) response pathway. Introducing the deletion of *mrc1* into $\Delta hhp1$ cells results in a similar MMS sensitivity as in case of the $\Delta hhp1$ single mutant. All plates were incubated at 30°C or 37°C for 4 days.

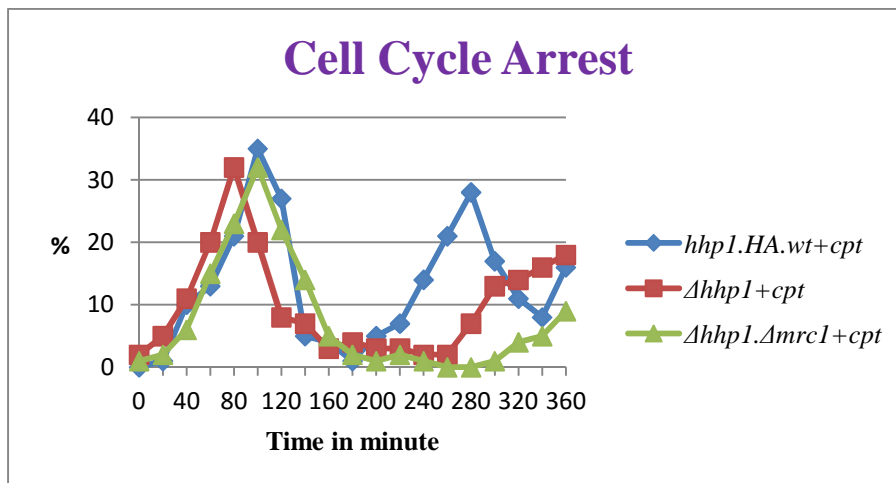


Figure: 3.3.2.4: Loss of Mrc1 extends the G2 delay in $\Delta hhp1$ cells. Wild type (*hhp1-HA*), $\Delta hhp1$ and $\Delta mrc1 \Delta hhp1$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

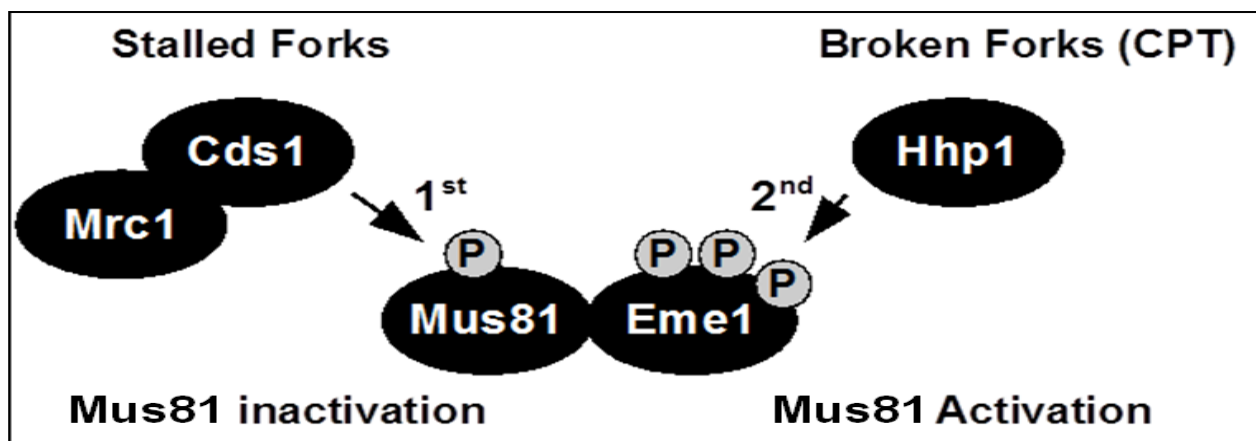


Figure: 3.3.2.5: Model for the co-operation of Hhp1 (CK1) kinase with Cds1 and Mus81. Hhp1 is required for the activation of the endonuclease Mus81-Eme1 when replication forks break. Upon replication fork arrest (stalled forks), Cds1 initially phosphorylates Mus81 to remove it from the nucleus. Mrc1 recruits Cds1 to stalled forks. When stalled forks break, this inhibition may be reversed by a second phosphorylation event executed by Hhp1 kinase. In this context, Cds1 would act as the priming kinase for Hhp1.

3.3.3. Hhp1 Kinase acts jointly with Srs2 DNA helicase

Given the recent finding that Srs2 DNA helicase associates with Mus81-Eme1 to process recombination intermediates in *S.cerevisiae* (517), the genetic relationship between $\Delta hhp1$ and $\Delta srs2$ was tested. Binding of Srs2 stimulates the endonuclease activity of Mus81, and Srs2 removes Rad51 from single-stranded DNA so that Mus81 can cleave the recombination structure.

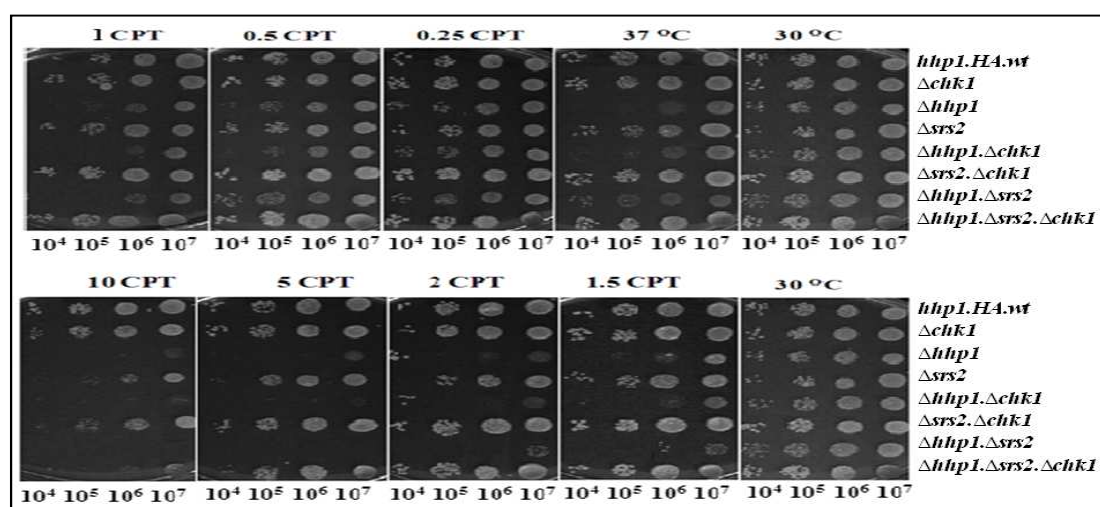


Figure: 3.3.3.1: Genetic association test for *hhp1*, *chk1*, and *srs2*. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT (camptothecin) concentrations. Plates were incubated for 4 days at 30°C. *hhp1.HA.wt* is used as positive control but here at this graph association among *hhp1* kinase and *srs2* helicase is an epistatic, *hhp1* kinase and *chk1* kinase is an epistatic as well, and *chk1* kinase and *srs2* helicase is epistatic, too.

Loss of *srs2* in a wild type background had little impact on the CPT sensitivity (Figure: 3.3.3.1) which suggests that the helicase is not important when DNA replication forks break. Deletion of *srs2* in the $\Delta hhp1$ mutant ($\Delta srs2.\Delta hhp1$) did not further increase the high sensitivity of the $\Delta hhp1$ strain. This places the DNA helicase and Hhp1 (CK1) in the same CPT response pathway. Interestingly, loss of Chk1 kinase in the $\Delta srs2.\Delta hhp1$ double mutant ($\Delta srs2.\Delta chk1.\Delta hhp1$) resembled the $\Delta srs2$ single mutant (Figure: 3.3.3.1). This unexpected rescue implies that Chk1 kinase becomes unregulated in the absence of both, Srs2 DNA helicase and Hhp1 kinase. Since the extended G2 arrest of $\Delta hhp1$ cells in the presence of CPT (Figure: 3.1.5: B) indicates a repair defect when DNA replication forks break, the experiment was repeated with the $\Delta srs2.\Delta hhp1$ strain. As in the case of the CPT survival test, loss of the DNA helicase had very little impact on the extended G2 arrest of $\Delta hhp1$ cells (Figure: 3.3.3.2).

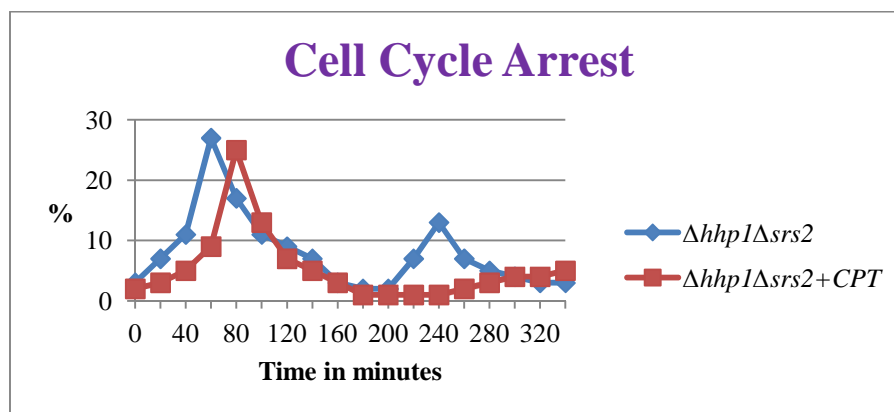


Figure: 3.3.3.2: The G2 cell cycle arrest of the *Δhhp1.Δsrs2* double mutant. The indicated strains were synchronised in G2 by lactose gradient centrifugation and exposed to 40 μ M CPT (camptothecin) at 30°C. Aliquots were withdrawn every 20 min and kept in methanol overnight. The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

While these findings do not rule out a role of Srs2 DNA helicase in the repair of broken DNA replication forks, it is evident that loss of Hhp1 kinase has a much stronger impact on the repair process compared to the DNA helicase. A role of Srs2 in the recombinational repair of broken replication forks is however supported by a previous publication (243). It is generally assumed that the recombinational repair of broken replication forks is postponed until the majority of the genome has been replicated. For example, while loss of the S-phase checkpoint kinase Cds1 has little impact on the CPT sensitivity of *S.pombe* cells, deletion of the *chk1* gene has a more pronounced effect although not as profound as UV or MMS damage (235). This implies that collapsed forks are repaired once cells have switched from the intra-S (Cds1) to the G2-M (Chk1) checkpoint. This conclusion is in line with the finding that *S.cerevisiae* cells repair damaged forks once they have completed S-phase (234).

What was however unexpected is the effective suppression of the CPT sensitivity of the *Δsrs2.Δhhp1* double mutant by the loss of Chk1 kinase. Since this implies that Chk1 becomes aberrantly active in the absence of the DNA helicase and Hhp1 kinase, the genetic link between Chk1 and Hhp1 was investigated.

3.4. Hhp1 Kinase acts jointly with Chk1 Kinase

To test the requirement of Chk1 kinase for the repair of broken forks in the context of Hhp1 kinase, the *chk1* gene was deleted in the *Δhhp1* strain (*Δchk1.Δhhp1*). Loss of Chk1 renders cells sensitive to higher CPT concentrations (Figure: 3.4.1). The *Δchk1.Δhhp1* double mutant was as CPT sensitive as the *Δhhp1* strain implying that both kinases act in the same pathway.

Although the CPT sensitivity of the $\Delta chk1$ strain is not as high as the sensitivity of the $\Delta hhp1$ mutant, an epigenetic relationship is possible since loss of Chk1 kinase suppresses the heat sensitivity of the $\Delta hhp1$ strain (Figure: 3.4.1, Figure: 3.4.2).

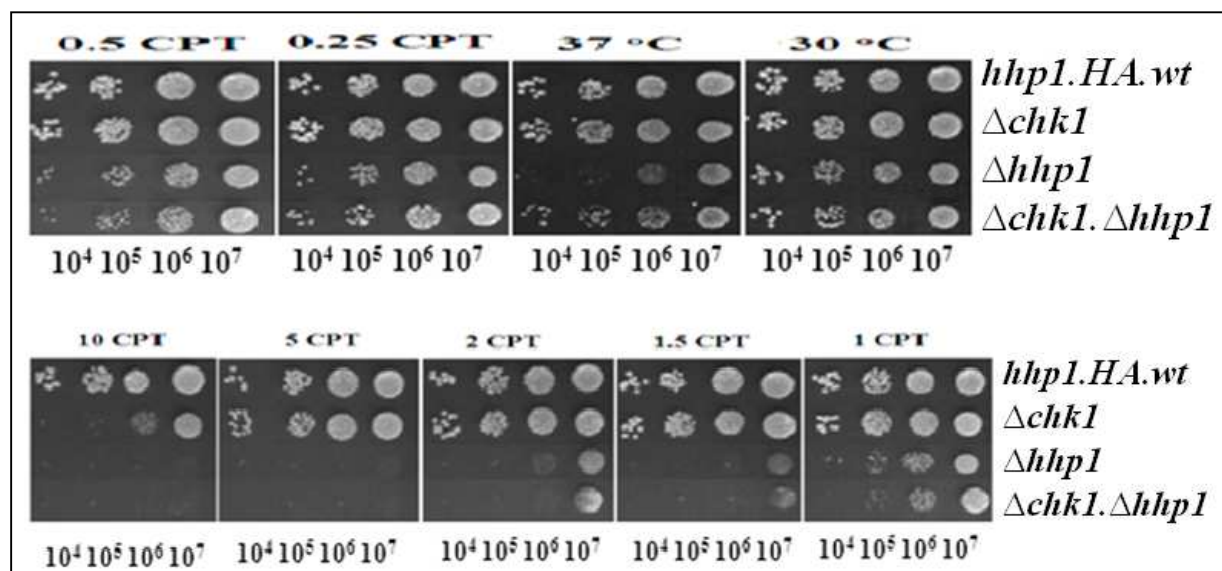


Figure: 3.4.1: Drop test for the $\Delta chk1.\Delta hhp1$ strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT (camptothecin) concentrations. Plates were incubated for 4 days at 30°C. *hhp1.HA.wt* wild type is used as a positive control.

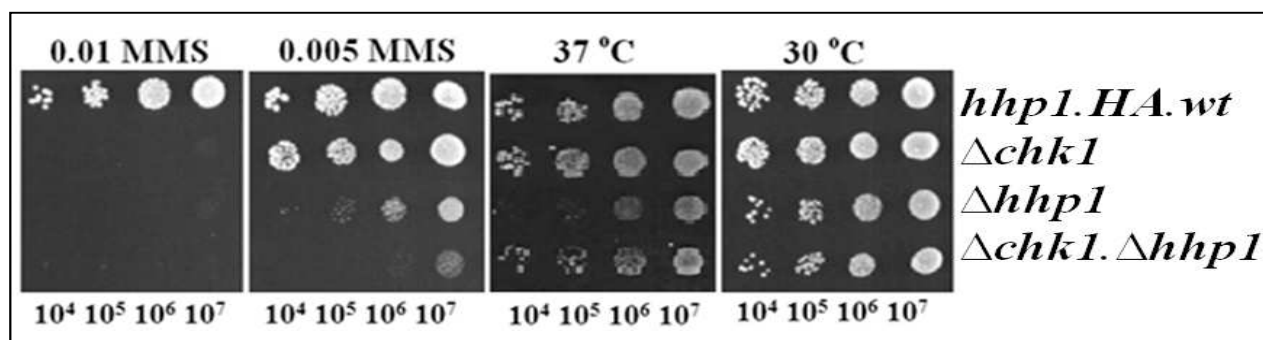


Figure: 3.4.2: Testing Hhp1 kinase and Chk1 kinase. Drop test with the indicated strains on YEA plates containing 0.005% or 0.01% methyl methanesulfonate (MMS). The plates were incubated at 30°C for 4 days. One YEA plate was incubated at 37°C.

The epigenetic relationship between Chk1 and Hhp1 may be specific to CPT as it does not extend to DNA alkylation damage by methyl-methanesulfonate (MMS). When cells were treated with this drug, the $\Delta chk1.\Delta hhp1$ double mutant was more sensitive than either of the single mutants (Figure: 3.4.2). The suppression of the heat sensitivity was again observed in this experiment. This difference suggests that Chk1 and Hhp1 cooperate when replication forks break

but not when alkylation damage is removed by base excision repair. Since Hhp1 acts jointly with Mus81-Eme1 under these conditions (Figure: 3.3.1.1, Figure: 3.3.2.5), the link between Chk1 and Hhp1 would place Chk1 also in the Mus81 pathway (Figure: 3.4.1, Figure: 3.4.3). This is a novel conclusion as there is so far only one publication linking *S.pombe* Chk1 with the phosphorylation of the Mus81-Eme1 complex (518).

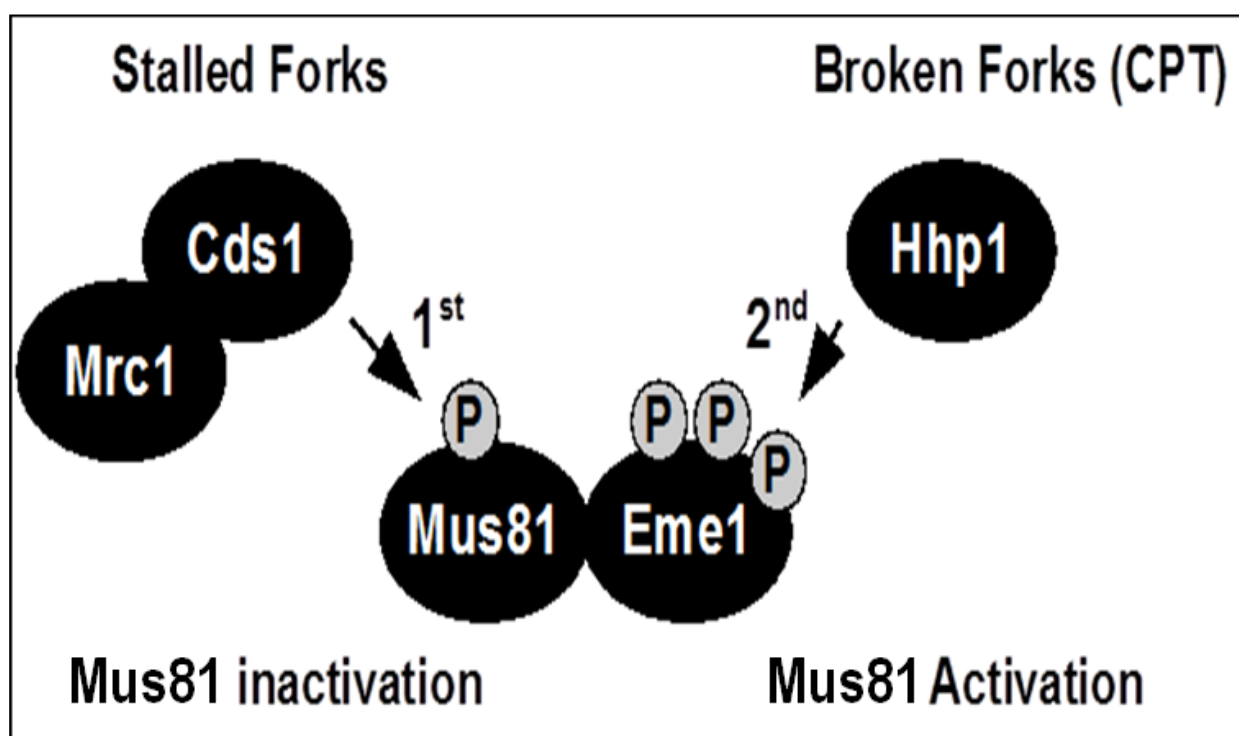


Figure: 3.4.3: Model for the co-operation of Hhp1 with Chk1 and Cds1. Hhp1 is required for the activation of the endonuclease Mus81-Eme1 when replication forks break. Upon replication fork arrest (stalled forks), Cds1 initially phosphorylates Mus81 to remove it from the nucleus. Mrc1 recruits Cds1 to stalled forks. When stalled forks break, this inhibition may be reversed by a second phosphorylation event executed by Hhp1 kinase. In this context, Cds1 would act as the priming kinase for Hhp1. However, under CPT (camptothecin) condition Chk1 also phosphorylates Eme1 (518) which may explain the genetic link between Hhp1 and Chk1.

To find out whether Chk1 kinase impacts on the repair of broken forks, $\Delta chk1$ cells were synchronised in G2 and released into rich medium with and without 40 μ M CPT. Loss of *chk1* had no significant impact on the normal G2 arrest as $\Delta chk1$ cells delayed progression through the second G2 phase for approximately 40 min (Figure: 3.4.4) as observed for wild type cells (Figure: 3.1.5: A).

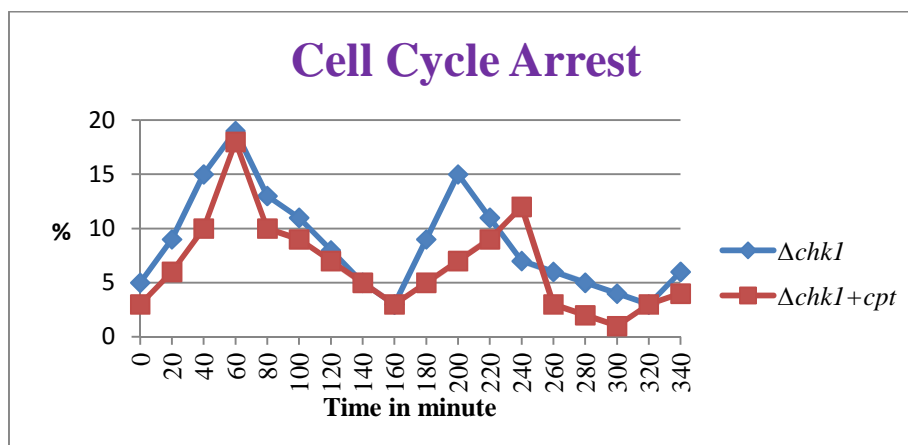


Figure: 3.4.4: Cell cycle arrest for $\Delta chk1$ strain. $\Delta chk1$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

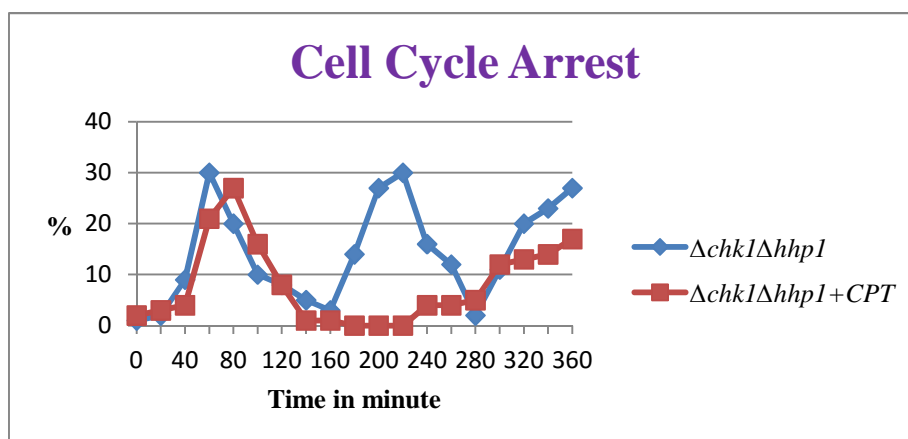


Figure: 3.4.5: Cell cycle arrest for $\Delta chk1.\Delta hhp1$ strain. $\Delta hhp1$ cells without $chk1$ ($\Delta chk1$) were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

The same assay was applied to the $\Delta chk1.\Delta hhp1$ double mutant which arrested in G2 for approximately 80 min before slowly coming out of the arrest thus resembling the extended arrest of the $\Delta hhp1$ single mutant (100 min – 120 min, **Figure: 3.4.5**).

Taken together, these observations suggest that Hhp1 affects cell cycle regulation under these conditions, whereas Chk1 does not despite its epigenetic relationship with Hhp1. One possible role of Chk1 could be to regulate Cds1 kinase when replication forks break in CPT medium since Cds1 inhibits Mus81 when replication forks stall (509). The hand-over between Cds1 and Chk1 is not well understood but needs to happen when stalled forks cannot be recovered. Since Cds1 and Chk1 are both activated by Rad3 (ATR) kinase in *S.pombe* (226) and because both kinases are epistatic with *hhp1* (e.g. loss of *cds1* suppresses the MMS sensitivity and loss of *chk1*

is epistatic in the presence of CPT), a *Δrad3.Δhhp1* double mutant was constructed and analysed. Consistent with the close relationship between Hhp1 and the two down-stream kinases (Cds1 and Chk1), the *hhp1* deletion was epistatic with loss of *rad3* (Figure: 3.4.6).

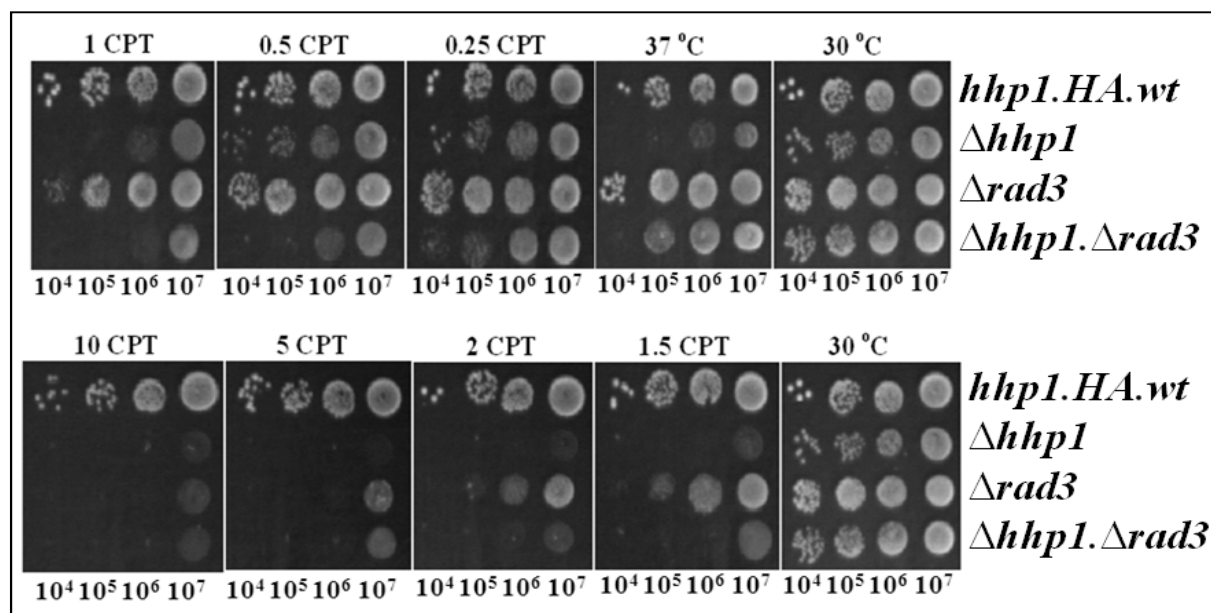


Figure: 3.4.6: CPT (Camptothecin) drop assay for the *Δrad3Δhhp1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

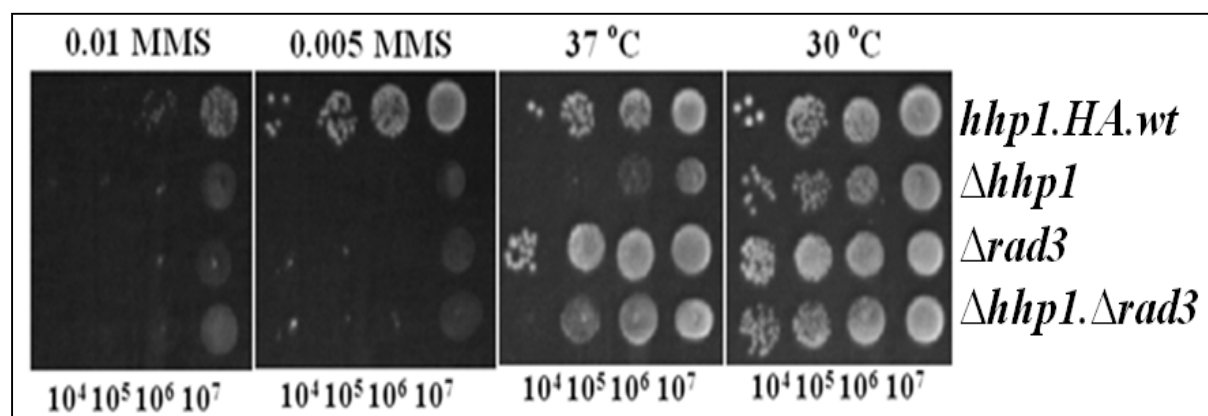


Figure: 3.4.7: MMS drop assay for the *Δrad3Δhhp1* strain. The indicated strains were applied to YEA plates containing 0.005% or 0.01% methyl methanesulfonate (MMS). The plates were incubated at 30°C for 4 days. One plate was incubated at 37°C.

To obtain further evidence for a role of Hhp1 in the repair of broken forks, cells without the main recombination protein Rad51 were studied. Rad51 is loaded onto 3'-single stranded DNA

by Rad52 (Rad22 in *S.pombe*) which also facilitates the invasion of the sister chromatid by the Rad51-ssDNA nucleoprotein filament (519). Interestingly, Mus81 acts closely with Rad22 (Rad52) in a Rad51-independent manner when DNA replication forks break in the presence of CPT (520). Based on the previous finding, which placed Hhp1 in the same pathway as Mus81, it was anticipated that the $\Delta rad51.\Delta hhp1$ double mutant is more CPT sensitive than the $\Delta rad51$ single mutant. This was indeed the case as shown in Figure: 3.4.8. The double mutant was also more heat sensitive than the $\Delta hhp1$ strain.

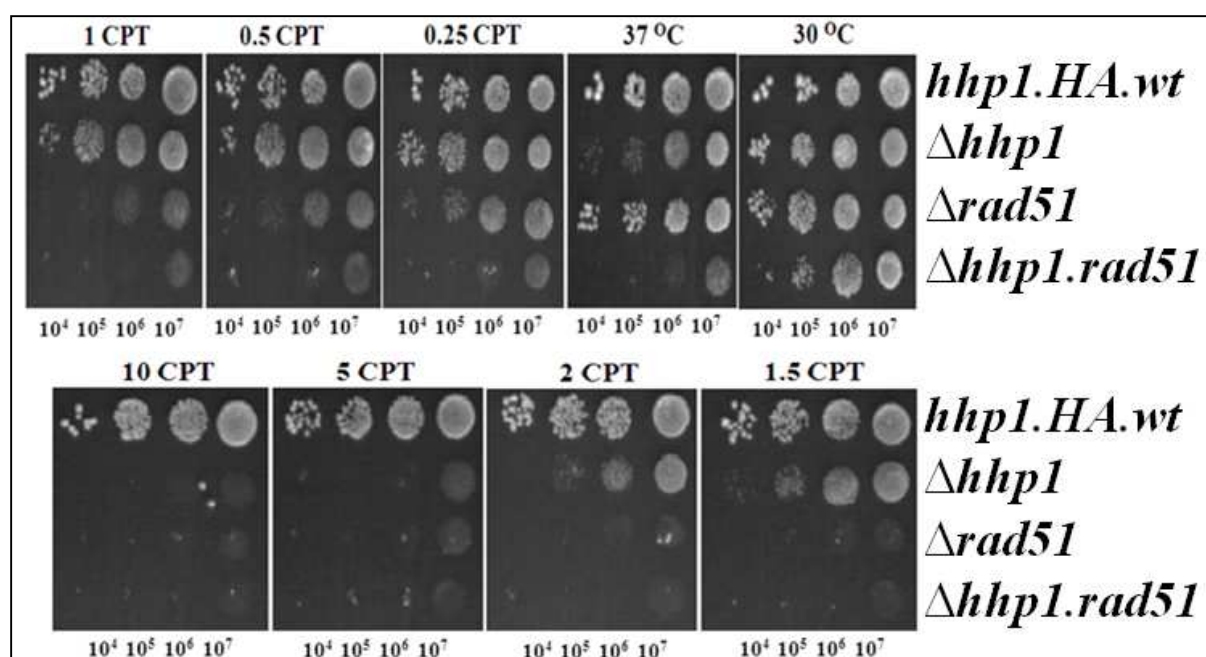


Figure: 3.4.8: Hhp1 and Rad51 act not in the same CPT (camptothecin) repair pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

This is an interesting finding as it implies that the Hhp1-Mus81 DNA repair function at broken replication forks requires Rad22 (Rad52) but not Rad51. Since Rad51 forms the 3'-ssDNA nucleoprotein filament, which acts at the heat of homologous recombination, it was also tested whether loss of Ctp1 (CTIP in human cells, Sae2 in *S.cerevisiae*), which is crucial for the resection of a DNA double-stranded break into the 3'-ssDNA Rad51 substrate (521, 522), would also increase the CPT sensitivity of the *hhp1* deletion strain. As shown in Figure: 3.4.9, the $\Delta ctp1.\Delta hhp1$ double mutant was more CPT sensitive than the single $\Delta ctp1$ mutant. This increase in sensitivity extended also to MMS induced DNA damage (Figure: 3.4.10).

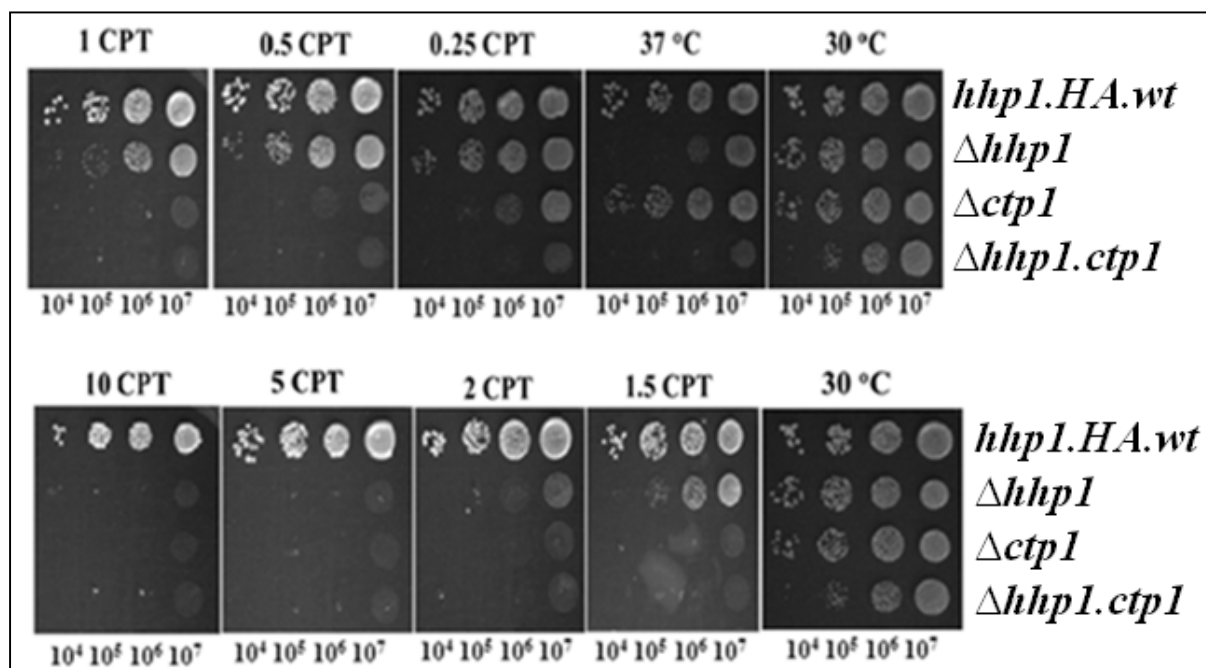


Figure: 3.4.9: Ctp1 and Hhp1 do not act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

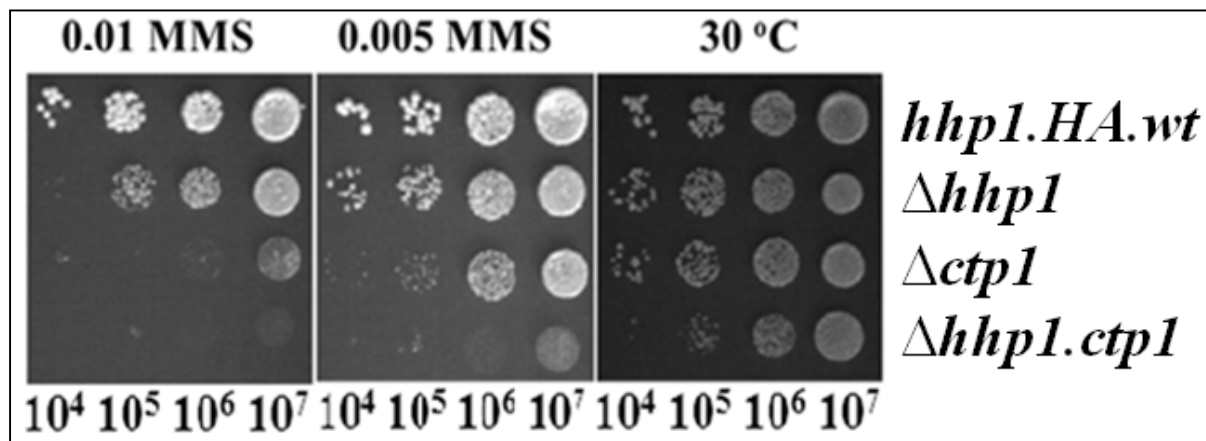


Figure: 3.4.10: Ctp1 and Hhp1 do not act in the same CPT response pathway. The indicated strains were applied to YEA plates containing 0.005% or 0.01% methyl methanesulfonate (MMS). The plates were incubated at 30°C for 4 days. One plate was incubated at 37°C.

Given that the Ku70-Ku80 heterodimer competes with the end processing enzymes like Ctp1 for access to a broken chromosome (203), the genetic link between Hhp1 and Ku70 was tested. As shown in [Figure: 3.4.11](#), loss of Ku70 ($\Delta ku70$) had no impact on the CPT sensitivity of $\Delta hhp1$ cells. The same applied to the MMS sensitivity of the $\Delta ku70.\Delta hhp1$ double mutant ([Figure: 3.4.12](#)). The extended G2 arrest of $\Delta hhp1$ cells in the presence of CPT was also not affected by

loss of *ku70* (Figure: 3.4.13). Taken together these data imply that an increased access to broken DNA replication forks in the absence of Ku70 does not affect the repair role of the Hhp1-Mus81 pathway.

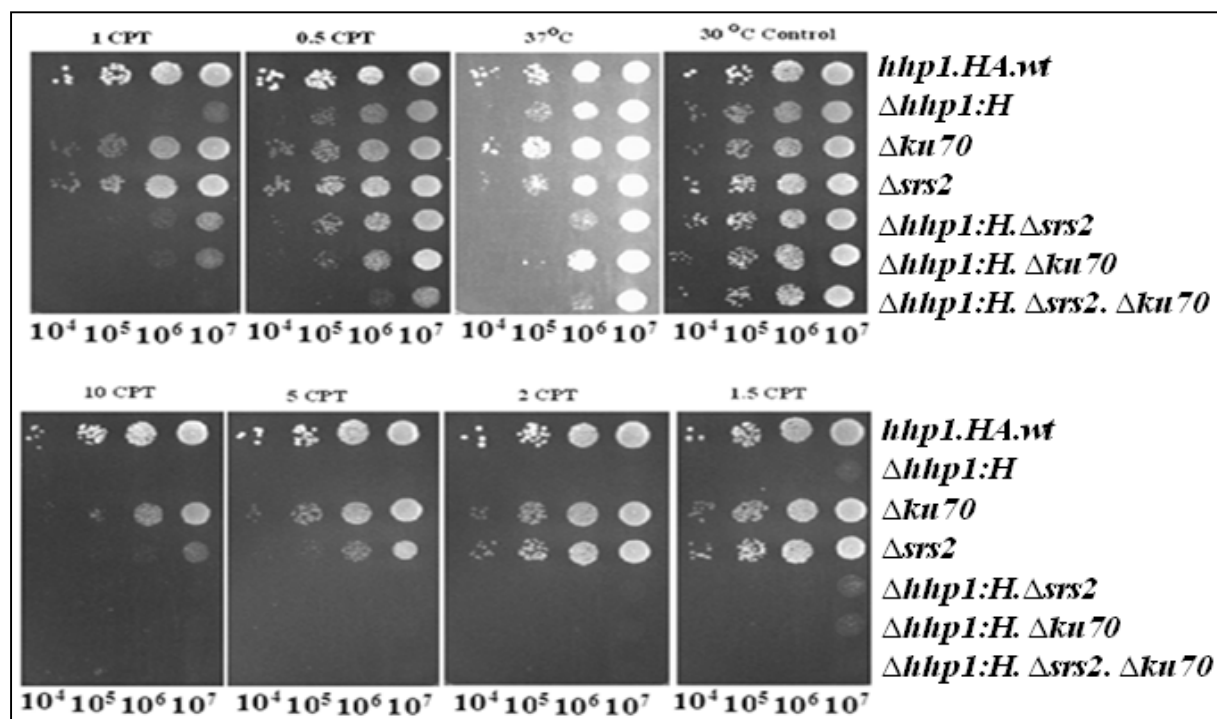


Figure: 3.4.11: The DNA binding protein Ku70 does not act in the same CPT (camptothecin) pathway as Hhp1 kinase. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

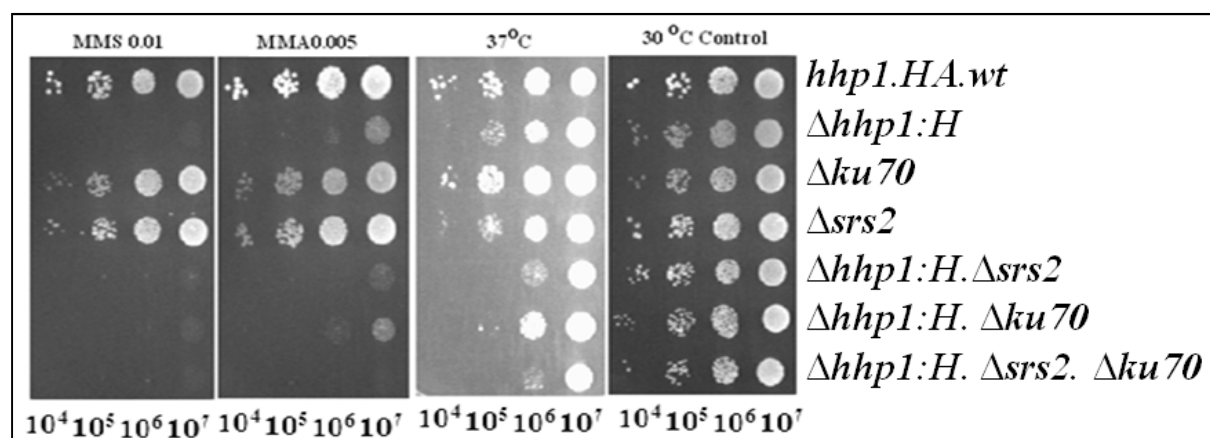


Figure: 3.4.12: The DNA binding protein Ku70 does not act in the same CPT pathway as Hhp1 kinase. The indicated strains were applied to YEA plates containing 0.005% or 0.01% methyl methanesulfonate (MMS). The plates were incubated at 30°C for 4 days. One plate was incubated at 37°C.

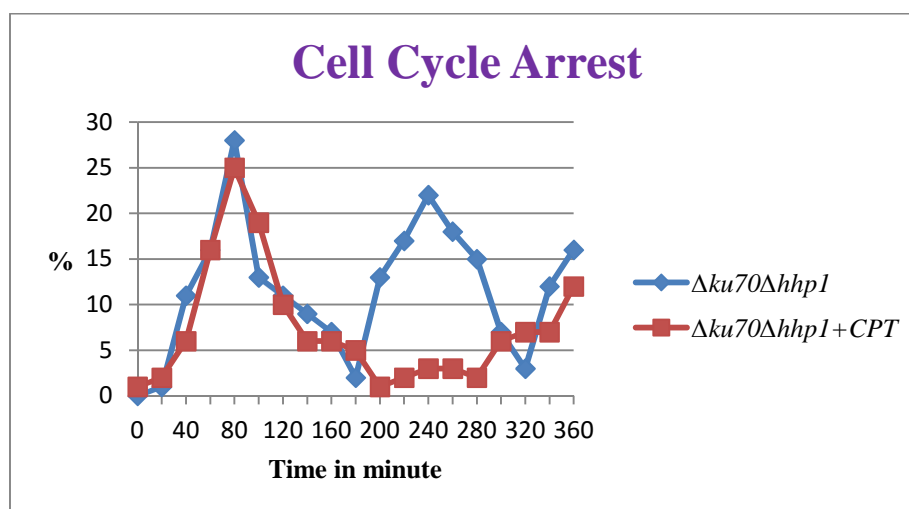


Figure: 3.4.13: Cell cycle G2-delay for $\Delta hhp1\Delta ku70$ cells. $\Delta hhp1$ cells without Ku70 ($\Delta ku70$) were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

3.5. Hhp1 Kinase acts jointly with Hsk1 (Cdc7) Kinase

S.pombe Hsk1 (Cdc7) kinase belongs to a group of enzymes which is related to cyclin-dependent kinases (531). The regulatory subunit of Hsk1 is Dfb1/Him1. Expression of Dfb1 peaks at the G1-S transition and the activity of the Hsk1-Dfb1 complex is required for the onset of DNA replication by phosphorylating subunits of the replicative MCM2-7 DNA helicase (532). The complex exerts also an important activity during DNA replication as it is a target of the intra-S checkpoint kinase Cds1 and maintains the structural integrity of replication structures (357, 533). The kinase acts jointly with the Swi1-Swi3 DNA replication complex in the response to MMS damage (511), and releases the Rad9-Rad1-Hus1 complex from chromatin at the end of the DNA damage checkpoint response (534). Cds1 and Hsk1 associate both the scaffold protein Mrc1 which also binds Swi1 (535). Since the Swi1-Swi3 dimer recruits Mrc1 to stalled DNA replication forks (516), the Hsk1-Dfb1 complex may fine tune the DNA damage checkpoint response executed by Cds1 and the Rad9-Rad1-Hus1 ring in S-phase. So far there are no data linking Hsk1 kinase to Hhp1 or Mus81-Eme1. Given the genetic link between Cds1, Mrc1 and Hhp1 (Figure: 3.4.3), a temperature sensitive allele of the essential *S.pombe hsk1* gene (*hsk1-1312*, S314I) was used to study the connection to Hhp1 kinase. The *hsk1-1312* allele encodes a kinase with a serine-to-isoleucine substitution at codon 314 (TCT to ATT) (357).

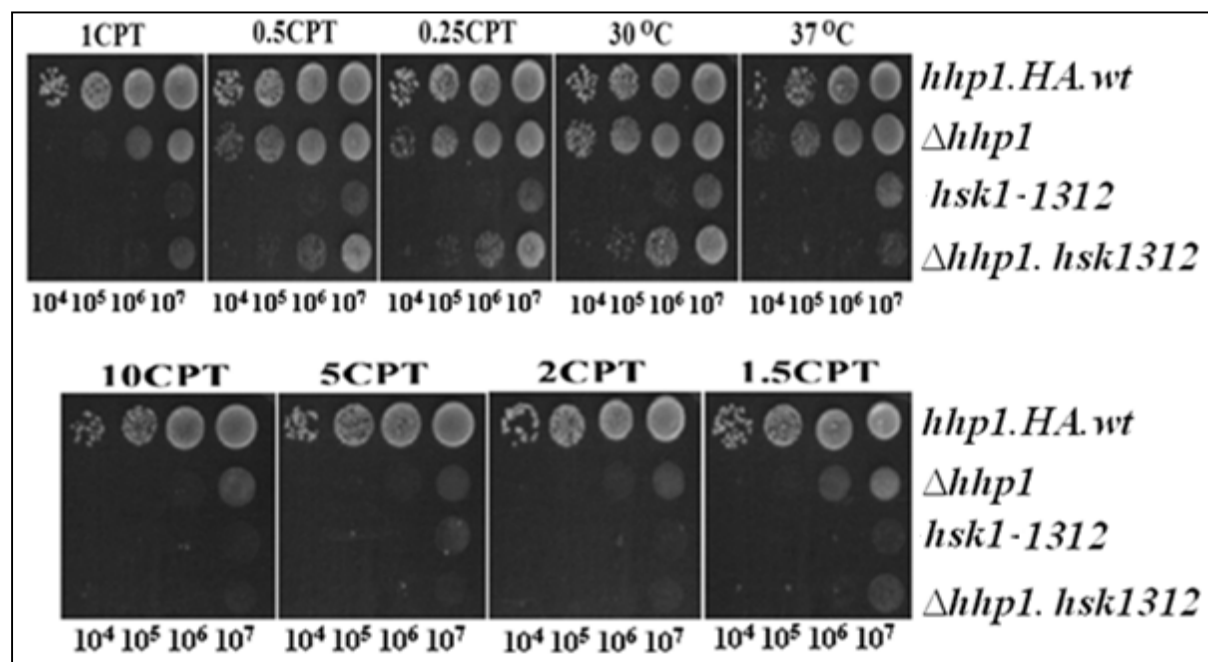


Figure: 3.5.1: Hsk1 (Cdc7) kinase and Hhp1 act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

Even at the semi-permissive temperature of 30°C *hsk1-1312* cells are highly CPT sensitive (Figure: 3.5.1). Interestingly, loss of Hhp1 suppressed the slow growth phenotype at 30°C but not at 37°C. The *hsk1-1312.Δhhp1* double mutant grew also better in the presence of low CPT concentrations. It is however difficult to decide whether this increased CPT resistance is a consequence of a faster growth of the double mutant or a true increase in resistance.

To distinguish between the two possibilities, an acute CPT test was performed. As shown in Figure: 3.5.2, the *hsk1-1312.Δhhp1* double mutant was as CPT sensitive as the *hsk1-1312* single mutant when cells were exposed to 40μM of the topoisomerase 1 inhibitor. This suggests that Hhp1 and Hsk1 act in the same CPT response pathway. The same epigenetic relationship extended also to MMS induced DNA damage (Figure: 3.5.3).

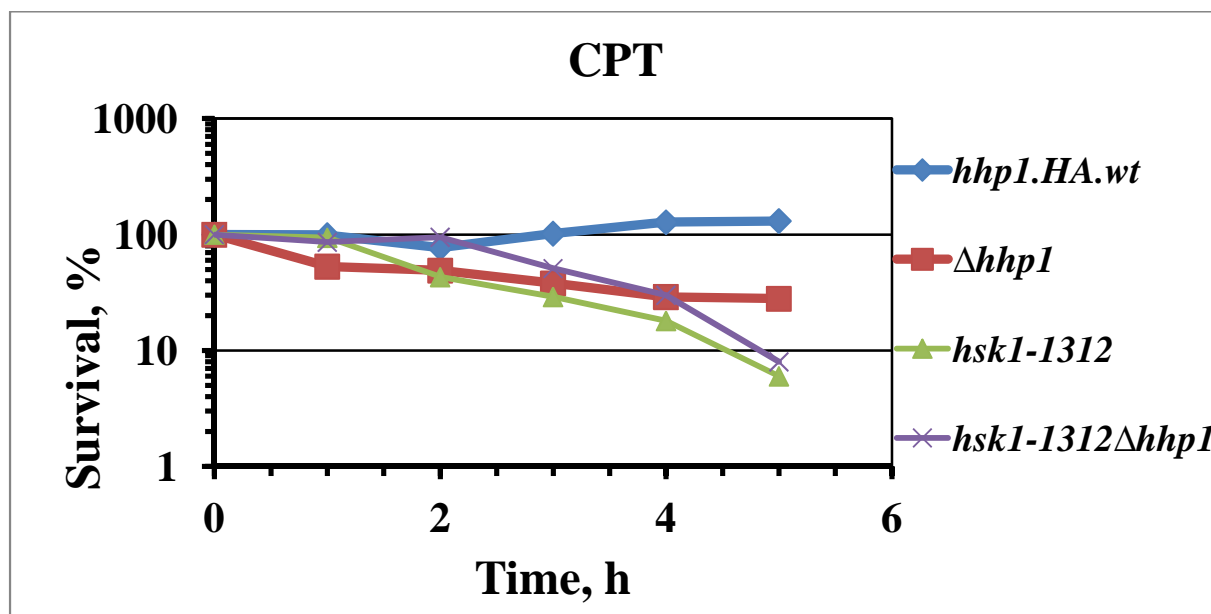


Figure 3.5.2: Hsk1 kinase and Hhp1 are epistatic for CPT (camptothecin). *Hhp1.HA.wild type*, Δ *hsk1-1312* Δ *hhp1*, Δ *hhp1*, and Δ *hsk1-1312* yeast strains were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40 μ M CPT for five hours at 30° C. Aliquots of 75 μ l were collected every hour and plated on one YEA plate.

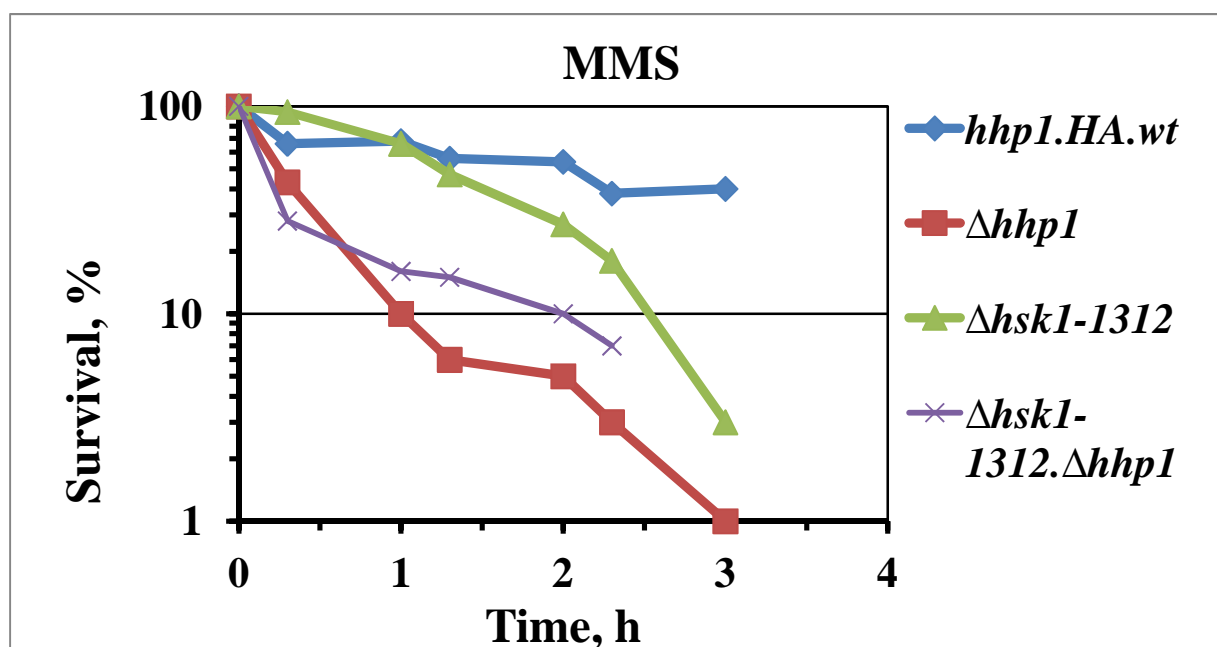


Figure 3.5.3: Hsk1 kinase and Hhp1 are epistatic for MMS (methyl-methanesulfonate). *Hhp1.HA.wild type*, Δ *hsk1-1312* Δ *hhp1*, Δ *hhp1*, and Δ *hsk1-1312* yeast strains were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 0.05% MMS for three hours at 30° C. Aliquots of 75 μ l were collected every hour and plated on one YEA plate.

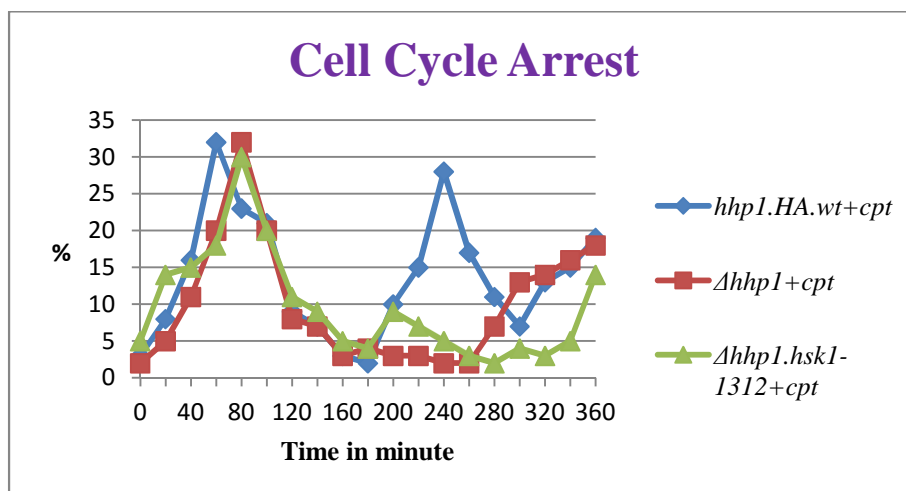


Figure: 3.5.4: G2 arrest of the *hsk1-1312* Δ *hhp1* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

The *hsk1-1312*. Δ *hhp1* double mutant was also synchronised in G2 and released into medium containing 40 μ M CPT. Interestingly a reduction in Hsk1 activity at the semi-permissive temperature of 30°C in the absence of Hhp1 kinase (Δ *hhp1*) extended the G2 arrest of Δ *hhp1* cells by approximately one hour (Figure: 3.5.4). This implies a more complex genetic relationship between the two kinases as they are epistatic for CPT survival but not for the cell cycle arrest. The extended G2 arrest in the *hsk1-1312*. Δ *hhp1* double mutant may be caused by an increased deficiency in the repair of broken forks or it could be a problem with the termination of the checkpoint signal at the end of repair as Hsk1 is required to remove the Rad9-Rad1-Hus1 complex from the chromatin (534). It is noteworthy that a similar extended G2 arrest was evident in the Δ *mrc1*. Δ *hhp1* double mutant (Figure: 3.5.5, and Figure: 3.4.3). Given that Hsk1 associates with Mrc1 (535), and phosphorylates Cds1 (357), this places the Hsk1-Dfb1 complex in the CPT response pathway which may be required to prime the Mus81-Eme1 complex for the phosphorylation by Hhp1 (Figure: 3.5.6).

This conclusion is supported by the epistatic relationship between Swi1 and Hhp1 in the presence of CPT induced DNA damage (Figure: 3.5.7).

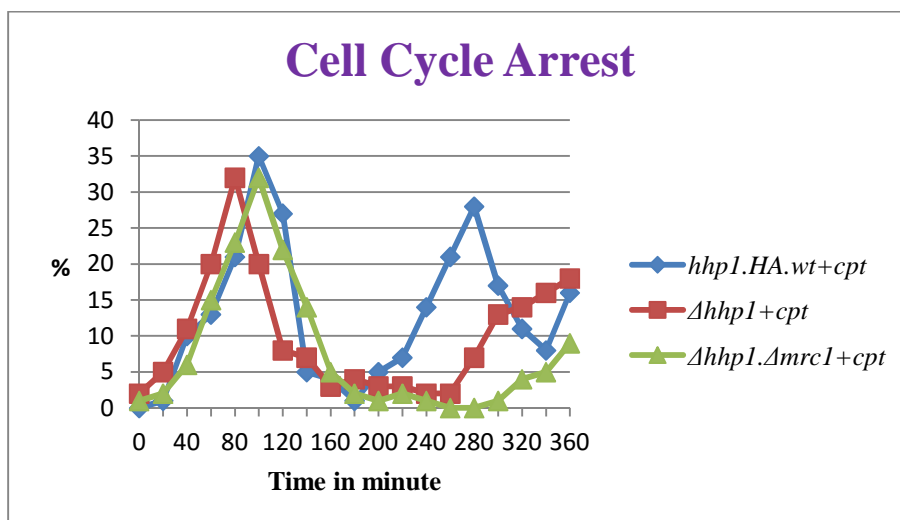


Figure: 3.5.5: Cell cycle delay of $\Delta mrc1\Delta hhp1$ cells. $\Delta hhp1$ cells without Mrc1 ($\Delta mrc1$) were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

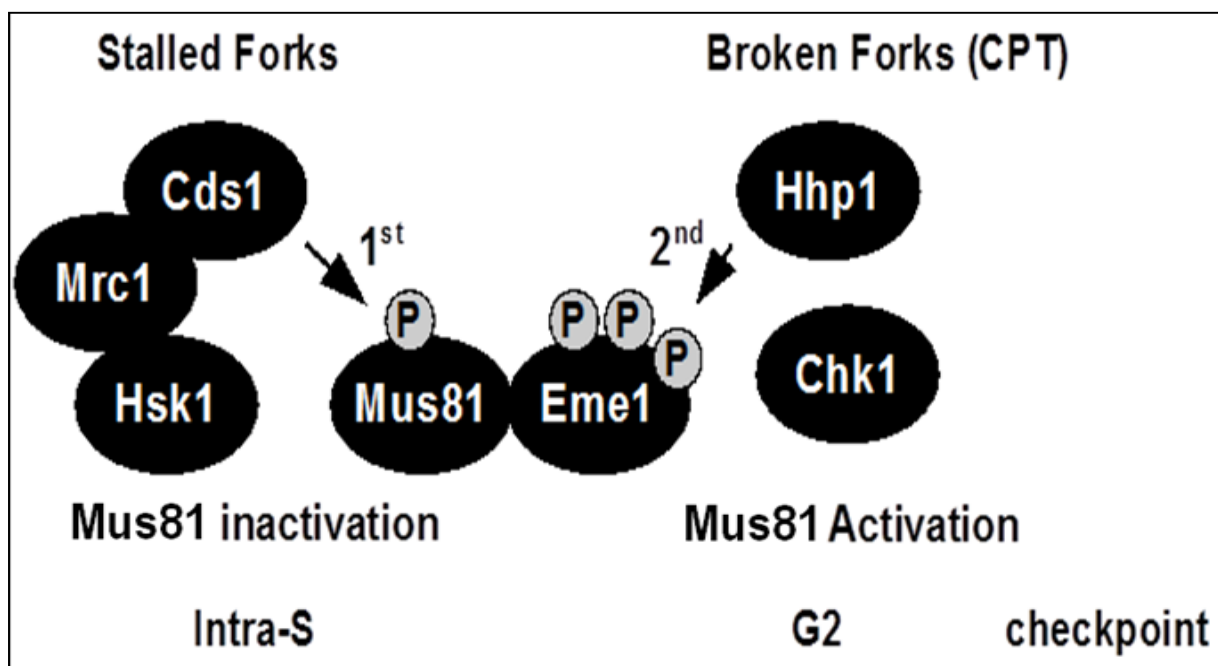


Figure: 3.5.6: Model of the roles of Hhp1 (CK1) in the regulation of Mus81-Eme1. Hhp1 is required for the activation of the endonuclease Mus81-Eme1 when replication forks break. Upon replication fork arrest (stalled forks), Cds1 initially phosphorylates Mus81 to remove it from the nucleus. Mrc1 recruits Cds1 to stalled forks. When stalled forks break, this inhibition may be reversed by a second phosphorylation event executed by Hhp1 kinase. In this context, Cds1 would act as the priming kinase for Hhp1. Hsk1 kinase, which binds to Mrc1, may switch the activity of Cds1 from its intra-S mode to its G2 mode. This may be important to reverse the inhibition of the Mus81-Eme1 complex.

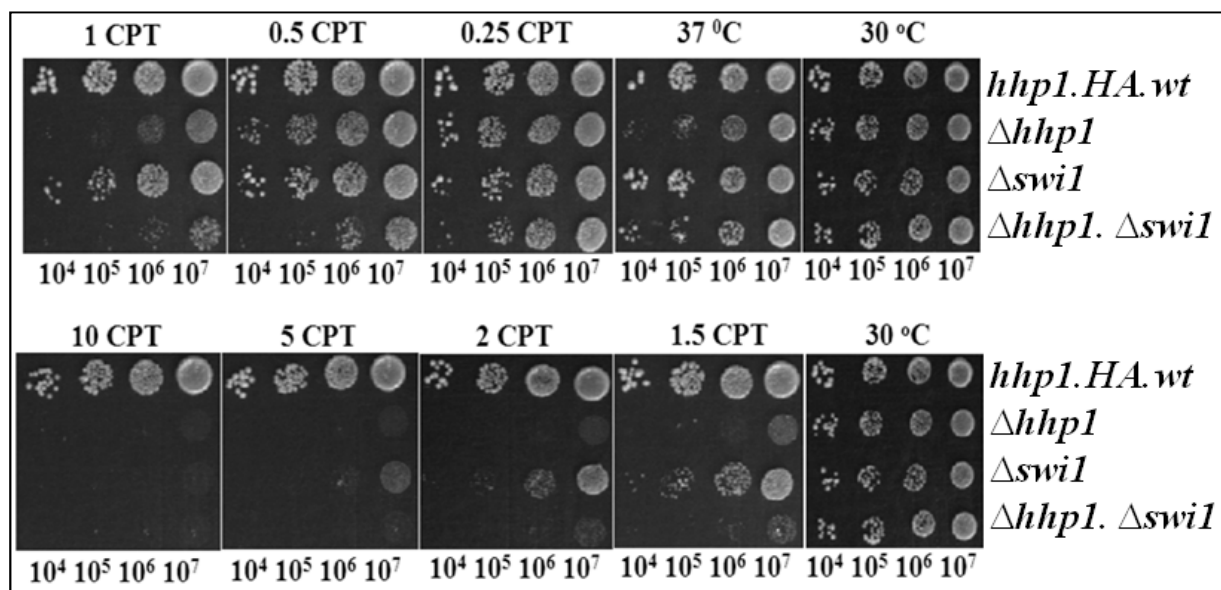


Figure: 3.5.7: Swi1 (Timeless) and Hhp1 act in the same CPT (camptothecin) pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C.

In summary these findings support a role of Hhp1 kinase in the regulation of the Mus81-Eme1 structure specific endonuclease when DNA replication forks break upon their collision with the immobilised topoisomerase 1 enzyme (CPT induced). Since the repair response is postponed till cells exit S-phase (234), and coincides with the activation of Chk1 kinase (235). It is quite possible that the Mrc1-Cds1-Hsk1-Swi1 complex at the damaged fork is involved in switching the response from the intra-S (Cds1-dependent) to the G2 (Chk1-dependent) DNA damage checkpoint (Figure: 3.5.6). How this transition is initiated is not yet known. One important outcome of this change is the activation of the Mus81-Eme1 endonuclease which is needed for the repair of collapsed forks but inactivated by Cds1 (509, 536). This switch could be triggered by the appearance of a DNA break at a stalled fork and the subsequent recruitment of DNA repair and DNA damage checkpoint proteins, or it could be dependent on the cyclin-dependent kinase Cdc2 which switches the repair response to broken chromosomes from Non-Homologous End Joining (NHEJ) to Homologous Recombination (HR) when cells pass from G1 to G2 (537). The possible role of the cell cycle regulator Cdc2 will be discussed in the next chapter.

Gene	Gene product	Impact on CPT Sensitivity of <i>Δhhp1</i>	Impact on G2 arrest of <i>Δhhp1</i> in CPT medium
<i>hsk1</i>	Serine Protein Kinase	epistatic	Extended by 60 min
<i>Δtel1</i>	Serine/Threonine Protein Kinase, ATM Checkpoint Kinase	Not epistatic	Extended by 60 min
<i>Δcds1</i>	Serine/Threonine Protein Kinase, Replication Checkpoint Kinase	epistatic	Like <i>Δhhp1</i> (140 min)
<i>Δsrs2</i>	ATR-dependent DNA helicase	epistatic	Like <i>Δhhp1</i> (120 min)
<i>Δmus81</i>	Crossover junction endonuclease Mus81, Holliday junction resolvase subunit	epistatic	-
<i>Δmus7</i>	DNA repair protein	epistatic	-
<i>Δmrc1</i>	Mediator of replication checkpoint protein 1	epistatic	Extended by 40 min
<i>Δchk1</i>	Serine/Threonine Protein Kinase	epistatic	Extended by 20 min
<i>Δrad3</i>	Serine/Threonine Protein Kinase, ATR Checkpoint Kinase	epistatic	-
<i>Δrad51</i>	DNA repair protein RAD51, RecA family recombinase Rad51	Not epistatic	-
<i>Δctp1</i>	Double-strand break repair protein ctp1, CtIP-related endonuclease	Not epistatic	-
<i>Δku70</i>	ATP-dependent DNA helicase II subunit 1, Ku domain protein Pku70	epistatic	Like <i>Δhhp1</i> (120 min)
<i>Δswi1</i>	Transcription regulatory protein Swi1, replication fork protection complex subunit swi1	Not epistatic	-

Table: 3.5.1: Summary table Chapter 3. Activities of Hhp1 kinase has tested with other cell proteins, the aim was to investigated how those proteins cooperated with Hhp1 kinase when DNA replication forks broken as response to introduced CPT (camptothecin) to cells. As result the kinase is in epistatic interaction with some proteins (i.e. *hsk1*, *cds1*, *srs2*, *mus81*, *mus7*, *rad3*, and *ku70*) and other not (i.e. *tel1*, *rad51* *ctp1*, and *swi1*).

Chapter 4: A novel Role of Crb2 in the regulation of DNA Repair at broken Replication Forks

Some sections of this Chapter were published, see appendix 2

Salah Adam Mahyous Saeyd, Katarzyna Ewer-Krzemieniewska, Boyin Liu, and Thomas Caspari. (2014). Hyperactive Cdc2 kinase interferes with the response to broken replication forks by trapping *S.pombe* Crb2 in its mitotic T215 phosphorylated state. *Nucleic Acids Research*, (42): 7734-7747.

Chapter Summary

The phosphorylation of Crb2 (53BP1) at threonine 215 by Cdc2 in mitosis was known for several years to be important for the coordination of the DNA damage response. The results summarised in this chapter strongly support a new role for this DNA binding protein and its T215 modification. The premature accumulation of Cdc2 activity in a *cdc2.1w* mutant (G146D), which is insensitive to Wee1 inhibition, results in an extended G2 arrest when DNA replication forks break in the presence of the topoisomerase 1 inhibitor camptothecin (CPT). Since the mutation of T215 to an alanine residue abolishes this arrest, it is postulated that the hyperactive Cdc2 kinase prolongs the mitotic T215 phosphorylation well into the next cell cycle. The aberrantly modified Crb2 protein may then block the activation of the endonuclease Mus81-Eme1 thereby delaying the repair of broken forks. As it has been reported that the full activation of Mus81-Eme1 depends on Srs2 DNA helicase, Cdc2 and Chk1 kinase, the results in this chapter support the idea that Crb2 has to be removed when Mus81-Eme1 cannot be activated by Srs2. Interestingly, loss of Crb2 abolishes also the extended G2 arrest of CPT-treated *hhp1* deletion cells which is consistent with the results reported in Chapter 3. Like Srs2, Hhp1 is important to activate Mus81-Eme1 probably phosphorylation Eme1. The inability to perform this modification may prolong the CPT-induced G2 arrest since the endonuclease is not fully active. The deletion of *hhp1* is epistatic with the deletion of *srs2*, the deletion of *chk1* and the *cdc2.1w* mutation. In summary, the results support the conclusion that Cdc2, Srs2, Chk1 and Hhp1 all regulate the activity of Mus81-Eme1 in a Crb2-dependent manner. Since Crb2 binds directly to chromatin it may shield broken forks from Mus81-Eme1 in the absence of Srs2 or Hhp1, or when Cdc2 activity is aberrantly high.

4.1. Introduction

Work over recent years established an important role of Cyclin-dependent kinases (CDKs) in the regulation of the DNA damage response (DDR) (197, 198, 199). Early experiments in *S.pombe* revealed a role of cyclin B in homologous recombination (HR) (198) which was later confirmed by findings in *S.cerevisiae* showing that Cdc28 (Cdc2, CDK1) kinase activity is important for the conversion of double-stranded DNA breaks in 3'-single stranded DNA at the start of HR (197). The temporal regulation of HR by CDK1-Cyclin B restricts this process to late S-phase and G2-phase when the sister chromatid is available for repair. Interestingly, the second break repair pathway, Non-homologous End Joining (NHEJ), is active throughout the cell cycle in higher eukaryotic cells, but down-regulated when yeast cells pass through G2-phase (199). The choice of the repair pathway is governed by the competitive binding of the Ku heterodimer (Ku70-Ku80) and the Rad50-Mre11-Nbs1 (MRN) complex (203). Work in *S.cerevisiae* showed that end resection is a two-step process where initially between 50 and 200 base pairs are removed by the MRN complex in association with the endonuclease Sae2 (Ctp1, CtIP) (521). The second step is the extensive resection of up to several kilobases by Exonuclease 1 (Exo1) or the DNA helicase complex Sgs1-Top3-Rmi1 (STR) jointly with the nuclease DNA2 (204, 521). Inactive Sae2 forms large oligomeric structures, which are converted into active Sae2 dimers upon its phosphorylation at serine-267 by Cdc28 kinase (523). The inability to phosphorylate Sae2 leads to a high camptothecin (CPT) sensitivity and faulty DNA end processing. The Ku heterodimer has a low affinity for single-stranded DNA and is removed by the endonuclease activity of Mre11 in the MRN complex to give Sae2 access to the broken chromosome (324). The second important execution point for Cdc28 kinase is the phosphorylation of Dna2 nuclease, which causes its relocation from the cytoplasm to the nucleus in S/G2 (524). In human cells, CDK promotes DSB end resection through the phosphorylation of NBS1 in the MRN complex and the Sae2 related protein CtIP (525).

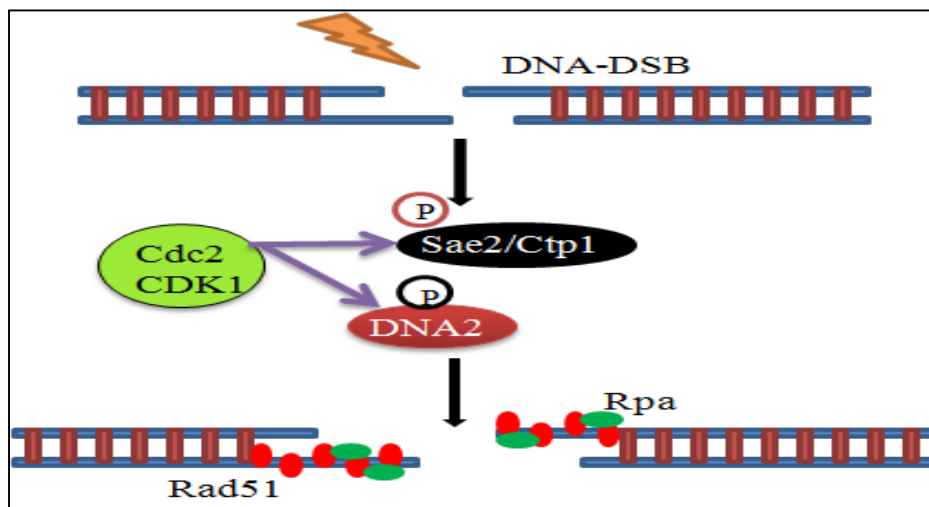


Figure: 4.1.1: CDK1 regulation of Sae2 and DNA2 in *S.cerevisiae*. CDK1 phosphorylates the nucleases Sae2 and DNA2 to produce ssDNA coated by RPA (green circles) at the break site. RPA is then replaced by Rad51 (red circles) to initiate homologous recombination.

CDK1 may regulate Sae2 and DNA2 in *S.cerevisiae*, CDK1 phosphorylates the nucleases Sae2 and DNA2 to produce ssDNA coated by RPA (green circles) at the break site (Figure: 4.1.1). RPA is then replaced by Rad51 (red circles) to initiate homologous recombination (529, 530). The cell cycle regulation of DNA end resection is achieved in a slightly different way in fission yeast. The Sae2 related protein Ctp1 is controlled at its gene expression level rather than by direct Cdc2 phosphorylation as its transcription peaks during the G1/S transition (315). A key role in this regulatory process is dependent on the Cdc2 dependent phosphorylation of the scaffold protein Crb2 (53BP1). Crb2 is phosphorylated by Cdc2-cyclin B at threonine-215 when cells pass through mitosis (215, 289) which is important to promote HR in S/G2 and to sustain the activated Chk1 dependent checkpoint signal (198, 215) (Figure: 4.1.2). The threonine-125 together with a second Cdc2 phosphorylation event at threonine-235 primes Crb2 for a third Cdc2 phosphorylation at the non-canonical site threonine-187. This enables the modified Crb2 protein to assemble with the DNA replication and checkpoint factor Rad4 (TopBP1) (290). The Crb2-Rad4 complex recruits then the DNA damage checkpoint kinase Chk1 (526). While the initial modification at T215 occurs in mitosis at the peak of Cdc2-cyclin B activity, this modification declines when cells progress through S phase. The latter may be important to allow binding of the G2 DNA damage kinase Chk1. Independently of these phosphorylation events, Crb2 can bind directly to DNA. Its tudor domain recognises K20-methylated histone H4 and its

BRCT domains bind to phosphorylated histone H2A (216, 218). This regulatory network is conserved in human cells as the Crb2 related protein 53BP1 is also a target of CDK1 (Cdc2) kinase (214). Consistent with the earlier finding that a mutation in cyclin B affects a late step in HR (230), *S.pombe* Cdc2 was recently shown to phosphorylate Eme1 which associates with the structure-specific endonuclease Mus81 to resolve late recombination intermediates in the absence of the DNA helicase Rqh1 (BLM) (515).

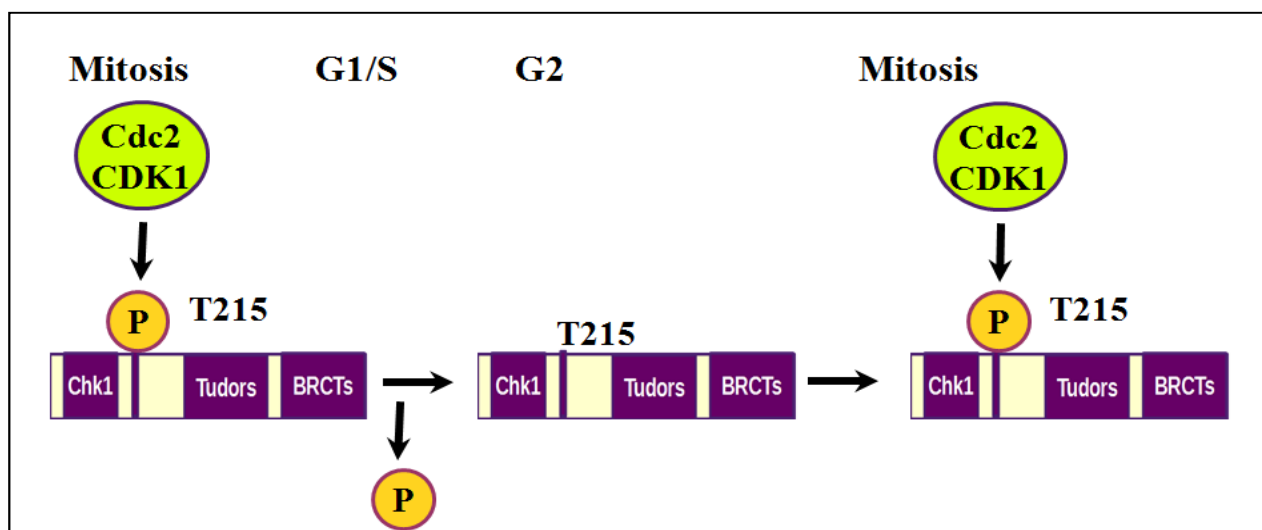


Figure: 4.1.2: Cdc2-dependent regulation of Crb2. Crb2 has one tutor domain and two C-terminal BRCT domains. Phosphorylation of threonine-215 during mitosis by the Cdc2-cyclin B complex primes Crb2 for its G1 activities. Dephosphorylation of T215 during the S phase may be important to promote binding of the G2 DNA checkpoint kinase Chk1.

In human cells, 53BP1 (Crb2) loads Rif1 onto broken DNA in G1 to block end resection. This step is later antagonised in S/G2 by BRCA1 in co-operation with CtIP (CtP1, Sae2) (205, 209, 211). While 53BP1 prevents end resection in G1-phase, it is required for this process in G2 in collaboration with BRCA1 (212, 213). It seems that human 53BP1 acts as a switch between NHEJ in G1 and HR in G2 (Figure: 4.1.3). Human BRCA1 is modified by CDK1 (Cdc2) at serine-1497, serine-1189 and serine-S1191 which is important for the intra-S DNA damage checkpoint response (527). BRCA2, which loads the recombination protein Rad51 onto single-stranded DNA is also modified by human CDKs at serine-3291, but this modification peaks in mitosis to block the BRCA2-Rad51 interaction (527). During S-phase, the human Mre11 subunit, which is part of the MRN (Mre11- Rad50-Nbs1) complex, recruits CDK2-Cyclin A to broken chromosomes to facilitate the formation of a complex between BRCA1 and the endonuclease CtIP (Ctp1, Sae2) to promote end resection (208, 209).

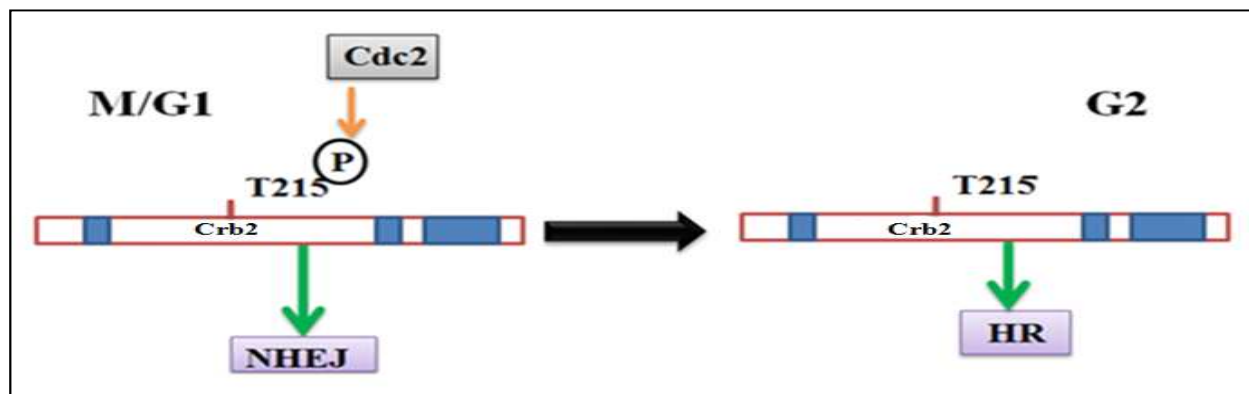


Figure: 4.1.3: Crb2 associated with Cdc2 to guide DNA repair. Cdc2 phosphorylated Crb2 at G1-phase and this phosphorylation is continued in G2-phase and result in use HR instead NHEJ (215, 198).

In summary, CDK phosphorylation events control the balance between NHEJ and HR at the level of end resection by promoting or preventing the recruitment of Sae2 (Ctp1, CtIP) endonuclease to the break. The MRN complex and the scaffold proteins Crb2 (53BP1) and BRCA1 do play key roles in this regulation. CDK enzymes affect HR also at a later step when recombination intermediates are processed by DNA Rqh1 (BLM) DNA helicase or the structure-specific endonuclease Mus81-Eme1. A third execution point appears to be in mitosis when HR proteins like BRCA2 are inactivated.

This chapter summarises the work on a hyperactive *cdc2* allele (*cdc2.1w*; *wee2-1*) in *S. pombe* which renders cells specifically sensitive CPT. The *cdc2.1w* mutant strain enters mitosis prematurely due to a dominant mutation in the vicinity of its ATP binding site (G146D) (222). This glycine-to-aspartate mutations renders Cdc2.1w kinase insensitivity to inhibition by Wee1 kinase (222, 223). Interestingly, loss of *wee1* results in a much wider DNA damage sensitivity profile than the *cdc2.1w* mutation (224, 226) although both mutant strains enter mitosis prematurely. This implies that the dominant G146D mutation in Cdc2.1w affects specifically the DNA damage response when DNA replication forks collide with immobilised topoisomerase 1 and break.

4.2. A hyper-active Cdc2.1w Kinase enhances the Camptothecin Sensitivity

Why the G146D mutation close to the ATP binding site of Cdc2 (Figure:4.2.1) renders the kinase insensitivity to Wee1 inhibition is not yet clear. To test the DNA sensitivity profile of *cdc2.1w* and Δ *wee1* deletion strains, a drop test was performed.

As shown in Figure:4.2.3, Figure:4.2.4, Figure:4.2.5, Figure:4.2.6, Figure:4.2.7, Figure:4.2.8, Δ *wee1* cells have a much wider DNA damage sensitivity spectrum than *cdc2.1w* cells despite the

fact that both mutants enter mitosis prematurely (224, 225, 226). Cells without Wee1 are also temperature sensitive. Hyper-activation of Cdc2 by the G146D mutation affects the response to CPT suggesting a role of the kinase in the repair of broken replication forks. To test whether Cdc2 and Wee1 act in the same DNA damage response pathway, a $\Delta wee1 cdc2.1w$ double mutant was tested. The data shown in Figure:4.2.5 and Figure:4.2.7 imply that both kinases act in the same pathway for CPT and MMS induced DNA lesions. To find out whether the CPT sensitivity of *cdc2.1w* cells is a direct consequence of the enhanced Cdc2 activity which accumulates prematurely in early G2 (245), a drop test on CPT plates was performed with a *cdc2.1w cdc25.22* double mutant. The point mutation in Cdc25 phosphatase, which removes the inhibitory Y15 phosphorylation from Cdc2 is expected to lower the impact of Cdc2.1w activity by prolonging G2 (221). Indeed, the double mutant was CPT resistant compared to the *cdc2.1w* single mutant (Figure: 4.2.9) which supports the conclusion that the premature activation of Cdc2 impairs the repair of broken replication forks. As reported previously (221), the *cdc2.1w cdc25.22* double mutant was less temperature sensitive because the hyper-active Cdc2.1w kinase partly overcomes the low Cdc25 phosphatase activity. Given that cells without Hhp1 kinase suffer from a defective response to broken replication forks (see Chapter 3), the genetic relationship between Wee1 and Cdc2.1w and Ck1 was explored. Repeated attempts to cross the deletion of *hhp1* with the deletion of *wee1* failed to produce the correct strain which suggests that loss of both kinases may be a lethal event. To circumvent this problem, the *wee1-50* temperature sensitive loss-of-function allele was used. Even at the semi-permissive temperature of 30°C, *wee1-50* cells are MMS and ionising radiation sensitive (164, 538). As shown in Figures: 4.2.5 and Figures: 4.2.7, the $\Delta hhp1 wee1-50$ strain was as CPT and MMS sensitive as the $\Delta hhp1$ single mutant which places both kinases in the same pathway. A $\Delta hhp1 cdc2.1w$ strain was also constructed and tested (Figures: 4.2.9), consistent with the earlier finding that Cdc2.1w and Wee1 act in the same pathway, the $\Delta hhp1 cdc2.1w$ double mutant was as (CPT and MMS) sensitive as the $\Delta hhp1$ single mutant (Figures: 4.2.10, Figures: 4.2.11).

These observations place Hhp1 kinase firmly together with the cell cycle regulators Wee1 and Cdc2 in the same DNA damage response pathway. Since Hhp1 is also closely linked with the Mus81-Eme1 complex (Chapter 3), it is very likely that Wee1 and/or Cdc2 regulate this endonuclease. This conclusion is supported by the physical association of Wee1 and Mus81 in human cells (539), and by the requirement of Cdc2 for the phosphorylation of Eme1 when

fission yeast cells progress through G2 (515) (Figures: 4.2.2). Whether Cdc2 and Wee1 target the Mus81-Eme1 endonuclease directly or indirectly via Hhp1 and/or Chk1 kinase is not yet known.

To get an insight into this, total extracts were prepared from Hhp1-HA cells and Hhp1-HA *cdc2.1w* cells either from untreated cultures or cultures treated with 40 μ M CPT for 4 hours. All extracts were then subjected to isoelectric focusing on a linear pH gradient from 3 to 10. As shown in Figure: 4.2.12, two spots with distinct isoelectric points were present in untreated Hhp1-HA extracts. In contrast to the earlier experiments shown in Chapter 3, the three alkaline Hhp1 forms closer to the negative end of the strip did not separate well as they merged into one signal. Elevated Cdc2 activity (*hhp1-HA cdc2.1w*) led to an increase in the more acidic form (number 2 in Figure: 4.2.12) which suggests that the post-translational modification pattern of Hhp1 kinase changes when Cdc2 activity increases. Interestingly, treatment of the *hhp1-HA cdc2.1w* strain with CPT resulted in the appearance of hyper-modified forms which had a more negative isoelectric point running closer to the positive end of the strip (Figure: 4.2.12). This indicates that Hhp1 is aberrantly modified when cells with high Cdc2 activity experience CPT damage. This also suggests that Cdc2 may directly target Hhp1.

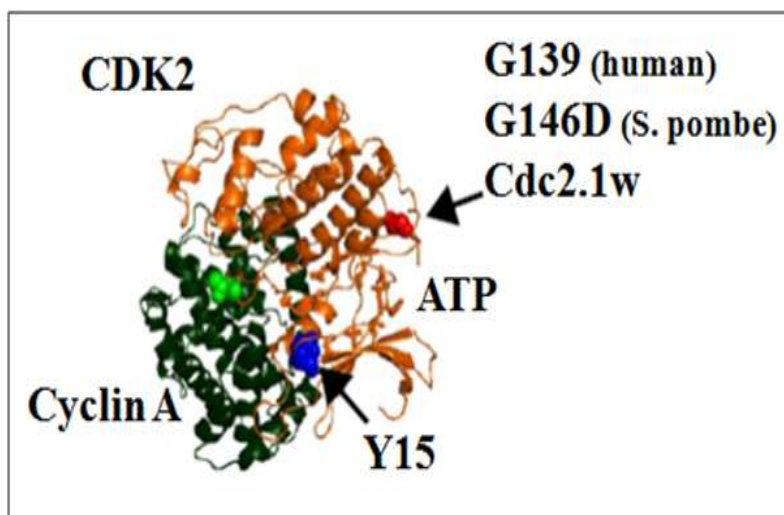


Figure: 4.2.1: Cdc2.1w kinase is mutated at glycine 146 (G146D). The aspartate mutation affects a loop at the entrance to the ATP binding site. The inhibitory tyrosine 15 (Y15) phosphorylation site is indicated in blue. Wee1 and Mik1 kinase phosphorylate Y15 in fission yeast CDK1 (Cdc2). The closely related structure of the human CDK2-cyclin A complex (PDB ID:1FIN (253)) has been visualised using the Polyview 3D program (adopted from my paper (272)).

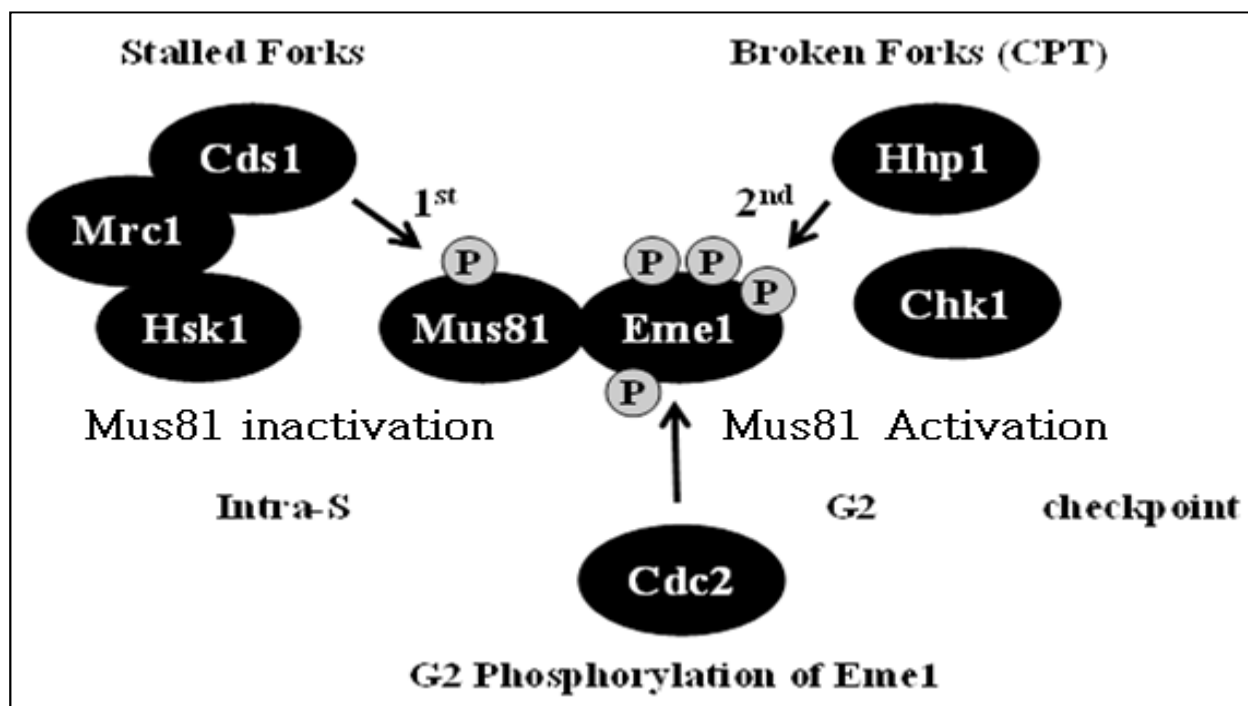


Figure: 4.2.2: The genetic linkage between Wee1, Cdc1 and Hhp1 could be explained by the regulation of the Mus81-Eme1 endonuclease. While the endonuclease is excluded from the nucleus in S phase upon its phosphorylation by Cds1, its activity is required for the repair of broken replication forks in G2.

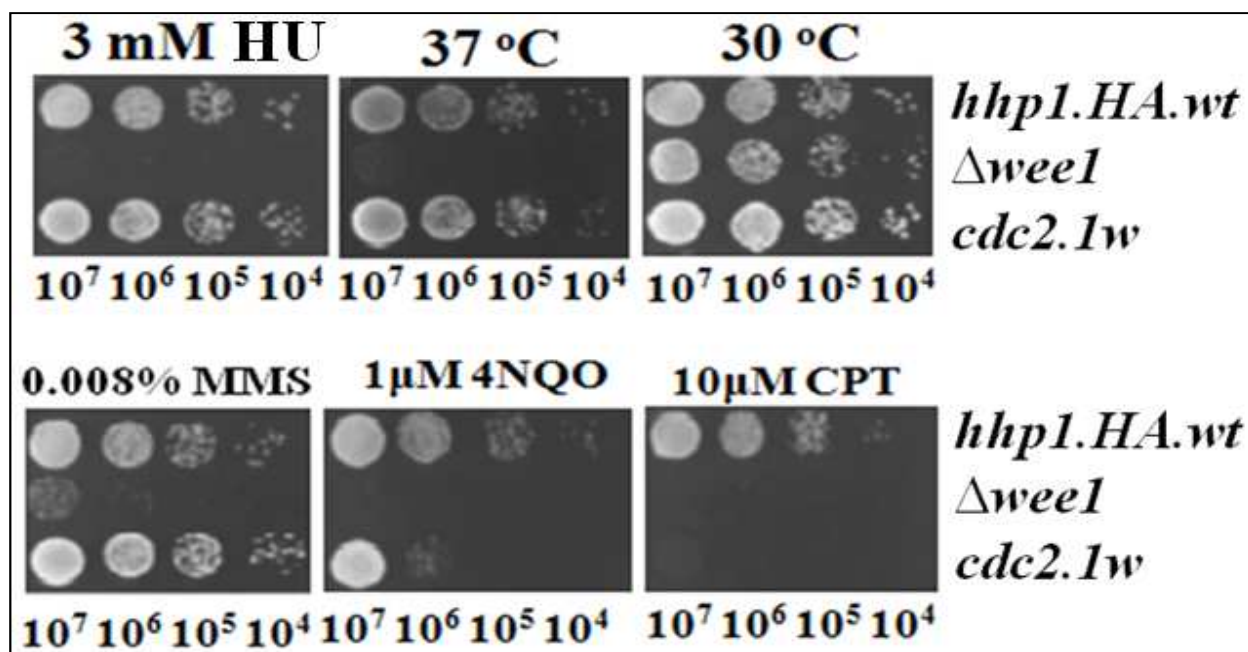


Figure: 4.2.3: *cdc2.1w* cells are mainly CPT sensitive. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, except one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity. CPT (camptothecin), HU (hydroxyurea), 4NQO (4-nitroquinoline oxide), MMS (methyl methanesulfonate).

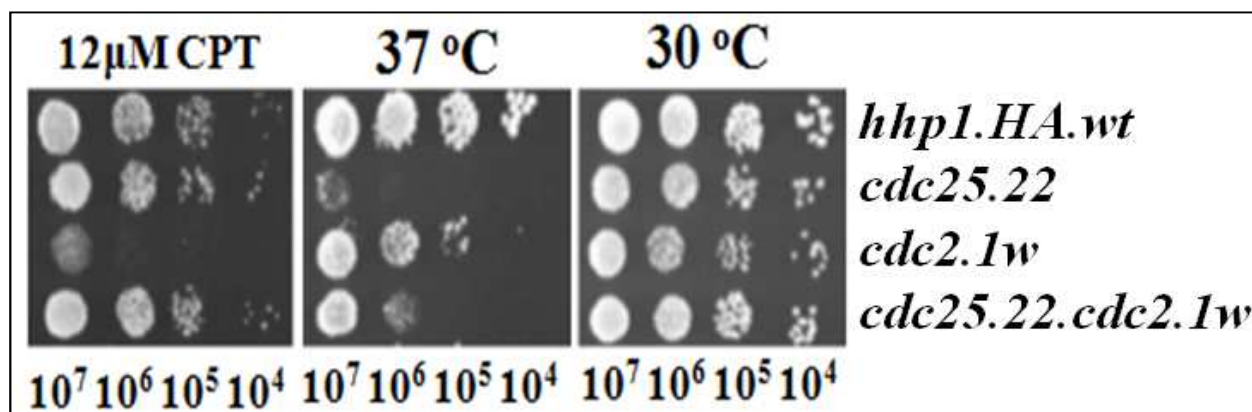


Figure: 4.2.4: A reduction in Cdc2.1w activity by a decrease of Cdc25 phosphatase activation (*cdc25.22*) suppresses the CPT (camptothecin) sensitivity. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, except one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity.

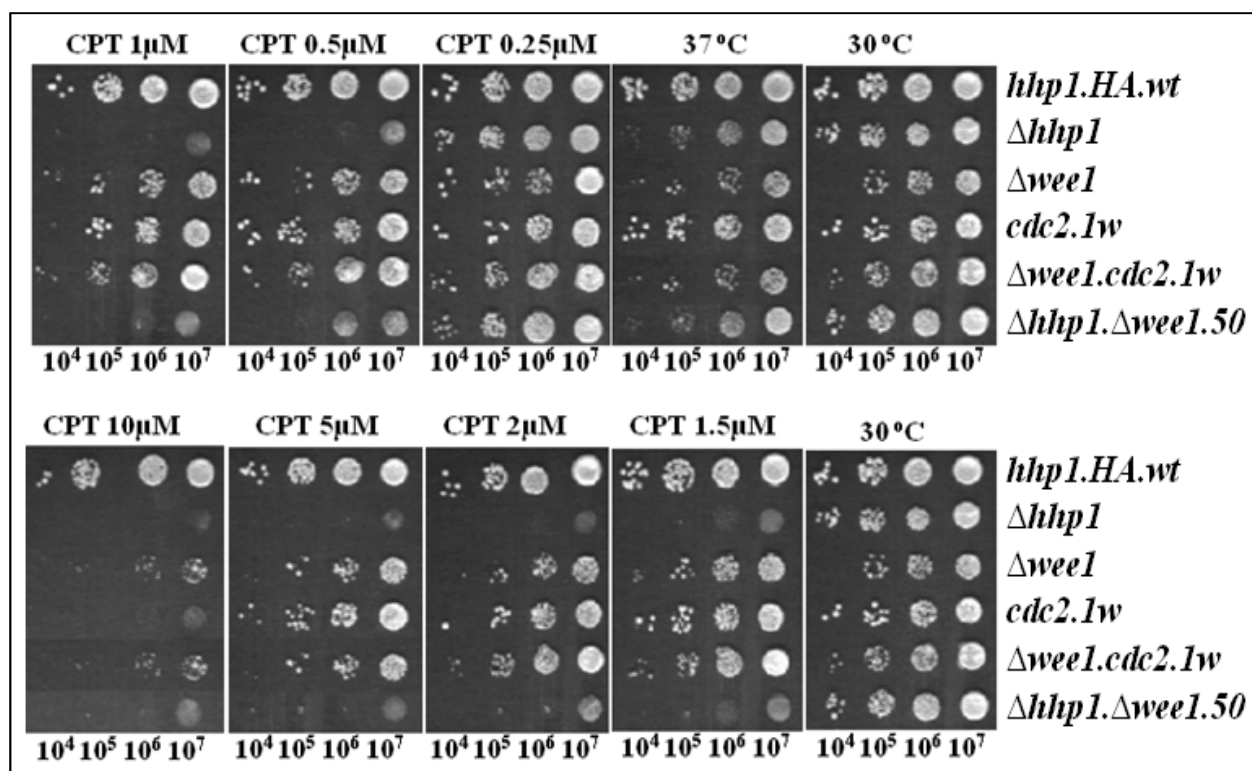


Figure: 4.2.5: Wee1 and Hhp1 act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, except one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity.

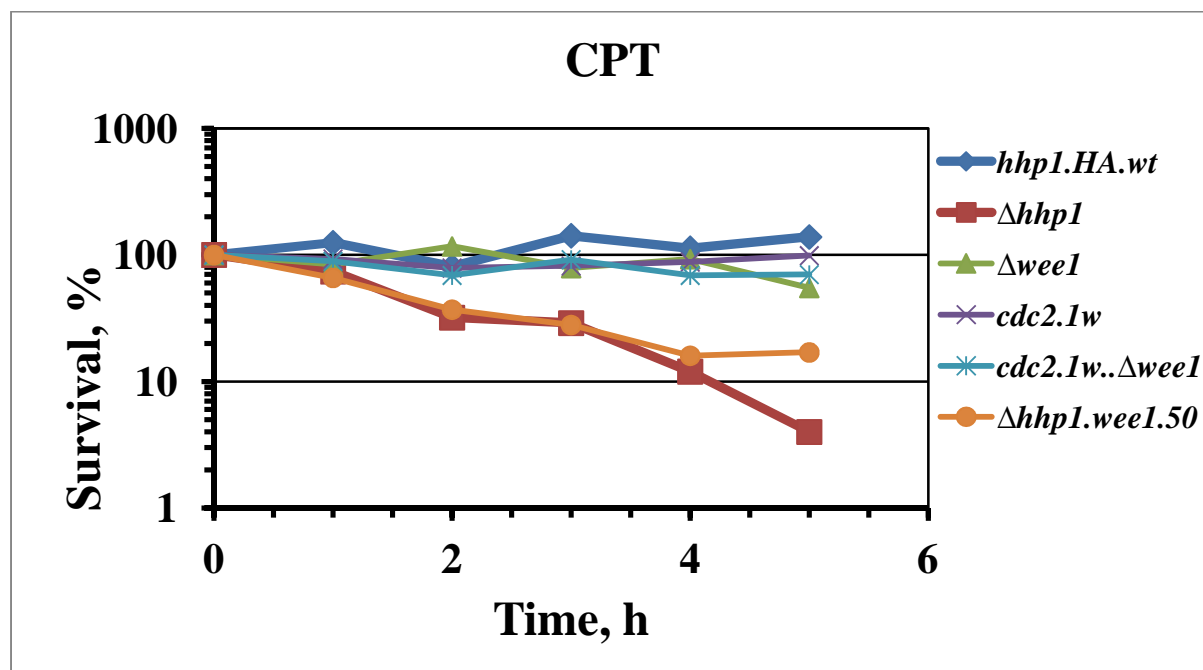


Figure: 4.2.6: Wee1 and Hhp1 act in the same CPT (camptothecin) response pathway. The indicated yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40μM CPT for five hours at 30 °C. Aliquots of 75 μl were collected every hour and the surviving colonies were scored after plating on YEA plates.

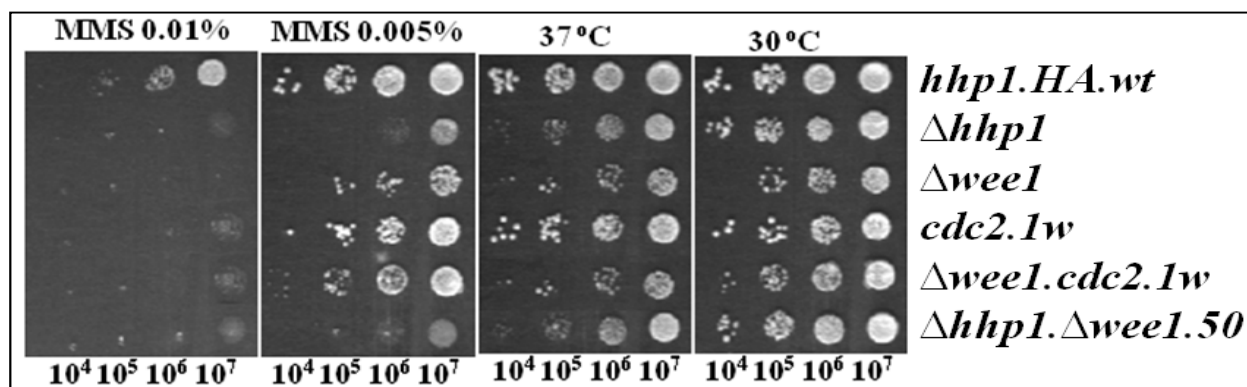


Figure: 4.2.7: Wee1 and Hhp1 act in the same MMS response pathway. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, except one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity. MMS (methyl-methansulfonate).

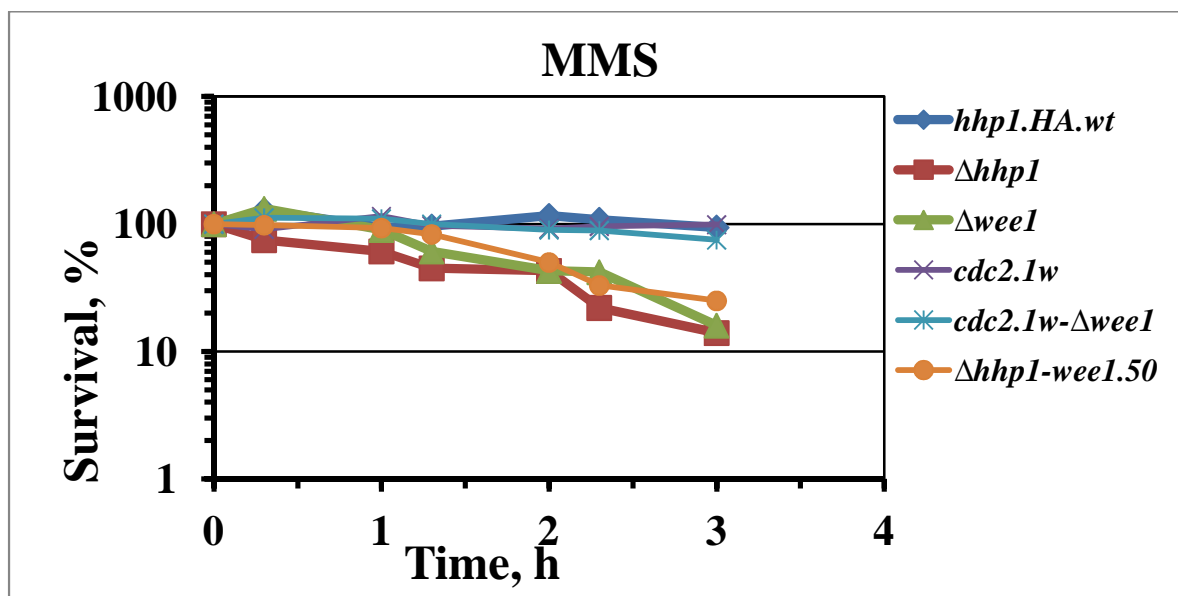


Figure: 4.2.8: Wee1 and Hhp1 act in the same MMS (methyl-methanesulfonate) response pathway. The indicated yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 0.05% MMS three hours at 30 °C. Aliquots of 75 μ l were collected at the indicated time points and the surviving colonies were scored after plating on YEA plates.

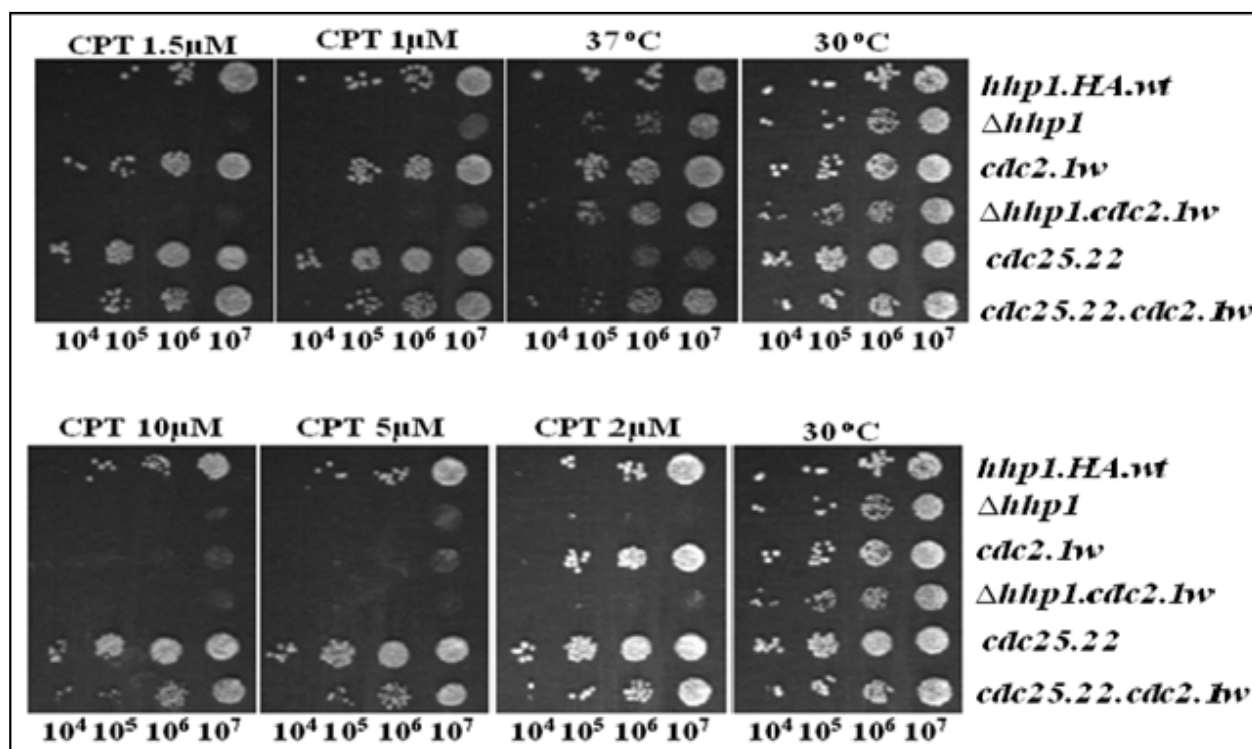


Figure: 4.2.9: Cdc2.1w and Hhp1 act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, except one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity.

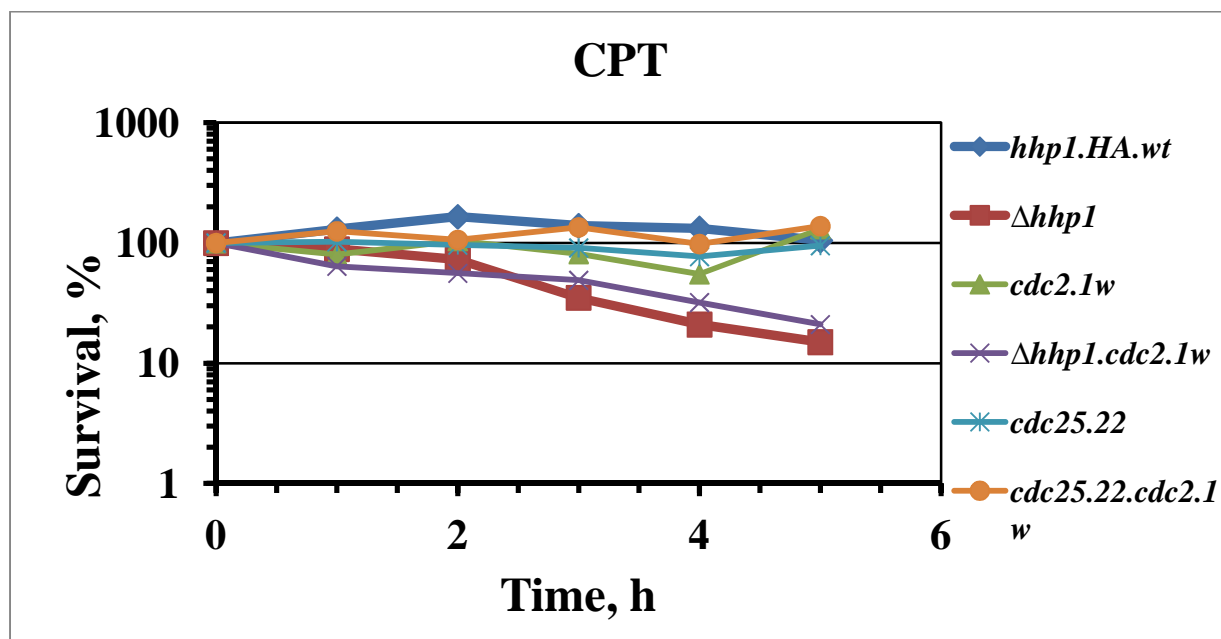


Figure: 4.2.10: Cdc2.1w and Hhp1 act in the same CPT (camptothecin) response pathway. The indicated yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40μM CPT for five hours at 30° C. Aliquots of 75 μl were collected every hour and the surviving colonies were scored after plating on YEA plates.

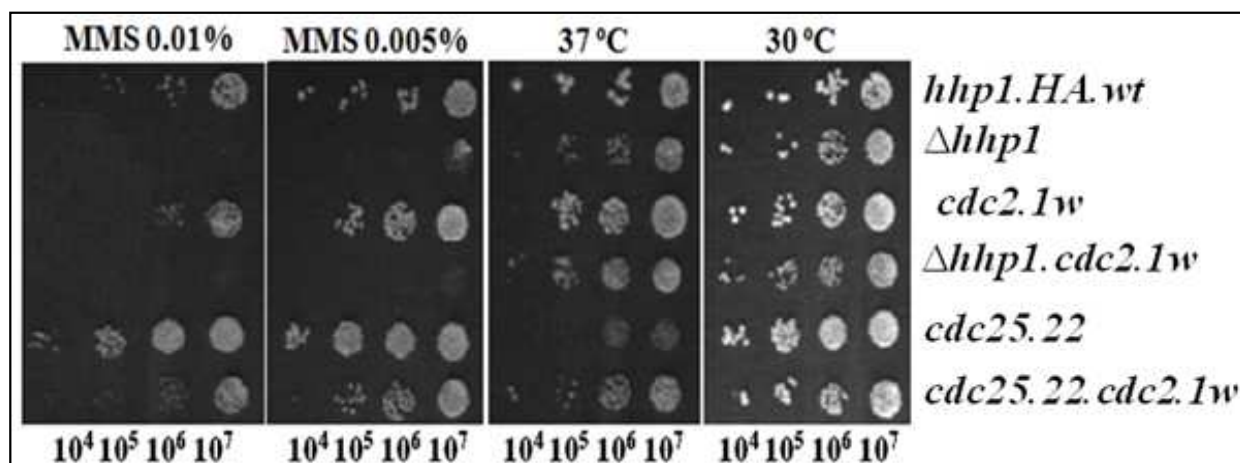


Figure: 4.2.11: Cdc2.1w has no impact on the methyl-methanesulfonate (MMS) sensitivity of cells without Hhp1. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, except one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity. Elevated Cdc2.1w activity improves growth of Δ *hhp1* cells at 37°C.

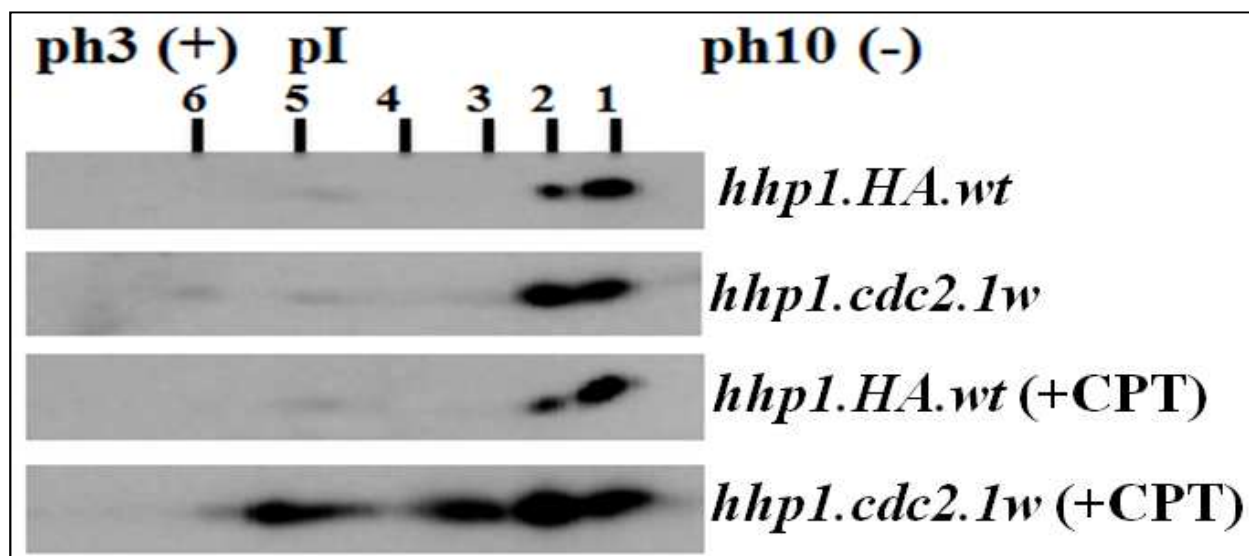


Figure: 4.2.12: Aberrant modification of Hhp1 kinase at elevated Cdc2 levels. Total protein extracts were first separated on a linear pH3-10 strip before being separated by size (proteins visualised by using an anti-HA antibody) on a 10% SDS PAGE (Western blot). Spot 1 is a combination of three Hhp1 forms which merged into one signal. The intensity of the more negative (acidic) signal 2 increases in *cdc2.1w* cells and more negative forms of Hhp1 appear when *hhp1-HA cdc2.1w* cells were treated with 40 μ M CPT (camptothecin) at 30 $^{\circ}$ C for 4 hours.

4.3. Elevated Cdc2 Activity Prolongs the G2 Arrest when Replication Forks Break

Since loss of Hhp1 kinase prolongs the G2 arrest when DNA replication forks break (Figure: 3.1.5.B), the duration of the G2 arrest was measured in wild type (*hhp1-HA*), Δ *chk1*, *cdc2.1w*, *cdc25.22* and *cdc2.1w cdc25.22* strains. Wild type cells arrest only briefly for 20-40 min in the second G2 phase in the presence of 40 μ M CPT (Figure: 4.3.1). The arrest happens in the second G2 phase since CPT acts only in S-phase and cells won't experience DNA breaks before they passed through the first round of DNA replication. Deletion of Chk1 kinase, which is activated by CPT treatment (193), abolishes this transient arrest (Figure: 4.3.2). Unexpectedly, the premature rise in Cdc2 activity in the *cdc2.1w* strain resulted in a prolonged G2 arrest for up to 100 min despite the early onset of mitosis in untreated cells (Figure: 4.3.3). This prolonged arrest resembles the extended G2 delay of Δ *hhp1* cells (100-120 min) (Figure: 3.1.5.B) and suggests that broken forks are either not efficiently repaired or that the arrest signal cannot be inactivated in the presence of elevated Cdc2 activity. This defect was not suppressed by the point mutation in *cdc25* as the *cdc2.1w cdc25.22* strain retained the prolonged arrest of the *cdc2.1w* single mutant (Figure: 4.3.4). The mutation in *cdc25* on its own had little impact on the short G2 delay of 20 min in the presence of CPT although the unperturbed cell cycle was delayed by 20-40 min

in untreated cells as expected from cells with a reduction in the activating activity of Cdc25 phosphatase (Figure: 4.3.5).

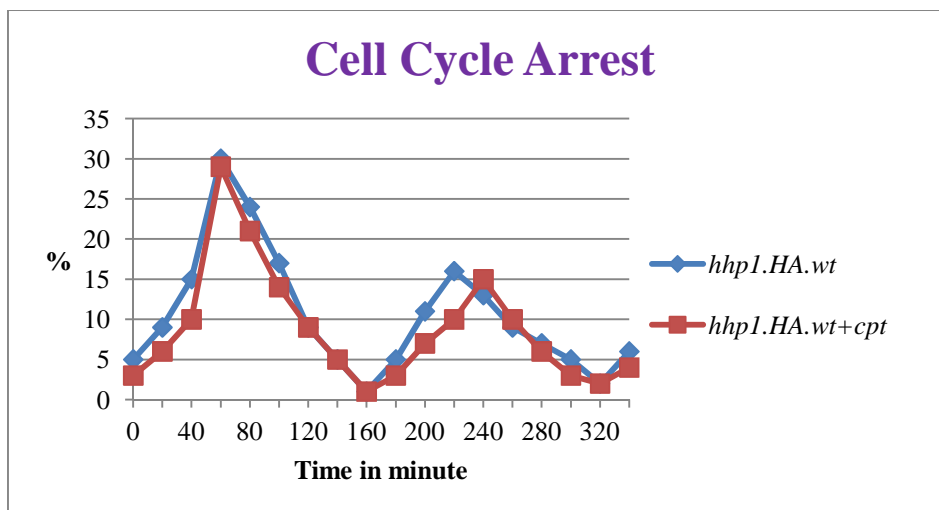


Figure: 4.3.1: CPT-induced (Camptothecin-induced) G2 arrest in wild type cells. Wild type cells (*hhp1.HA.wt*) only briefly delay in the second G2 in the presence of CPT. Wild type cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

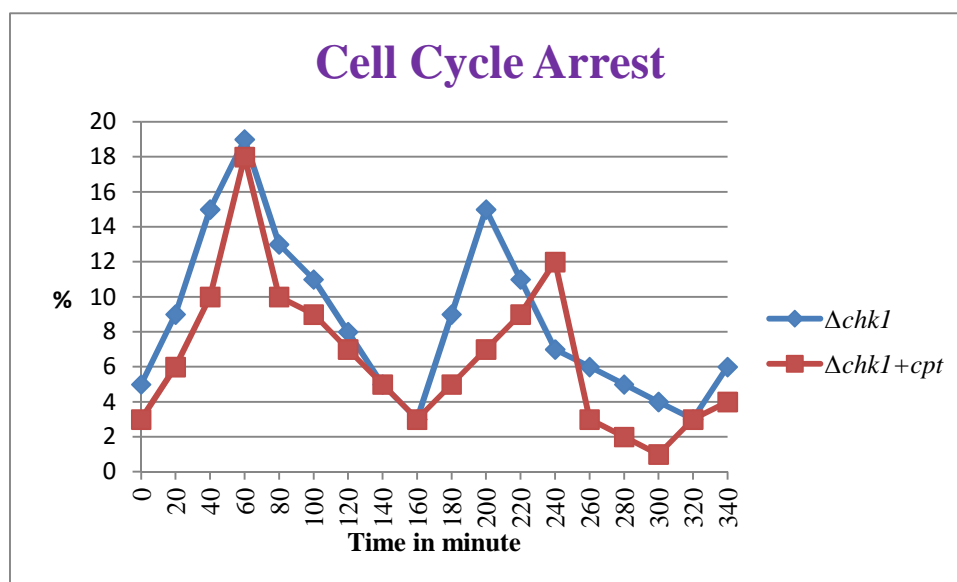


Figure: 4.3.2: Δ *chk1* cells and DNA replication. Here Δ *chk1* cells cultured with and without 40 μ M CPT (camptothecin). Treated cells had G2 delay about forty minutes at the second peak which is response to replication fork collapse.

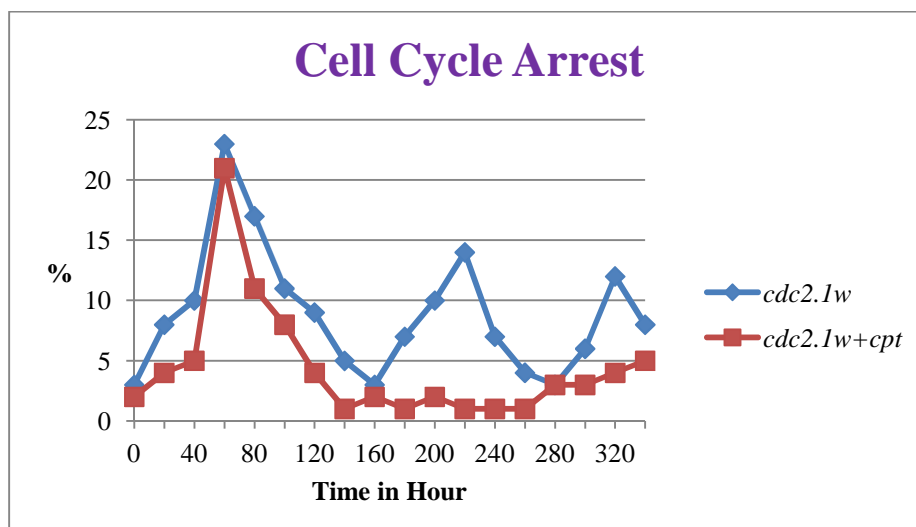


Figure: 4.3.3: High Cdc2 activity results in an extended G2 arrest in the presence of CPT. *cdc2.1w* cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

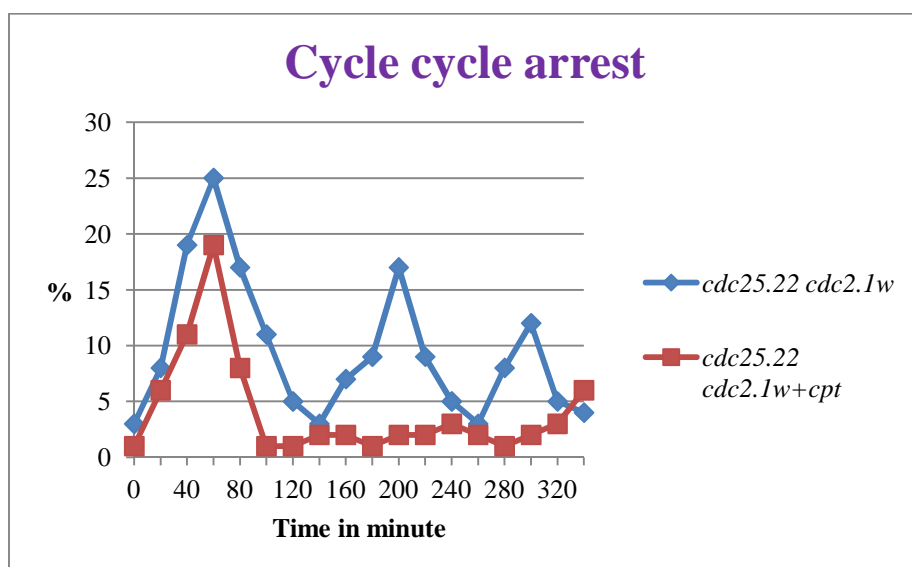


Figure: 4.3.4: A reduction in Cdc25 phosphatase activity does not abolish the extended G2 arrest in *cdc2.1w* cells. *cdc25.22 cdc2.1w* cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

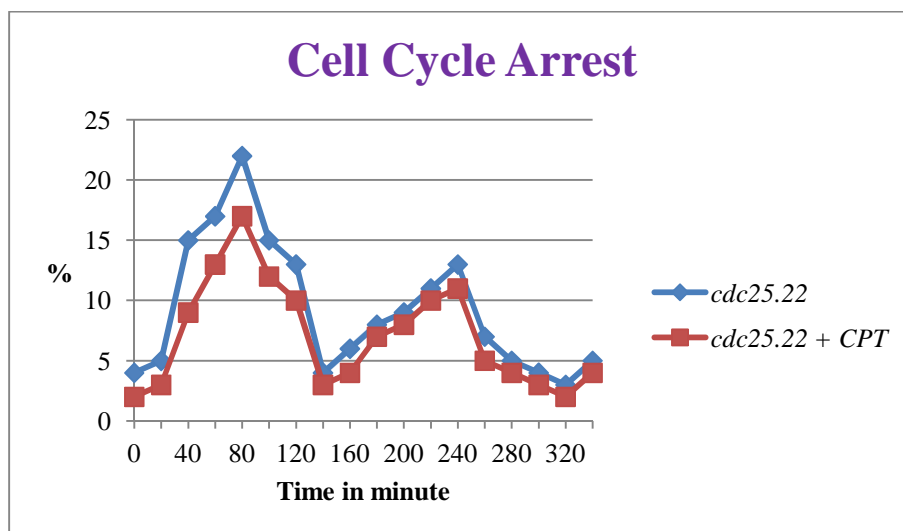


Figure: 4.3.5: The mutation in Cdc25 phosphatase has little impact on the CPT-induced G2 arrest, but cells delay progression through the unperturbed cell cycle. *cdc25.22* cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

4.4. Cds1 and Chk1 influence both the G2 arrest in *cdc2.1w* cells

The extended G2 arrest in *cdc2.1w* cells suggests that damaged replication forks are not efficiently repaired which would cause a prolonged DNA damage checkpoint signal. To test whether Chk1 or Cds1 are required for this arrest, both kinase genes were either individually deleted in *cdc2.1w* cells (Δ *chk1 cdc2.1w*, Δ *cds1 cdc2.1w*) or simultaneously removed (Δ *cds1* Δ *chk1 cdc2.1w*). Interestingly only the deletion of both kinases at the same time abolished the extended G2 arrest which suggests that both kinases are required (Figure: 4.3.3, Figure: 4.4.1, Figure: 4.4.2, Figure: 4.4.3). It is well established that Chk1 acts in G2 after the execution point of Cds1 in S (285, 540) which is normally explained by the appearance of DNA damage in the absence of Cds1 which then activates Chk1 (236). It is therefore possible that elevated Cdc2 activity triggers a problem at DNA replication forks which initially activates Cds1 and is then transferred to Chk1 if Cds1 is absent.

In summary, these experiments support the idea that the premature accumulation of Cdc2 activity early in the cell cycle interferes with the response to broken DNA replication forks in the presence of the topoisomerase 1 inhibitor camptothecin (CPT). This triggers the sequential activation of Cds1 and Chk1 resulting in a prolonged G2 arrest.

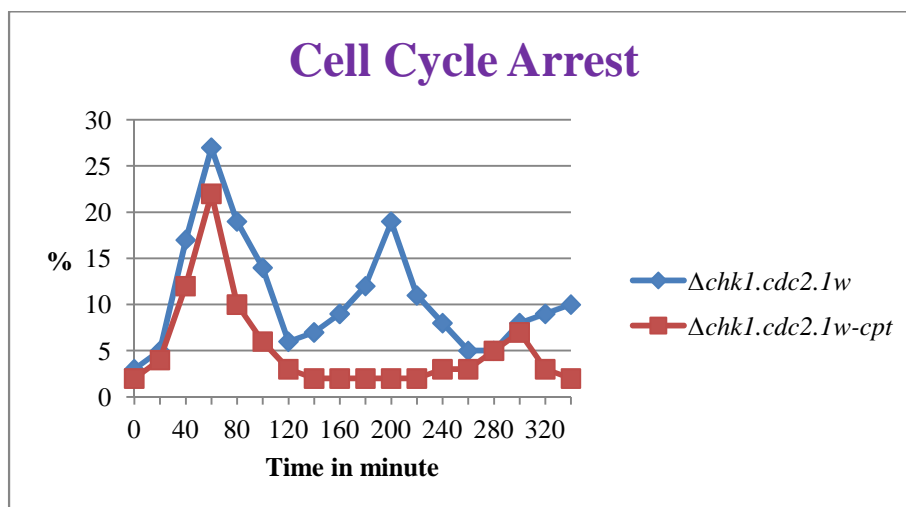


Figure: 4.4.1: Deletion of *chk1* does not reduce the G2 arrest in *cdc2.1w* cells. $\Delta chk1.cdc2.1w$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

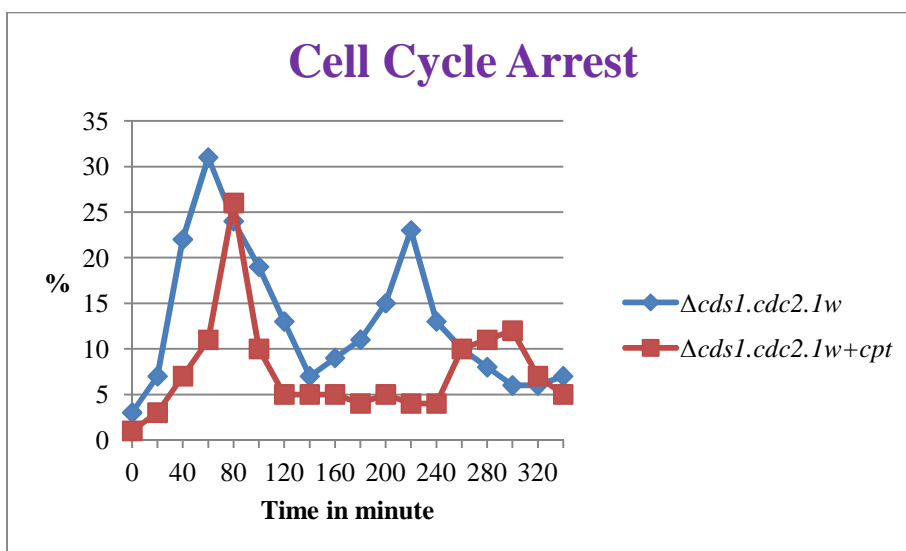


Figure: 4.4.2: Deletion of *cds1* does not reduce the G2 arrest in *cdc2.1w* cells. $\Delta cds1.cdc2.1w$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

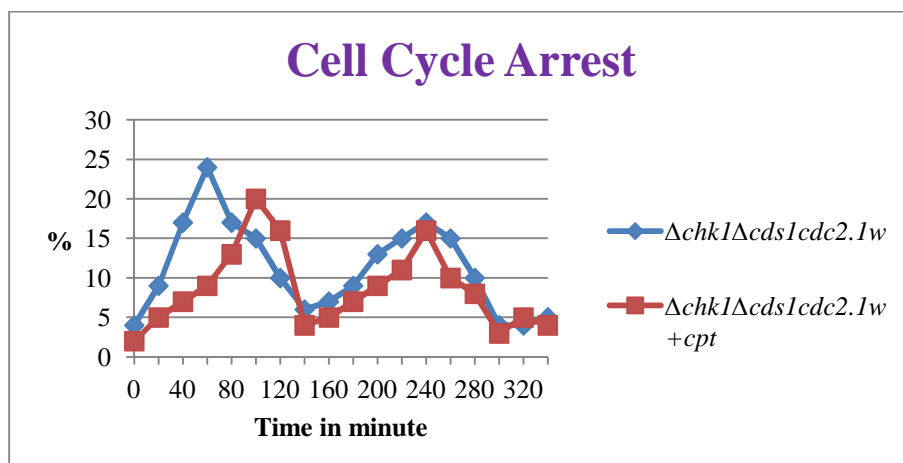


Figure: 4.4.3: Deletion of both, *chk1* and *cds1*, abolishes the G2 arrest in *cdc2.1w* cells. $\Delta chk1 \Delta cds1 cdc2.1w$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

4.5. Cdc2 targets the DNA Binding Protein Crb2 at Threonine-215 to regulate Cds1 and Chk1

To find a requirement of Cds1 for the extended G2 arrest in *cdc2.1w* cells (Figure: 4.4.2) was a surprise as Cds1 is normally only activated when replication forks stall (237). Given that Cdc2 phosphorylates the DNA binding protein Crb2 (53BP1) at threonine-215 (T215) (215), and since Crb2 associates with Chk1 (270), the requirement of Crb2 and the T215 phosphorylation for the extended G2 arrest in *cdc2.1w* cells was tested.

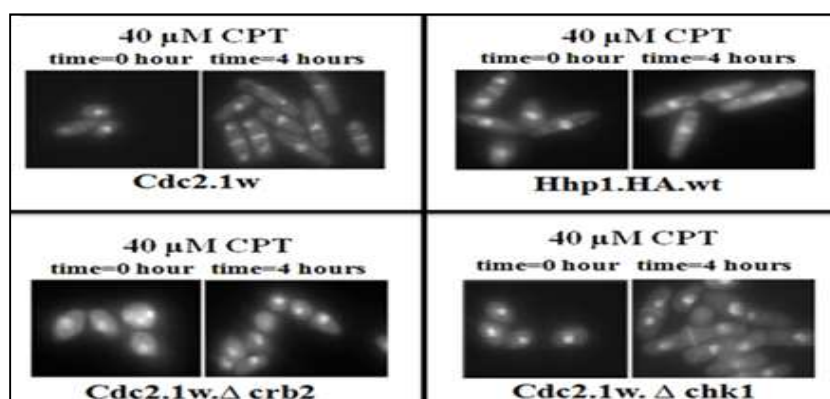


Figure 4.5.1: Fluorescence microscope screening *S.pombe* strains. The indicated strains were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40 μ M CPT (camptothecin) or left untreated for four hours at 30 °C. Cells were fixed in methanol, and the DNA and the cell wall were stained using Hoechst and calcofluor respectively (adopted from my paper (272)).

The *cdc2.1w.Δcrb2* strain had a very small and heterogeneous cell size as cells entered mitosis early (Figure: 4.5.1). Since this made it very difficult to synchronise cells, the asynchronous *cdc2.1w Δcrb2* strain was incubated in rich medium with 40 μ M CPT for 5 hours together with *hhp1-HA* wild type cells, the *cdc2.1w* strain and the double mutant *cdc2.1w.Δchk1*. Every hour samples were taken, fixed and stained with Hoechst (DNA) and calcofluor (new septum) to examine the cells under the microscope. As shown in Figure: 4.5.1, loss of *crb2* prevented elongation of the cells after they have been exposed to the topoisomerase 1 inhibitor CPT for 5 hours. Cell elongation, which was evident for the other three strains, indicates checkpoint activation and a G2 arrest (226). Consistent with the absence of a G2 arrest, the septation index of the *cdc2.1w.Δcrb2* strain did not drop while the *cdc2.1w* and *cdc2.1w.Δchk1* strains showed a clear decline (Figure: 4.5.2).

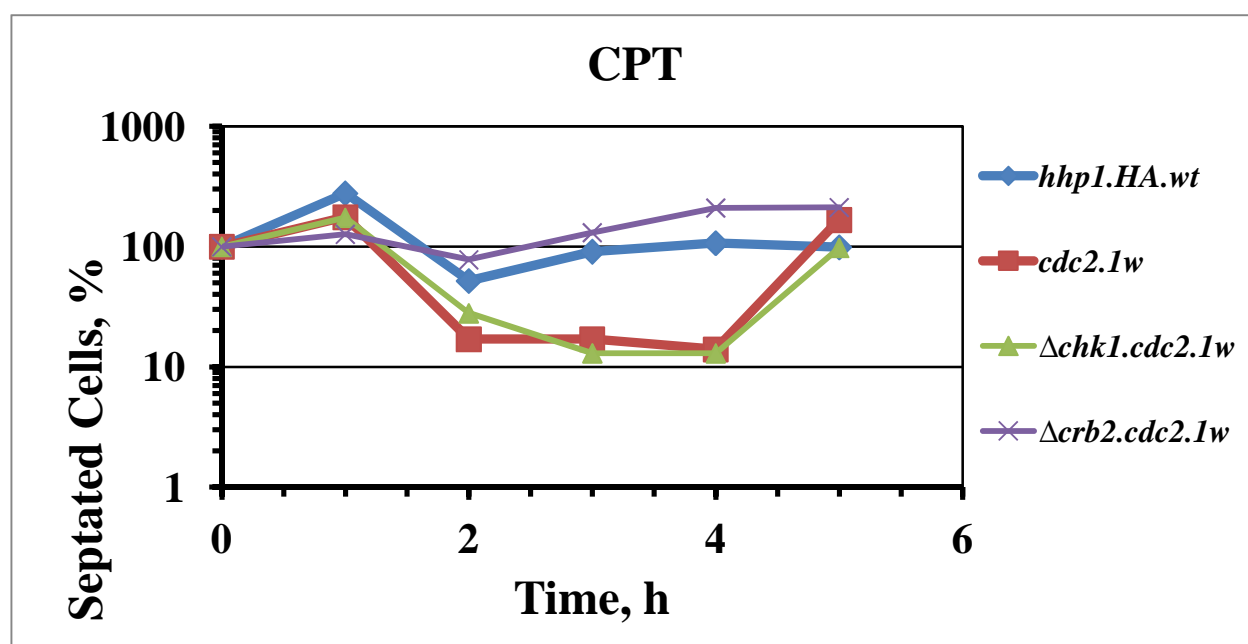


Figure: 4.5.2: Loss of Crb2 abolishes the extended G2 arrest in *cdc2.1w* cells. The indicated strains were exposed to 40 μ M CPT (camptothecin) at 30 °C for 5 hours. Samples were withdrawn every hour, fixed in methanol and stained with Hoechst and calcofluor. The percentage of septated cells is shown. A drop in the number of septated cells indicated a G2 arrest. Cells from the 4 hour time point are shown in Figure 4.5.1.

Wild type cells showed only a modest drop at the 2-hour time point consistent with the 20-40 min delay when cells are synchronised. The requirement of Crb2 for the extended G2 delay was very unexpected since loss of Chk1 had not the same impact. Interestingly, the ability to arrest for longer is not linked with the survival of the double mutants as both, and *cdc2.1w.Δchk1* and

cdc2.1w.Δcrb2, lost viability to a similar degree in the presence of 40 μ M CPT (Figure: 4.5.5). Both double mutants were more CPT sensitive than the single mutants and the *cdc2.1w.Δchk1* strain was also temperature sensitive (Figure: 4.5.3, Figure: 4.5.4). This implies that Cdc2.1w, Chk1 and Crb2 affect more than one pathway. The data also reveal that the T215 modification of Crb2 by Cdc2 is important to activate Cds1 and Chk1 in the *cdc2.1w* mutant since the *cdc2.1w crb2.T215A* mutant (Figure: 4.5.6) resembles the *cdc2.1w Δcds1 Δchk1* strain (Figure: 4.4.3).

Since Chk1 is phosphorylated at serine-345 in the presence of CPT (235), the phosphorylation status of Chk1 was tested in *chk1.HA* wild type cells, *chk1.HA.cdc2.1w* and *chk1.HA.cdc2.1w.Δcrb2* strains. As reported previously (229), Chk1 is phosphorylated even in the absence of DNA damaging agents in a *cdc2.1w* strain (Figure: 4.5.5). CPT treatment induced the checkpoint-dependent phosphorylation in all strains with the exception of the *chk1.HA.cdc2.1w Δcrb2* strain (Figure: 4.5.5). The requirement of Crb2 for the activation of Chk1 has been reported previously (246). Why Chk1 becomes phosphorylated in a *cdc2.1w* strain is not yet clear. Either the hyperactive Cdc2 kinase targets Chk1 directly or causes a form of DNA damage which indirectly activates Chk1.

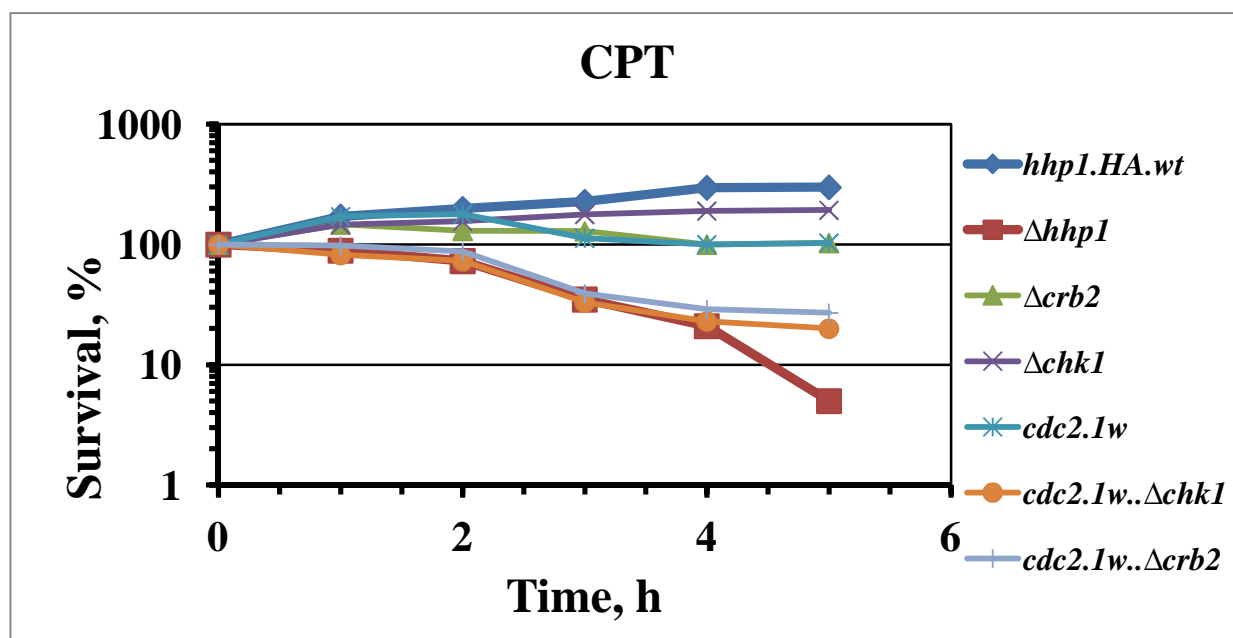


Figure: 4.5.3: *S.pombe cdc2.1w* cells without *chk1* or *crb2* are highly CPT sensitive. The indicated yeast strain cells were cultured in YEA medium overnight at 30 $^{\circ}$ C. Cells were harvested and treated with 40 μ M CPT for five hours at 30 $^{\circ}$ C. Aliquots of 75 μ l were collected every hour and the surviving colonies were scored after plating on YEA plates.

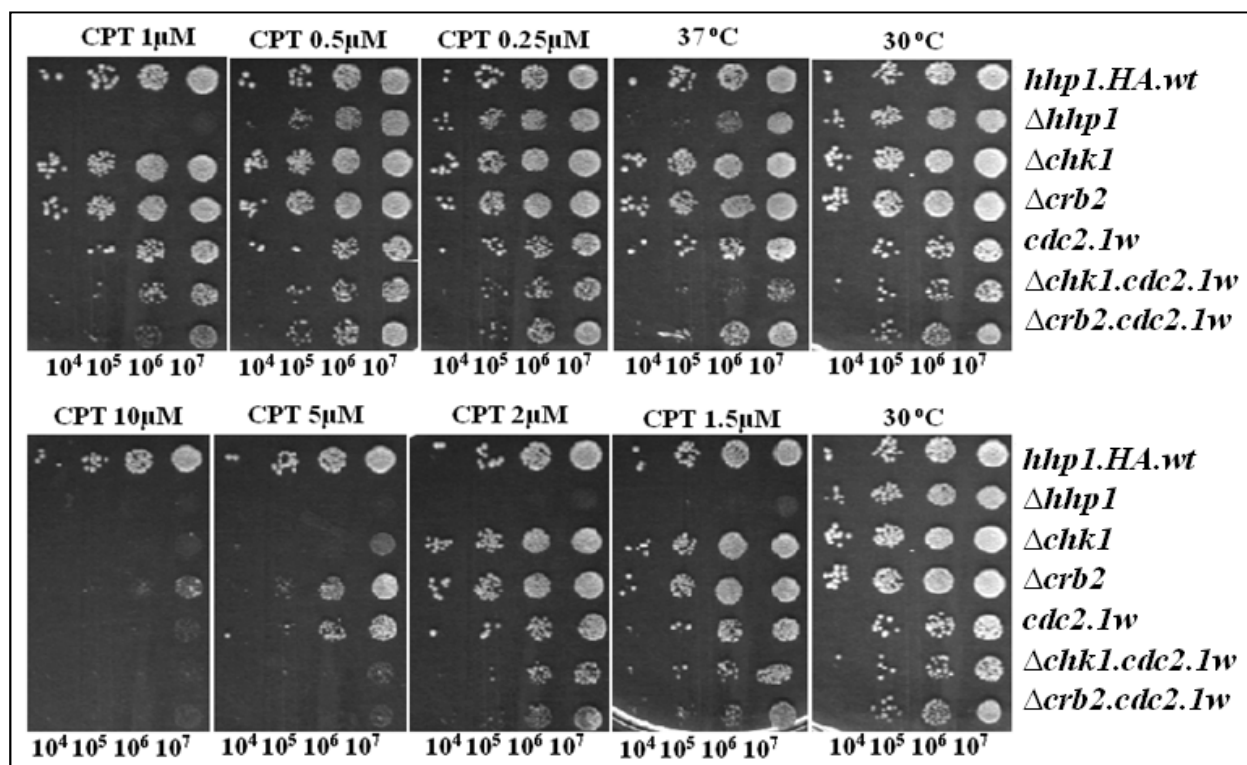


Figure: 4.5.4: Deletion of *chk1* or *crb2* increases the CPT sensitivity of *cdc2.1w* cells. Serial dilutions of the indicated strains were dropped on rich medium plates with or without drugs. All plates, but one, were incubated at 30°C for 4 days. One plate without a drug was incubated at 37°C to test for temperature sensitivity.

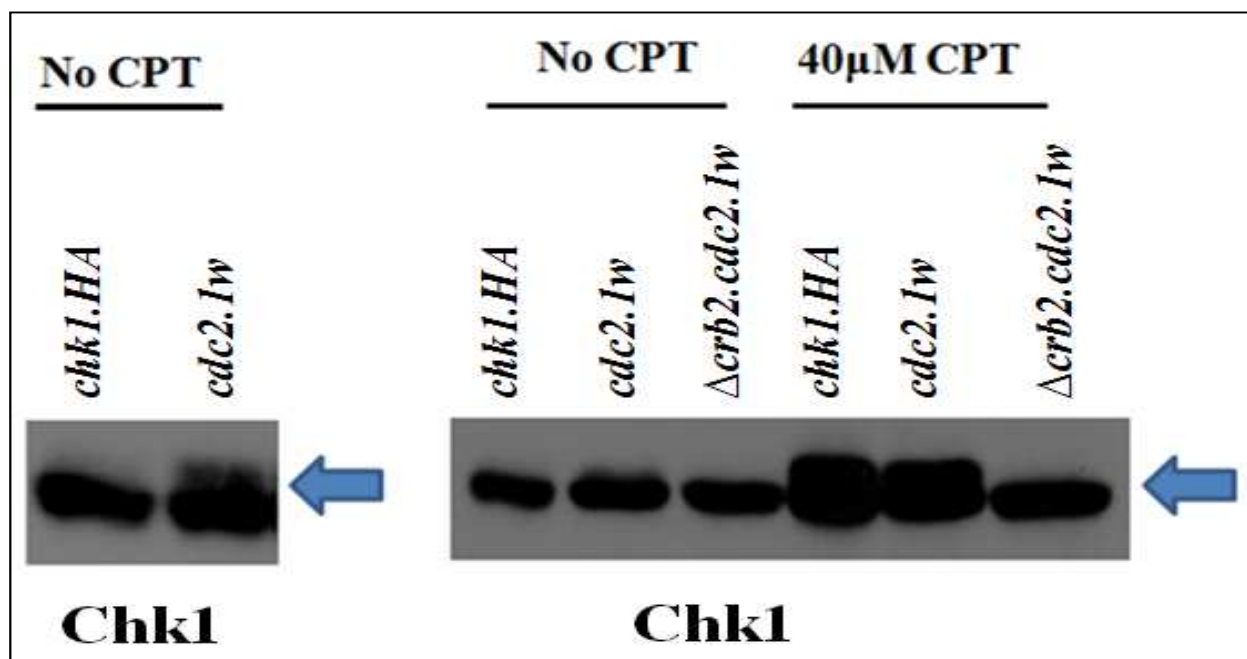


Figure: 4.5.5: Chk1 is phosphorylated in untreated *cdc2.1w* cells (*chk1-HA cdc2.1w*) and in the presence of CPT. Deletion of *crb2* abolishes the CPT-induced phosphorylation. After a 4 hour incubation in the presence of 40μM CPT in YEA medium, 15 μl of total protein extracts were run on a 10% acrylamide gel. The proteins were visualised by using an anti-HA antibody after Western blot (adopted from my paper (272)).

To test whether the Cdc2 dependent phosphorylation of Crb2 at T215 is required for the extended G2 arrest, the *cdc2.1w.crb2.T215A* strain was synchronised in G2 and released in rich medium with or without 40 μ M CPT. As shown in **Figure: 4.5.6**, loss of the phosphorylation site completely abolished the G2 arrest. Consistent with the close link between Cdc2 and Crb2, the *cdc2.1w.crb2.T215A* strain was as CPT and MMS sensitive as the *crb2.T215A* mutant (**Figure: 4.5.7**, **Figure: 4.5.8**, **Figure: 4.5.9**). This finding is important as it reveals how Cdc2.1w could affect the G2 arrest. Hyper-active Cdc2.1w could aberrantly modify Crb2 at T215 at a time in the cell cycle at which T215 should not be modified. The phosphorylation of Crb2 at T215 peaks when cells pass through mitosis and correlates with re-entry into the cell cycle after a G2 arrest induced by UV damage (215). Since Cdc2.1w becomes active much earlier in the cell cycle (245), the T215 modification could also appear earlier thereby interfering with the response to broken DNA replication forks break in the presence of CPT (**Figure: 4.5.10**). Since Cds1 and Chk1 are both required for this extended arrest (**Figure: 4.4.3**), the aberrantly T215 phosphorylated Crb2 protein may interfere not only with the activation of Chk1 but also with the regulation of Cds1.

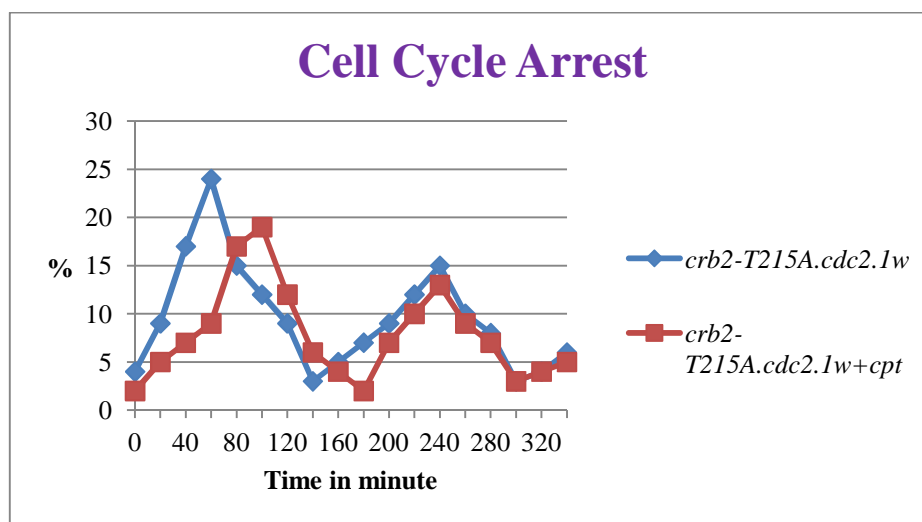


Figure: 4.5.6: Loss of the Cdc2 phosphorylation site threonine-215 (T215A) abolished the CPT-induced G2 arrest in *cdc2.1w* cells. *crb2-T215A cdc2.1w* cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

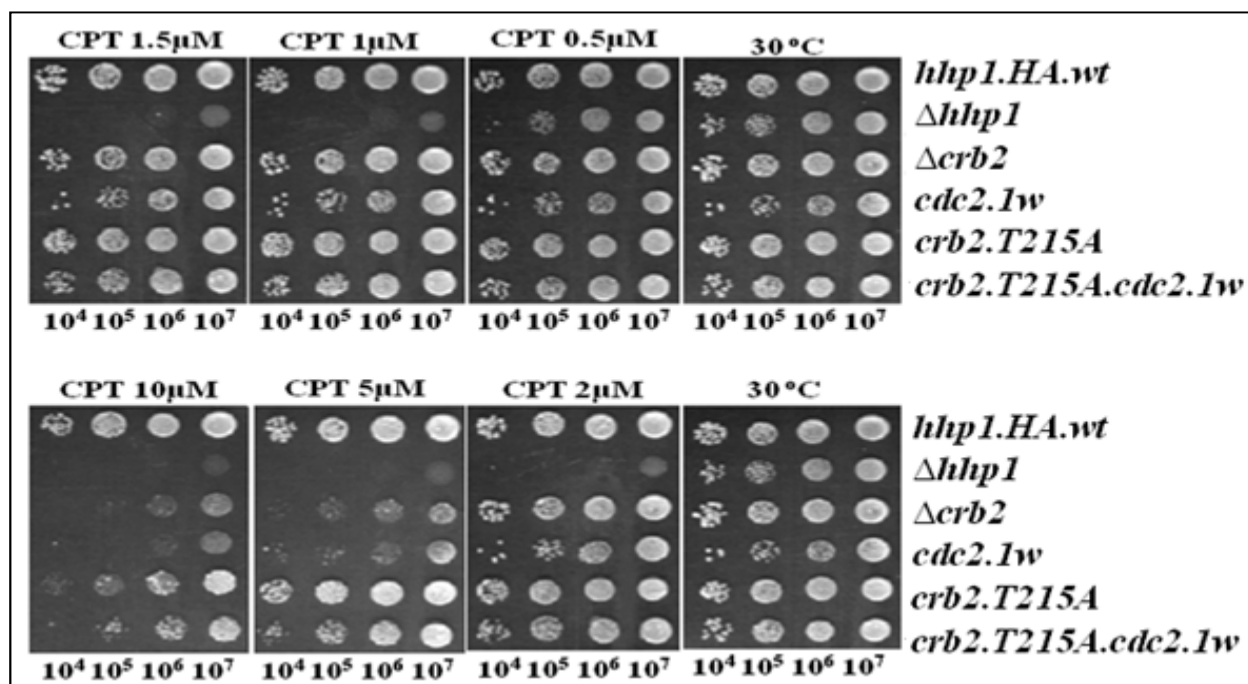


Figure: 4.5.7: *Crb2.T215A* and *Cdc2.1w* act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C.

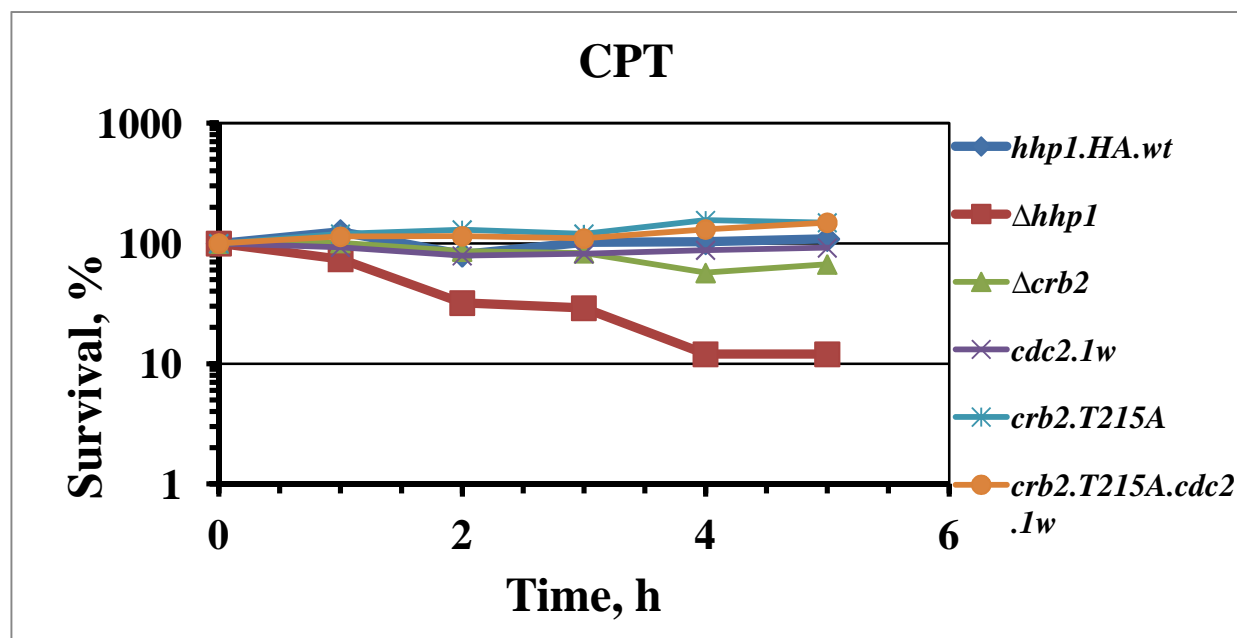


Figure: 4.5.8: *crb2.T215A.cdc2.1w* and *crb2.T215A* resistance to CPT (camptothecin) effects. The indicated yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40μM CPT for five hours at 30 °C. Aliquots of 75 μl were collected every hour and the surviving colonies were scored after plating on YEA plates.

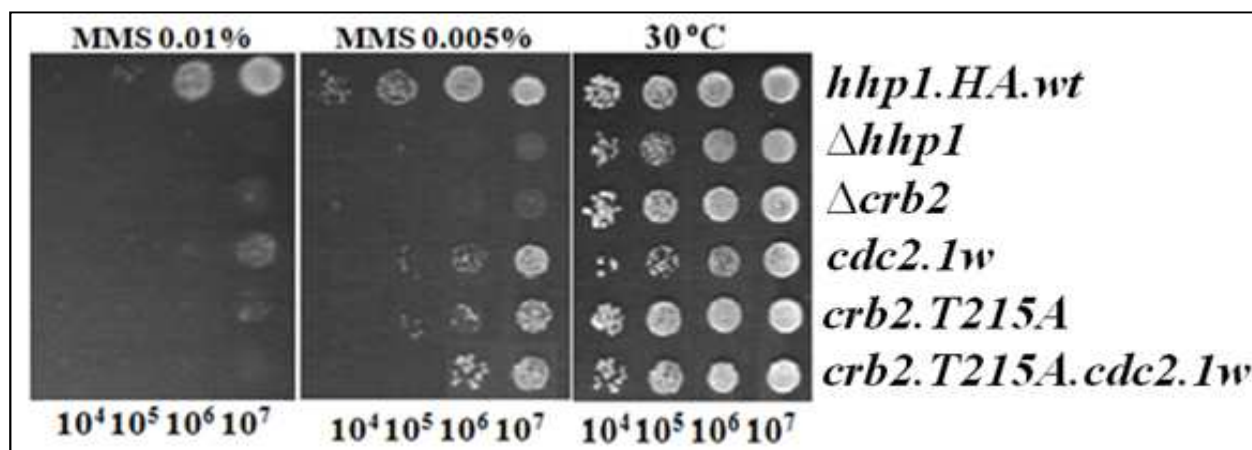


Figure: 4.5.9: Crb2.T215A and Cdc2.1w act in the same MMS (methyl-methanesulfonate) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C.

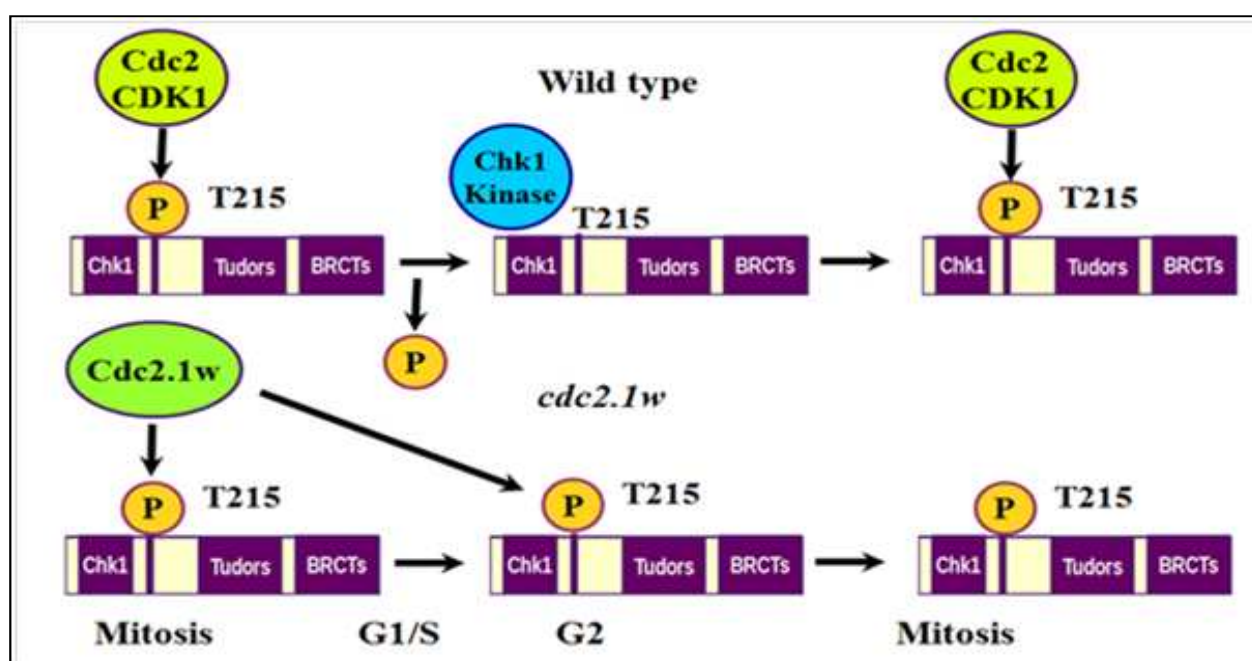


Figure: 4.5.10: Hyperactive Cdc2.1w interferes with the regulation of Crb2. Crb2 has three domains: the N-terminal Chk1 binding domain, the tudor domain which interacts with methylated histones and the two C-terminal BRCT domains which bind to phosphorylated histones (216). T215 is normally phosphorylated when cells pass through mitosis which is important for the normal regulation of Chk1 in G2 (215). The premature rise in *cdc2.1w* activity may extend the T215 phosphorylation into G1-S thereby interfering with the regulation of Chk1 and Cds1 kinase when DNA replication forks break in S phase.

4.6. Srs2 DNA Helicase acts in the Cdc2.1w Pathway

Since Srs2 DNA helicase is a Cdc2 target in *S.cerevisiae* where it is phosphorylated by Cdc28 (Cdc2) to promote the process of homologous recombination HR) (240), a *cdc2.1w.Δsrs2* strain was tested. In *S.pombe*, Srs2 removes the recombination protein Rad51 from single-stranded DNA thereby preventing unwanted HR, but the helicase can also promote recombinational repair in the presence of UV-induced DNA damage. Interestingly, Srs2 and Mus81-Eme1 act in the same UV repair pathway (243). As shown in Figure: 4.6.1, the *cdc2.1w.Δsrs2* strain is as CPT sensitive at the $\Delta srs2$ single deletion which places both proteins in the same CPT response pathway. The same epigenetic relationship applies also to MMS (Figure: 4.6.2)

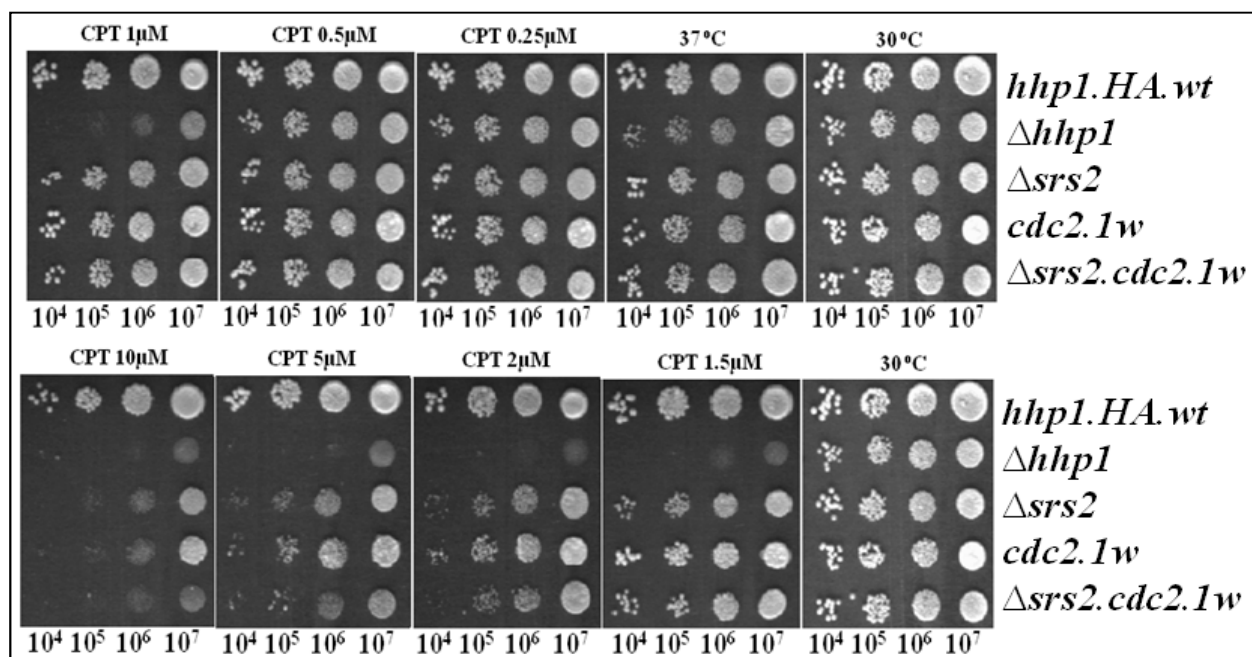


Figure: 4.6.1: Srs2 DNA helicase and Cdc2.1w act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

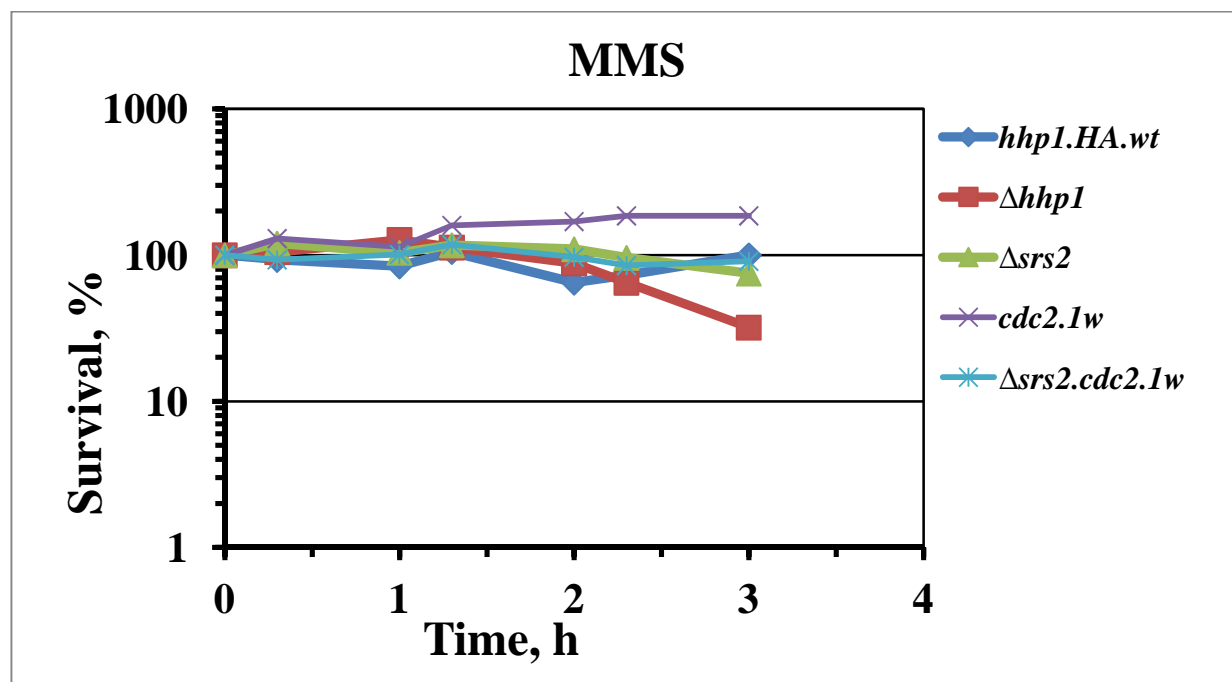


Figure: 4.6.2: Δ *srs2.cdc2.1w* response to 0.005% MMS (methyl-methanesulfonate) concentrations. The indicated yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40 μ M CPT for five hours at 30° C. Aliquots of 75 μ l were collected every hour and the surviving colonies were scored after plating on YEA plates.

Since the link with *Cdc2.1w* suggests a role for *Srs2* in the extended delay, the Δ *srs2* single deletion strain was synchronised and tested. Interestingly, loss of *srs2* extends the G2 arrest in the presence of CPT (Figure: 4.6.3), although not to the same extent as the *cdc2.1w* mutation. The combination of *cdc2.1w* with the deletion of *srs2* (*cdc2.1w.Δsrs2*) produced a long G2 arrest (Figure: 4.6.4) resembling the *cdc2.1w* single mutation. This supports the conclusion that *Srs2* DNA helicase and *Cdc2.1w* act in the same CPT response pathway. This idea is further supported by the reduction of the CPT-induced G2 delay of Δ *srs2* cells upon the introduction of the *crb2.T215A* mutation (*crb2.T215A.Δsrs2*) (Figure: 4.6.5).

Interestingly, as in the case of the *cdc2.1w Δchk1* mutant (Figure: 4.4.1), the Δ *srs2.Δchk1* double mutant displayed a G2 arrest which resembled the longer delay of the Δ *srs2* strain (Figure: 4.6.6). This is in line with the finding that only loss of *Crb2* but not loss of *Chk1* abolishes the extended CPT arrest in *cdc2.1w* mutant cells (Figure: 4.5.6). It also suggests that hyper-active *Cdc2* and loss of *Srs2* DNA helicase result in similar problems at a broken replication fork.

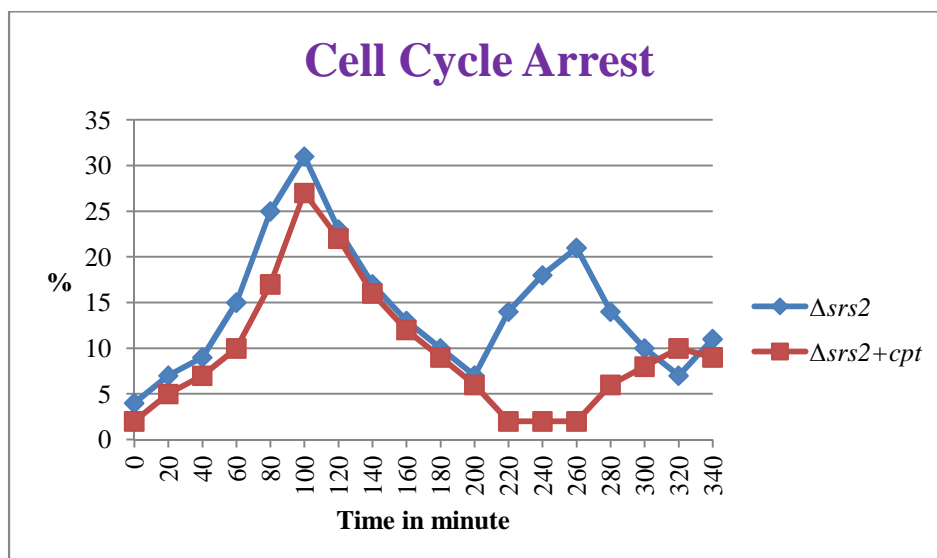


Figure 4.6.3: Loss of the DNA helicase *srs2* prolongs the G2 arrest in the presence of CPT (camptothecin). $\Delta srs2.cdc2.1w$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

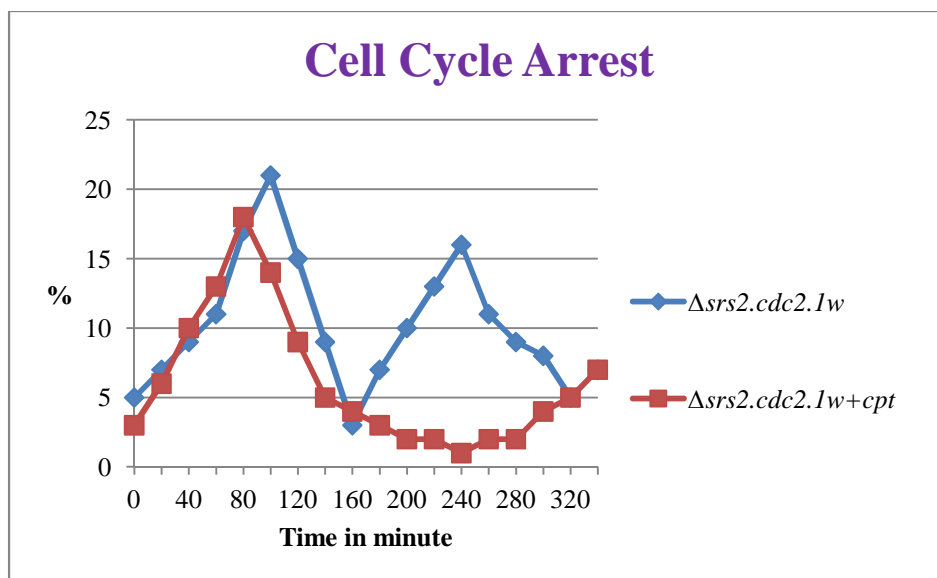


Figure 4.6.4: Loss of the DNA helicase *srs2* does not reduce the G2 arrest in *cdc2.1w* cells in the presence of CPT (camptothecin). $\Delta srs2.cdc2.1w$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

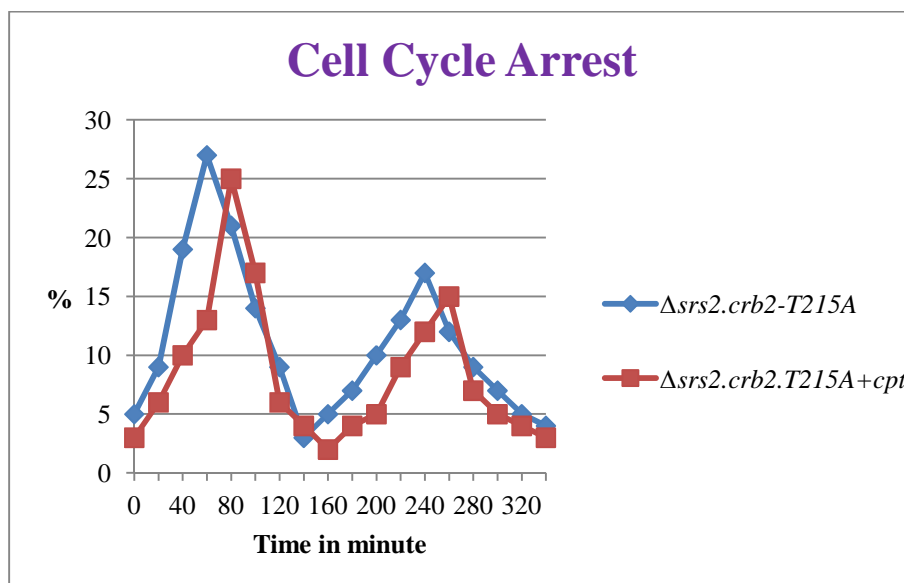


Figure: 4.6.5: Loss of the Cdc2 phosphorylation site threonine-215 (T215A) strongly reduces the G2 arrest in the presence of CPT (camptothecin). $\Delta srs2.crb2.T215A$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

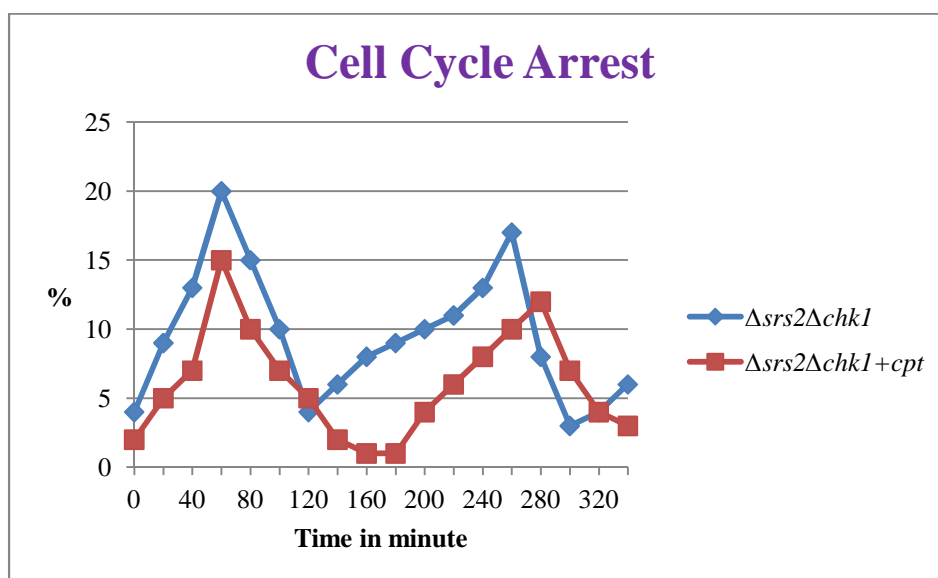


Figure: 4.6.6: Loss of Chk1 only reduces the G2 delay in $\Delta srs2$ cells in the presence of CPT (camptothecin). $\Delta srs2.\Delta chk1$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

This also implies that the longer delay in the absence of Srs2 DNA helicase depends on the Cdc2 phosphorylation of Crb2 at T215 but not on the interaction between Crb2 and Chk1. This raises the intriguing questions of how Srs2 could affect the response to broken DNA replication forks and what could go wrong in the Cdc2.1w strain? As explained earlier, the hyperactive Cdc2.1w kinase may extend the T215 phosphorylation well into S and G2 which could affect the ability of Crb2 to coordinate the response to broken forks. Since the T215 phosphorylation normally peaks in mitosis (215), this mitotic pattern of Crb2 activity could be extended into the next S phase and G2. Part of this mitotic imprint seems to be the inability to regulate Chk1 kinase. In relation to the results discussed in Chapter 3, which place Chk1 and Hhp1 kinase in the same repair pathway as Mus81, it is interesting to note that Srs2 and Mus81 physically interact and that Srs2 activates the endonuclease on a variety of DNA substrates. The DNA helicase activity of Srs2 is not necessary for this stimulation (517). As shown in Figure: 4.6.7, Cdc2 and Srs2 could come together at the level of the Mus81-Eme1 complex with Srs2 regulating the activity of Mus81 and Cdc2 modifying Eme1. The DNA binding protein Crb2 could either affect the activities of the kinases Cds1 and Chk1 or it could interact with the Mus81-Eme1 complex at the chromatin. Crb2 can associate with methylated and phosphorylated histones through its Tudor and BRCT domains, respectively (215, 218). The absence of Srs2 (Δ srs2) could extend the G2 arrest in the presence of CPT since Mus81 would not be activated which could delay the repair of broken forks. Premature activation of Cdc2 in the Cdc2.1w strain may have a similar effect either by aberrantly modifying Eme1 or by indirectly extending the mitotic phosphorylation pattern of Crb2. A third possibility is that hyper-active Cdc2.1w targets Srs2 directly as reported for Srs2 in budding yeast (240).

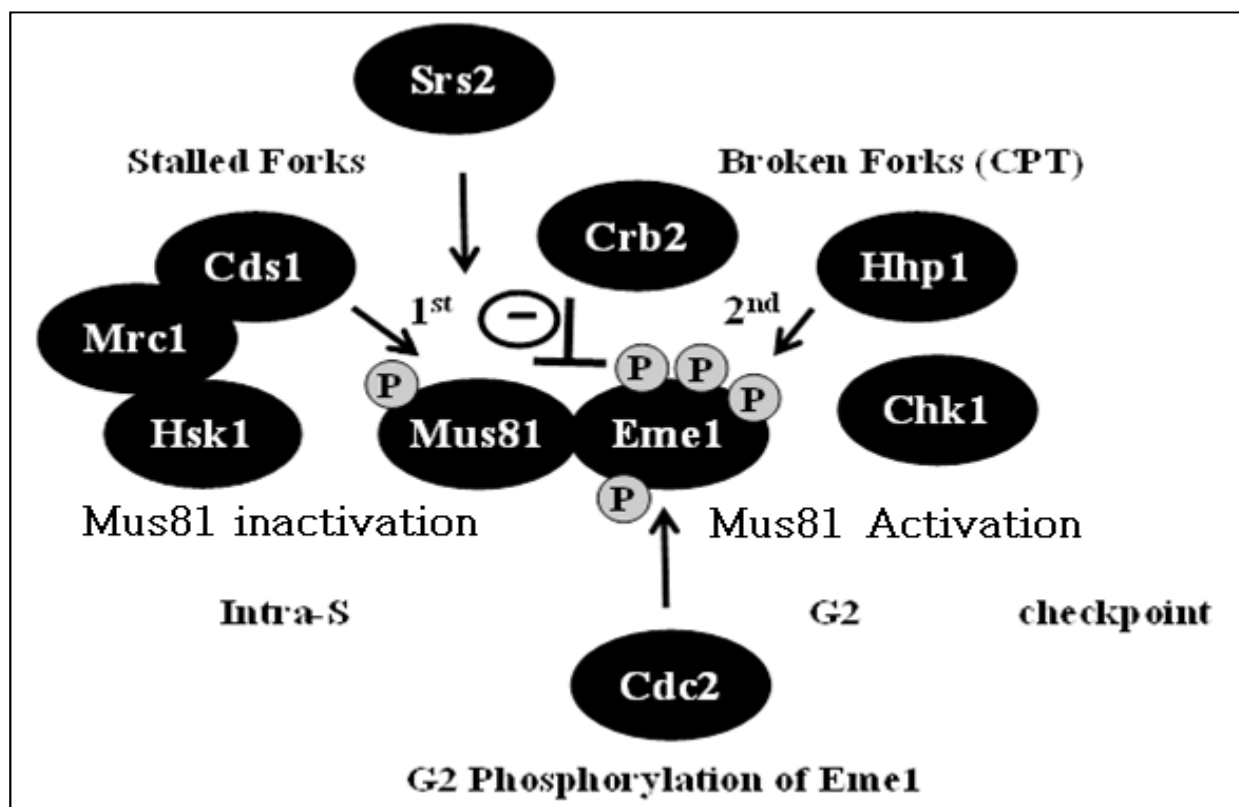


Figure: 4.6.7: Model of Mus81-Eme1 regulation by Srs2, Cdc2 and Hhp1. Srs2 DNA helicase and Cdc2 could both regulate the activity of the structure-specific endonuclease Mus81-Eme1 at broken DNA replication forks. Loss of Srs2 ($\Delta srs2$) or hyper-activation of Cdc2 ($cdc2.1w$) could block this enzyme thereby delaying the repair of broken forks which would explain the extended G2 arrest in the presence of the topoisomerase 1 inhibitor CPT (camptothecin).

4.7. Hhp1 Kinase acts with Srs2 and Cdc2.1w in the same CPT Response Pathway

Given the close genetic link between Hhp1 kinase, Cdc2 and the endonuclease Mus81-Eme1 (see Chapter 3), the relationship between Srs2 and Hhp1, and between Cdc2.1w and Hhp1 was studied. As shown in Figure: 4.7.1, the deletion of *srs2* in the $\Delta hhp1$ strain ($\Delta srs2.\Delta hhp1$) does not increase the CPT sensitivity. The same epigenetic relationship extends to MMS although the deletion of *srs2* is not MMS sensitive under these conditions (Figure: 4.7.2). Loss of Srs2 in a $\Delta hhp1$ mutant had no major impact of the extended G2 arrest in the absence of Hhp1 kinase (Figure: 4.7.3). In summary, these findings are in line with the earlier conclusion (see also Chapter 3) that Hhp1 kinase targets Eme1 (Figure: 4.6.7). This proposed modification by Hhp1 seems to be important for the activation of the Mus81-Eme1 complex, which also needs the Srs2 protein. To contribute effectively to the repair of broken DNA replication forks, the Mus81-Eme1 complex appears to require Srs2, Cdc2 and Hhp1. The role of Crb2 in this regulatory network is not yet clear, but as in the case of *cdc2.1w* (Figure: 4.5.6), the deletion of *crb2* in the

Δhhp1 strain abolished the extended G2 arrest (Figure: 4.7.4). Since loss of Crb2 shortened the long delay in *Δhhp1* and *cdc2.1w* cells, and because Cdc2 and Hhp1 may be required to activate Mus81-Eme1, it is possible that Crb2 blocks the endonuclease (i.e. deletion of *crb2* may overcome the absence of Srs2 or the inhibitory Cdc2 modification in the *cdc2.1w* mutant). This blockage could be direct since Crb2 binds to chromatin or it could be indirect if Crb2 is to blocking Cdc2 and/or Hhp1 kinase (Figure: 4.6.7).

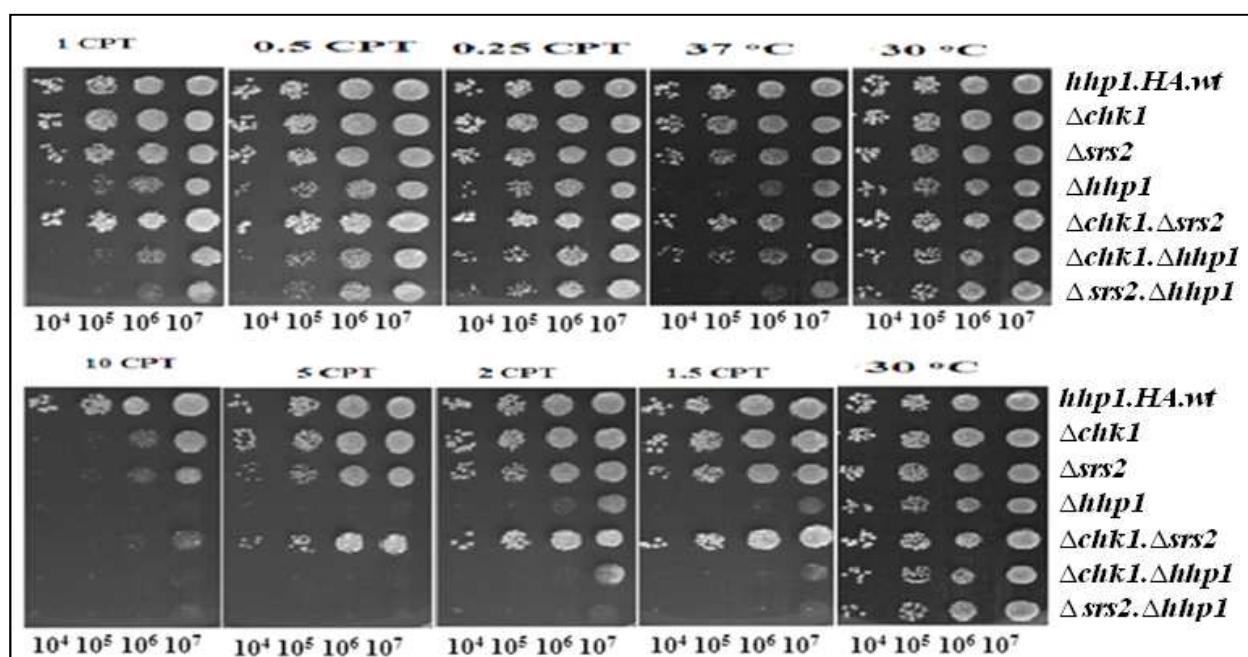


Figure: 4.7.1: Srs2 DNA helicase and Hhp1 act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

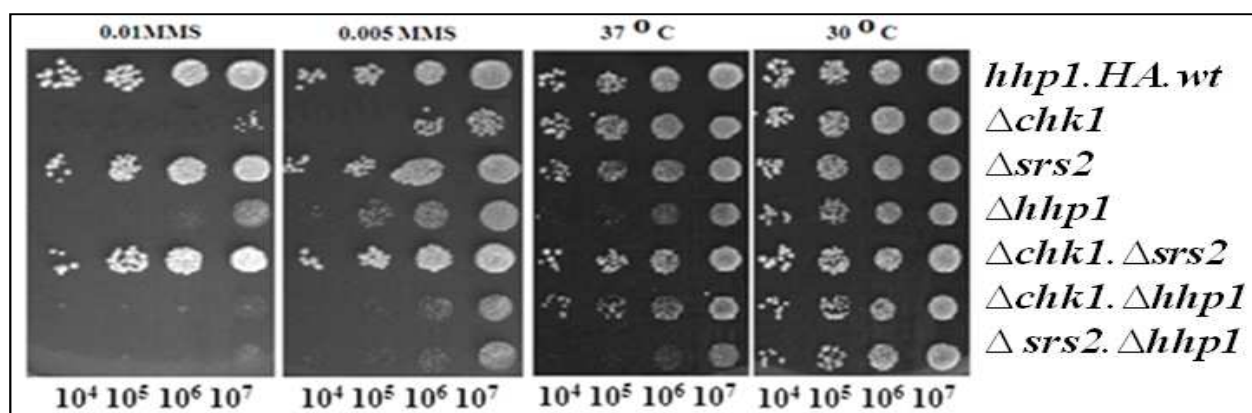


Figure: 4.7.2: Srs2 DNA helicase and Hhp1 act in the same MMS (methyl-methanesulfonate) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

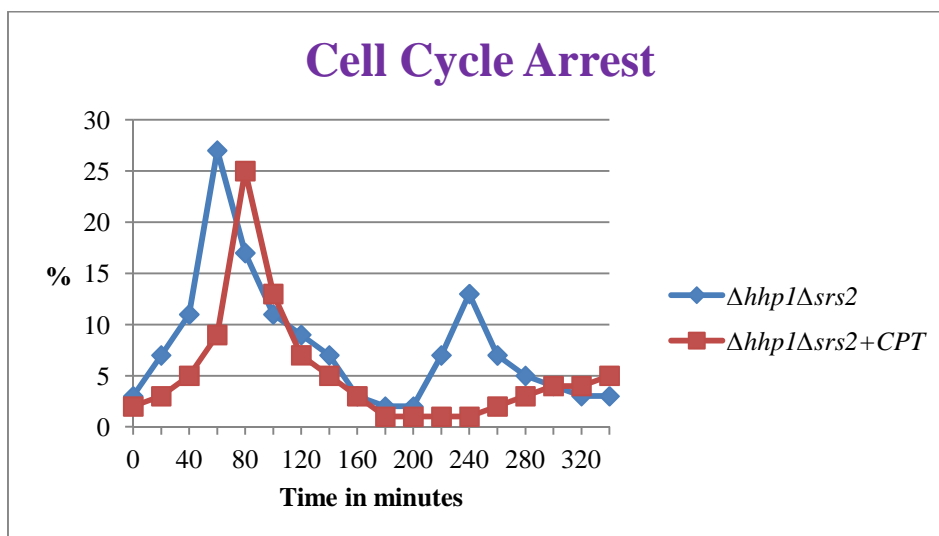


Figure: 4. 7.3: Loss of the DNA helicase Srs2 has little impact on the extended G2 arrest in the absence of Hhp1 kinase. $\Delta hhp1.\Delta srs2$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT (camptothecin) at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

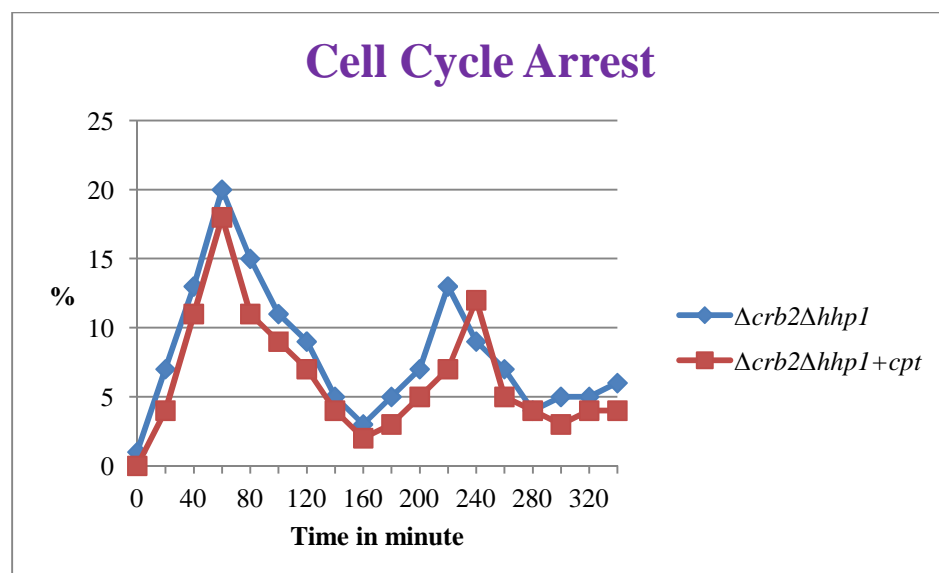


Figure: 4. 7.4: Deletion of *crb2* abolishes the extended G2 arrest in the absence of Hhp1 kinase when DNA replication forks break in CPT (camptothecin) medium. $\Delta crb2.\Delta hhp1$ cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were withdrawn at the indicated time points and the percentage of septated G1-S cells were scored. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

Consistent with this model, deletion of *crb2* in the $\Delta hhp1$ strain ($\Delta crb2.\Delta hhp1$) did not increase the CPT and MMS sensitivity of the $\Delta hhp1$ single mutant (Figure: 4.7.5, Figure: 4.7.6). The same epigenetic relationship applied to the *cdc2.1w* mutant (Figure: 4.7.7). Interestingly, the hyper-active *cdc2.1w* mutation improved the growth of cells without *hhp1* at elevated temperatures. This positive impact of elevated Cdc2 activity extended also to the short term exposure of cells to CPT. As shown in Figure: 4.7.8, the *cdc2.1w* $\Delta hhp1$ double mutant lost viability later compared to the $\Delta hhp1$ strain. A similar effect could also be produced when Cdc2 activity was increased by impairing its inhibition by Wee1 kinase (*wee1-50. $\Delta hhp1$*). This shows that high Cdc2 activity levels can partly override the loss of Hhp1 (Figure: 4.7.8).

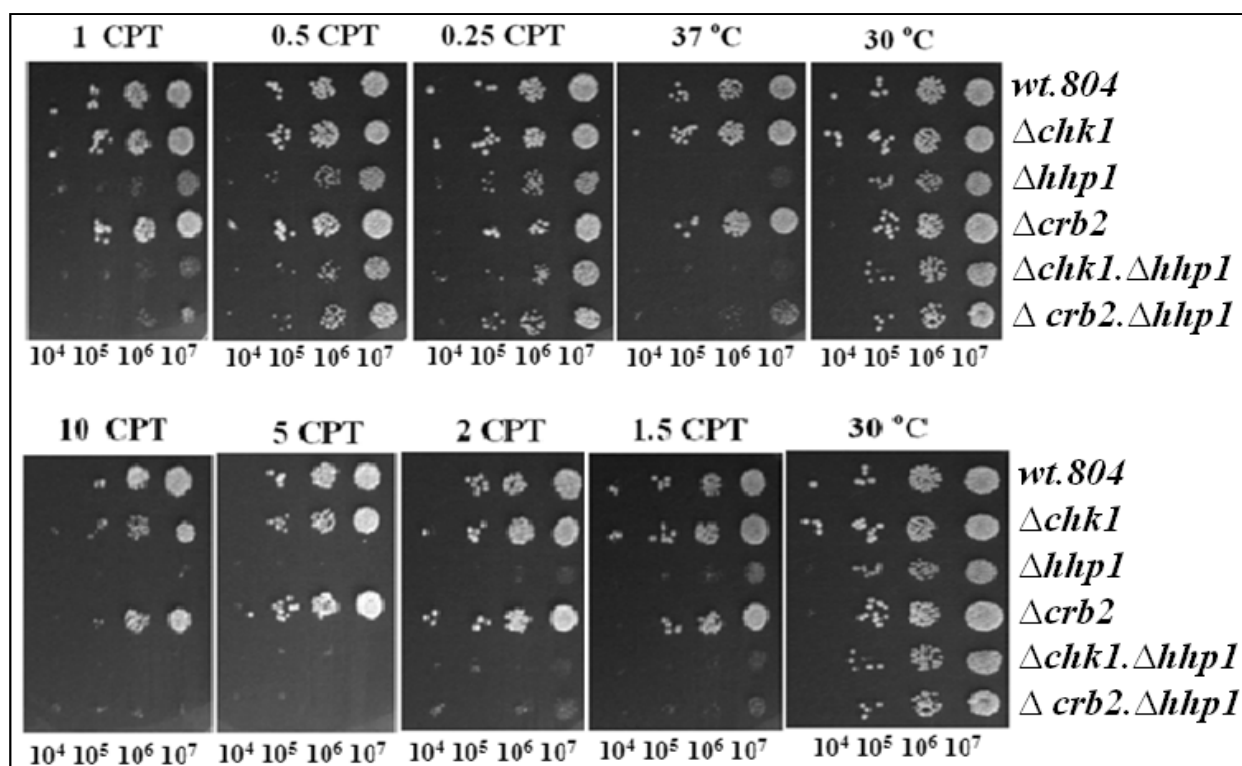


Figure: 4.7.5: Crb2 and Hhp1 act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

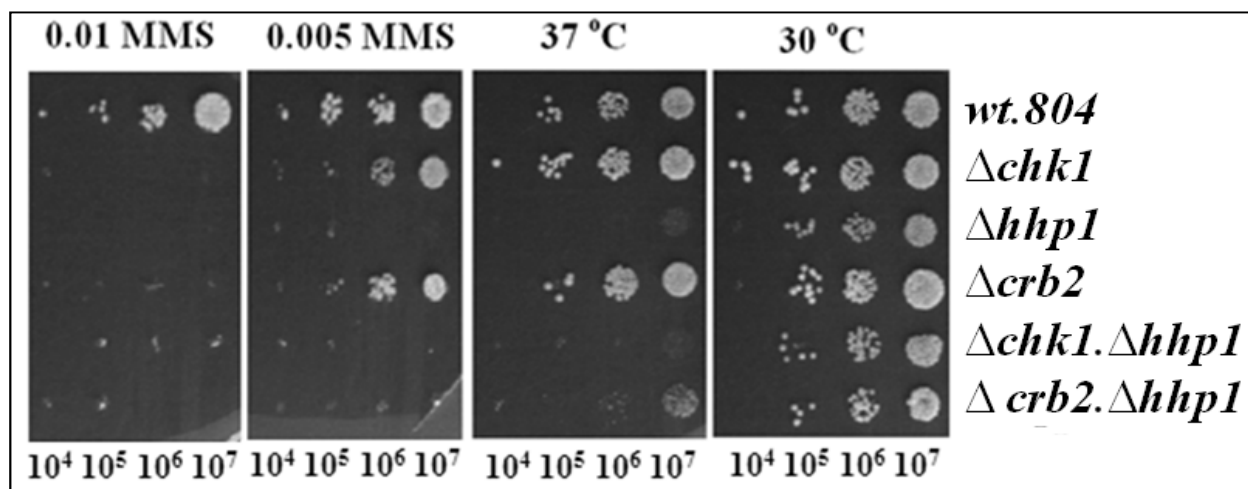


Figure: 4.7.6: Crb2 and Hhp1 act in the same MMS (methyl-methanesulfonate) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C

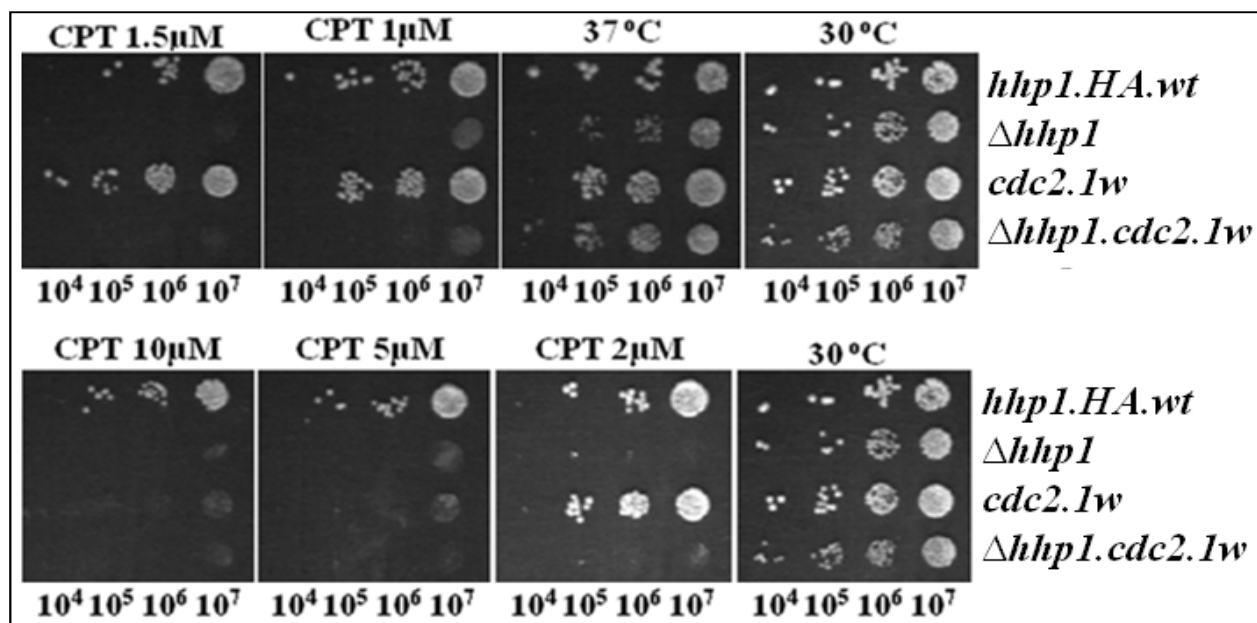


Figure: 4.7.7: Cdc2.1w and Hhp1 act in the same CPT (camptothecin) response pathway. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C

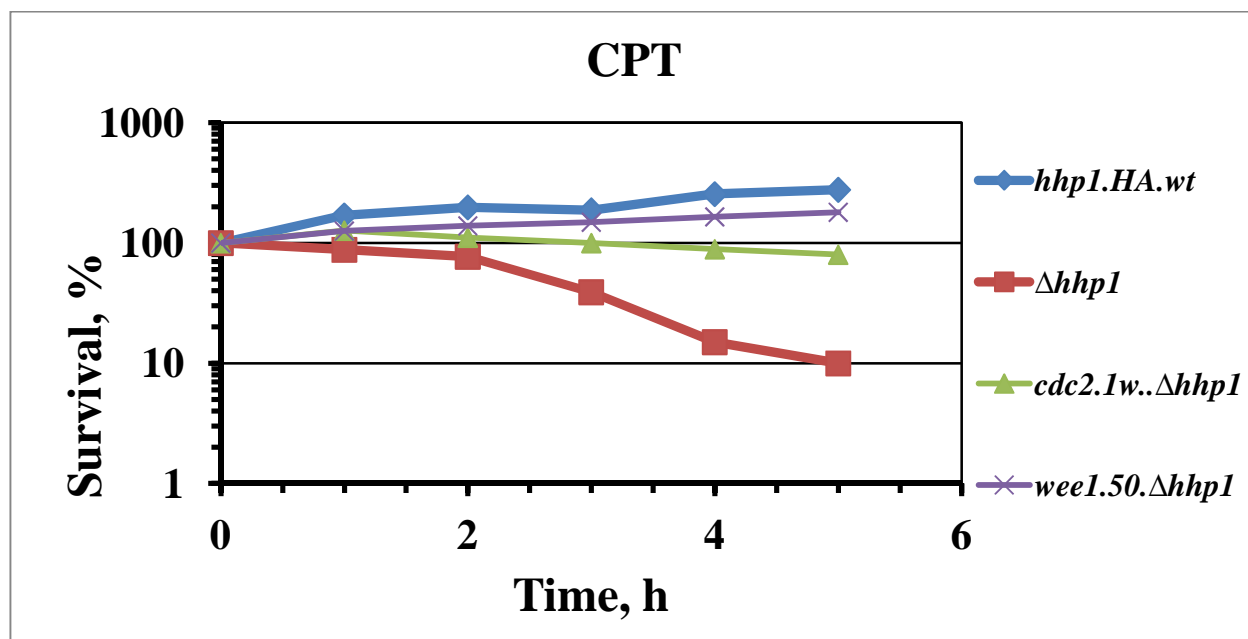


Figure: 4.7.8: Wee1 render Hhp1 resistant. The indicated yeast strain cells were cultured in YEA medium overnight at 30° C. Cells were harvested and treated with 40μM CPT (camptothecin) for five hours at 30 °C. Aliquots of 75 μl were collected every hour and the surviving colonies were scored after plating on YEA plates.

Chapter 5: Tyrosine 227 within the Nuclear Localisation Sequence may act as a switch between the DNA Repair and Cell Cycle Activities of Hhp1 Kinase

Chapter Summary

How CK1 regulate DNA repair and cell cycle progression is so far only poorly understood. This chapter presents results which identify tyrosine 227 within the nuclear localisation sequence (NLS) as a potential regulatory switch. Mutation of Y227 to a phenylalanine residue increases strongly the DNA damage sensitivity while having only a small effect on the cell cycle regulation of Hhp1. Deletion of the complete NLS in frame causes a similar phenotype. This implies that nuclear localisation is much more important for the DNA repair activities of Hhp1 than for its cell cycle functions. Interestingly, the high DNA damage sensitivity of the NLS mutants is partly suppressed upon deletion of the DNA damage checkpoint kinase 1 (Chk1). A model is presented in which Chk1 activates the DNA repair endonuclease Mus81-Eme1 and Hhp1 inhibits this enzyme. A similar phenotype applies also to a Hhp1 mutant kinase which lacks the potential Chk1 phosphorylation site serine 183. The chapter also contains results which identify the ATP analogue-sensitive M84G mutant as a separation-of-function mutant which affects the cell cycle but not the DNA repair activities of Hhp1.

5.1. The ATP-analogue sensitive Methionine-84 to Glycine Mutation is a Separation-of-Function Mutation

Casein kinase 1 epsilon (CK1 ϵ = Hhp1) is a member of the CK1 protein family of ubiquitously expressed, monomeric serine/threonine-specific kinases (14). Human cells express seven CK1 isoforms: CK1 α ; CK1 δ ; CK1 γ 1; CK1 γ 2; CK1 γ 3; CK1 β ; and CK1 ϵ (Hhp1), which can have additional splice variants. The structure of CK1 enzymes is highly conserved. The kinase domain is located in the smaller N-terminal domain (small lobe, Figure: 5.1.1), while the larger C-terminal domain encompasses the rest of the protein (large lobe) (1).

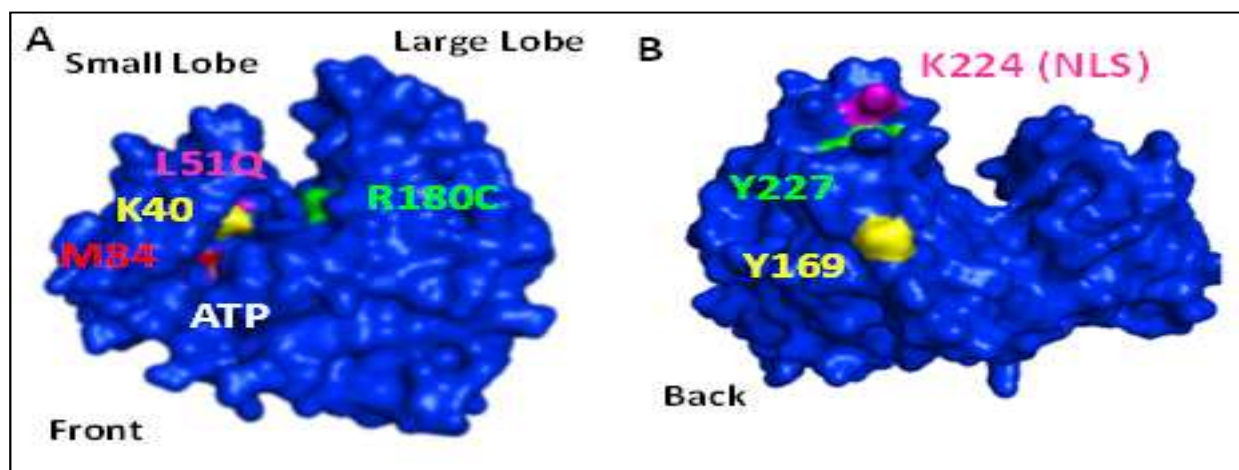


Figure: 5.1.1: Structure of Hhp1 kinase. The visualisation of Hhp1 kinase is based on the structure of the N-terminal 297 amino acids of the highly related *S.pombe* CK1 protein Cki1 (488). The conserved residues K40 (K41Cki1), L51 (L52Cki1), M84 (I84Cki1), Y169 (Y171Cki1), R180 (R183Cki1), K224 (K227Cki1) and Y227 (Y230Cki1) are shown. A: Front view: the ATP binding site in the small lobe with K40, L51Q and M84 is shown. R180C sits at the interface between the two lobes. B: Back view: The nuclear localisation domain with K224 and Y227 is located in a small groove at the top of the large lobe. Y169 is located at the side of the large lobe. Protein ID: 1CSN. The picture was generated with Polyview3D.

CK1 family members were identified in the nucleus, cytoplasm, and attached to the plasma membrane (1). CK1 isoforms phosphorylate a large number of substrates which regulate different cellular processes like cell differentiation (24), cell proliferation, apoptosis (42), circadian clock regulation (491), chromosome segregation (138), DNA repair (15) and vesicle transport (120). Mutations or changes in CK1 kinases have an impact on diseases including neurodegeneration (428) and cancer of the pancreas (490), the mammary gland (78), and in adenoid cystic carcinomas (76).

To understand more about the function and regulation of CK1 (Hhp1) in the context of the response to DNA damage especially when DNA replication forks break in the presence of the

topoisomerase 1 inhibitor camptothecin (CPT), five mutant versions of Hhp1 are discussed in this chapter. They include *hhp1.S183A*, *hhp1.M84G*, *hhp1.M84G.NLS.deletion*, *hhp1.M84G.Y227F*, *hhp1.M84G.Y169F*.

MALDLRIGNK	YRIGRKIGSG	SFGDLYLGTN	VVSGEEVAIK	LESTRAKHPQ
LEYEYRVYRI	LSGGVGIPFV	RWFGVECDYN	AMVM ^{M84G} DLLGPS	LEDLFNFCNR
KFSLKTVLLL	ADQLISRIEF	IHSKSFLHRD	IKPDNFLMGI	GKRGNQVNII
DFGLAKKYRD	HKTHLHIPY ^{Y169F} R	ENKNLTGTAR	YAS ^{S183A} INTHLGI	EQSRRDDLES
LGYVLVYFCR	GSLPWQGLKA	TTKKQKY ^{Y227F} EKI	MEKKISTPTE	VLCRGFPQEF
SIYLNRYTRSL	RFDDKPDYAY	LRKLFRLDFC	RQSYEFDYMF	DWTLKRKTQQ
DQQHQQQQLQQ	QLSATPQAIN	PPPERSSFRN	YQKQNFDEKG	GDINTTVPVI
NDPSATGAQY	INRPN			

Figure: 5.1.2: Amino acid sequence of *S.pombe* Hhp1. The location of the mutated amino acids has been indicated.

Methionine 84 is located inside the ATP binding site (Figure: 5.1.1) and has been previously mutated to a smaller glycine residue (M84G) to allow for the binding of a bulky ATP analogue which cannot be hydrolysed (381). This mutation was recreated in this study. Serine 183 was mutated since it resembles a potential Chk1 phosphorylation site (544) although the residue may not be accessible from the outside based on the structure of the highly related *S.pombe* CK1 enzyme Cki1 (Figure: 5.1.3).

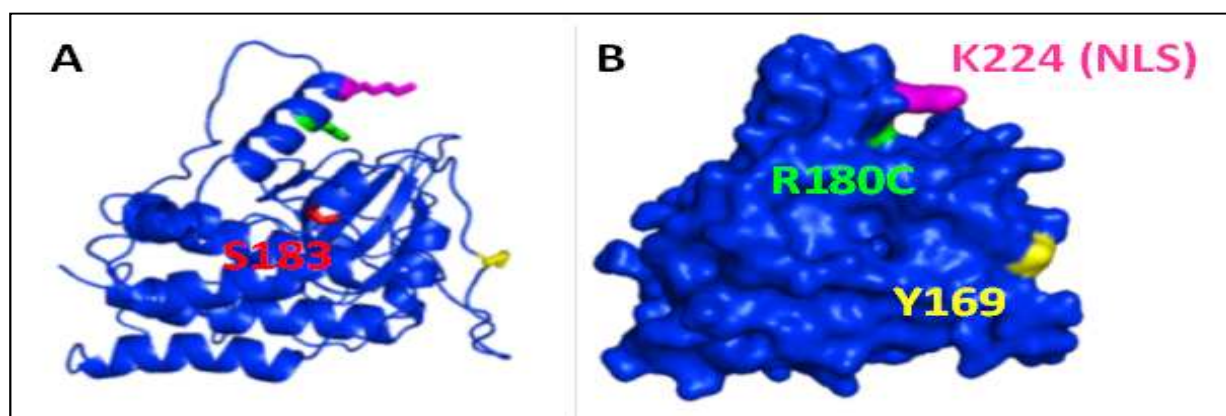


Figure: 5.1.3: Structure of Hhp1 kinase. The visualisation of Hhp1 kinase is based on the structure of the N-terminal 297 amino acids of the highly related *S.pombe* CK1 protein Cki1 (488). The conserved residues Y169 (Y171Cki1), R180 (R183Cki1), and S183 (S186Cki1) are shown. A: Cartoon view of the beta sheets, alpha helices and loops: the side chains of the indicated amino acids are shown. B: Surface view: Note that the side chain of S183 is not accessible from the outside in this conformation. The image was generated with Polyview3D.

The sequence 183-SINT-186 in *S.pombe* Hhp1 resembles the highly phosphorylated Chk1 motifs 176-SINF-179 in human cyclin G or 699-SIPAF-703 in human Mad1A (544). The nuclear localisation sequence of Hhp1 (222-TKKQKY-227) forms a groove at the tip of the larger C-terminal lobe (Figure: 5.1.2). The potential phosphorylation site Y227 sits at the bottom of this groove, while K224 protrudes into the space of the groove. The second potential phosphorylation site Y169 is located at the surface of the larger lobe.

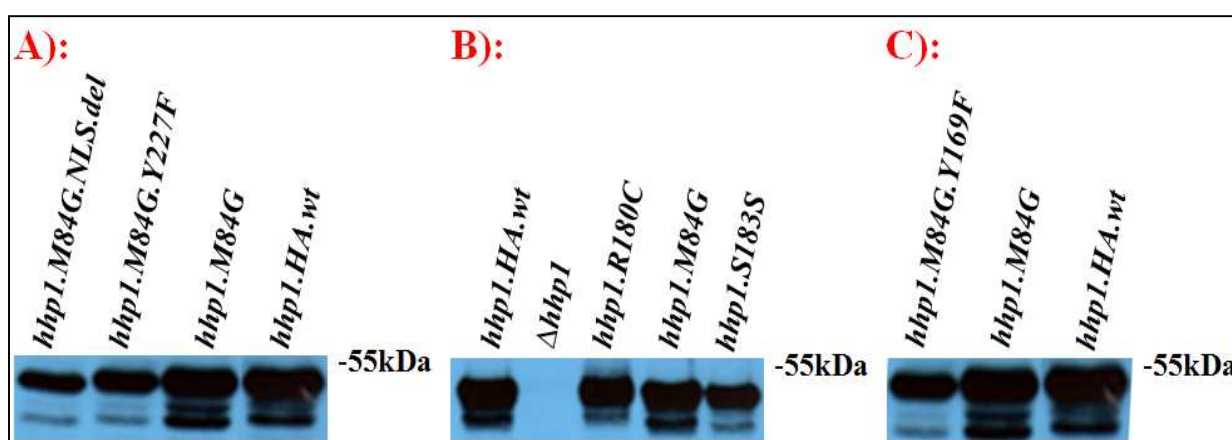


Figure: 5.1.4: Protein Expression Levels of Hhp1 kinase strains. Total protein was isolated, 15 μ l of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody. Two positive controls used were *hhp1HA.wt*, and *hhp1M84G* strains, and the negative control is the deletion strain (Δ *hhp1*). All strains express full-length Hhp1.

All mutant strains were constructed using the Cre-Lox base strain technology (258). The mutant alleles of *hhp1-HA* were generated by fusion-PCR and integrated at the endogenous *hhp1* locus on chromosome 2. All integrated and mutated *hhp1* genes were amplified from the isolated strains and the mutations were confirmed by DNA sequencing. Protein expression levels were then analysed by Western blot using an anti-HA antibody. As shown in Figure: 5.1.4, all mutant proteins are well expressed.

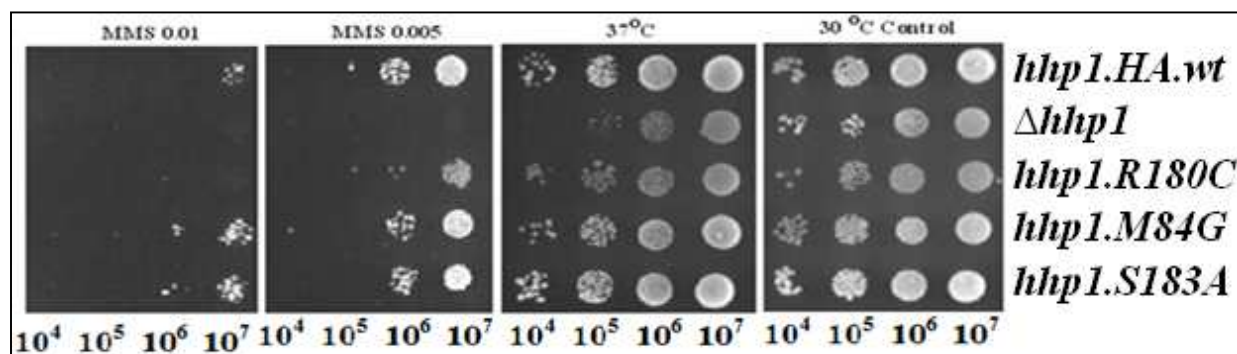


Figure: 5.1.5: MMS (methyl-methanesulfonate) drop test for the *hhp1.S183A* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

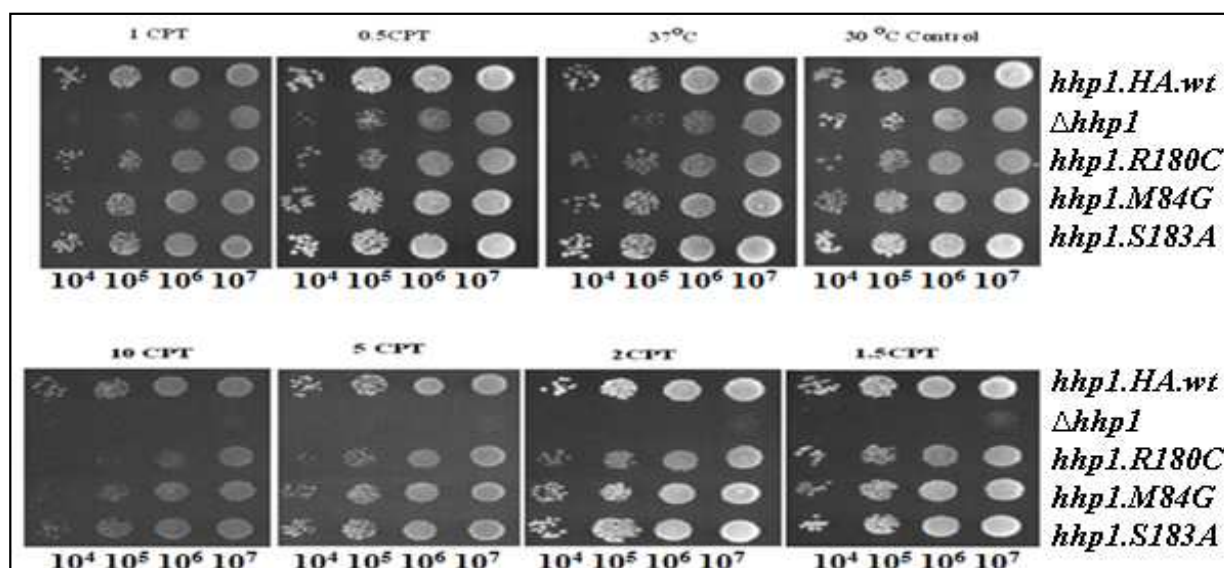


Figure: 5.1.6: CPT (camptothecin) drop test for the *hhp1.S183A* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

Cells expressing either *hhp1.M84G-HA* or *hhp1.S183A-HA* were as MMS and CPT resistant as *hhp1-HA* wild type cells (Figure: 5.1.5, Figure: 5.1.6). This shows that the widening of the ATP binding site (M84G) or the replacement of S183 by an alanine residue has no impact on cell viability in the presence of these drugs. This conclusion is in line with the previous report on M84G (382). To test whether either mutation has an effect on the CPT or heat induced G2 arrest, cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40μM CPT, or into rich medium at 30°C or 40°C. While heat stress arrested wild type cells in the first G2 phase for approximately 180 min (Figure: 5.1.10), CPT delayed the progression through the second G2 only briefly for 20-40 min after cells were damaged in the

previous S phase (Figure: 5.1.9). Cells with the M84G mutation initiated the G2 arrest at 40°C but did not maintain the arrest for the same duration as wild type cells. They started to re-enter the cell cycle after 60 min (Figure: 5.1.7) while wild type cells delay for up to 180 min (Figure: 5.1.10). Very unexpectedly, the mutation had the opposite effect on the CPT-induced G2 delay. In contrast to wild type cells, M84G cells delayed entry into mitosis throughout the duration of the experiment (Figure: 5.1.8). This shows that the widening of the ATP binding site strongly affects the cell cycle activities of Hhp1 but not the survival of cells in the presence of DNA damage. If the M84G mutation may reduce the kinase activity, this reduction does not impact on the survival of the cells in the presence of MMS or CPT, but on the ability of Hhp1 to regulate the G2 arrest when DNA replication forks break or when cells are exposed to heat stress. Why the mutation affects the heat and DNA damage-induced G2 delay in opposite ways is not yet clear. The main conclusion of these findings is that the analogue-sensitive M84G mutation behaves like a separation-of-function mutation.

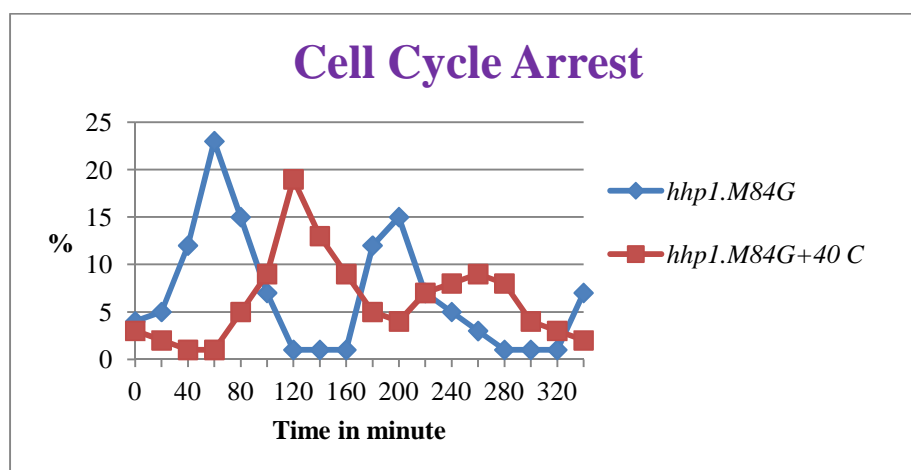


Figure: 5.1.7: Cell Cycle Arrest of the *hhp1.M84G* strain. Indicated cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C or 40 °C. Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S, was scored (%). Heat stress at 40 °C delayed the first G1/S peak by 40-60 min. Then the cell cycle pattern continued normally.

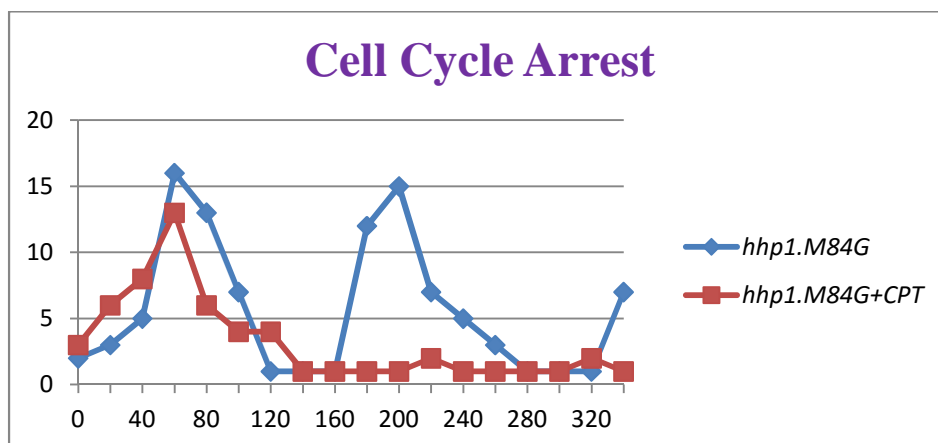


Figure: 5.1.8: Cell Cycle for *Hhp1.M84G* cells in the presence of CPT (camptothecin). Indicated cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). 40 μ M CPT cause a severe G2 arrest.

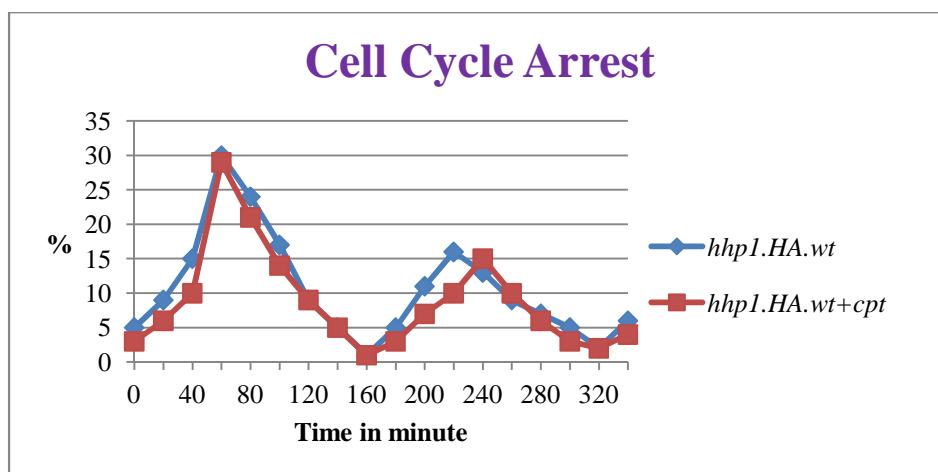


Figure: 5.1.9: Cell Cycle Arrest of *hhp1.HA*.wild type cells. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). *Hhp1.HA.wt* cells have a G2 delay of about 20-40 min due to broken replication forks.

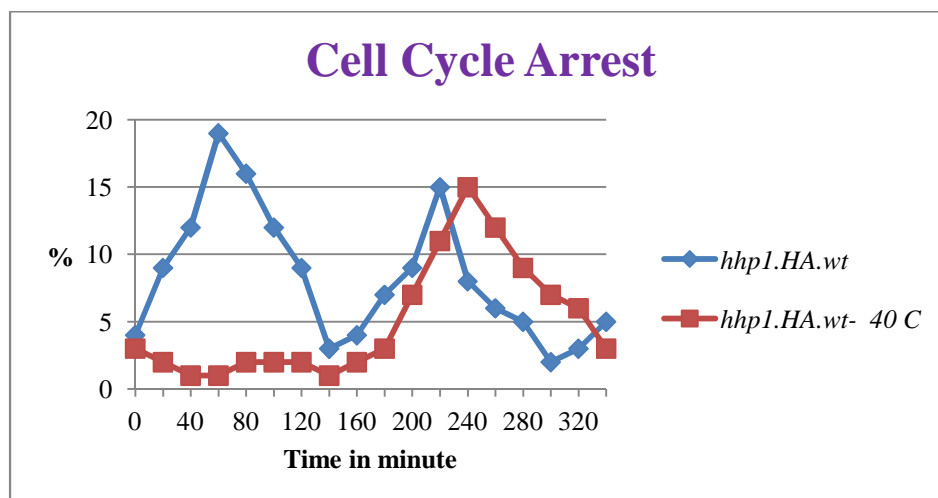


Figure: 5.1.10: Cell Cycle Arrest for the *hhp1.HA.wild type* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C or 40 °C. Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Heat stress at 40 °C delayed the first G1/S peak by approximately 160 min. Then the cell cycle pattern continued normally.

5.2. Mutation of the potential Chk1 Phosphorylation Site Serine-183 affects specifically the Cell Cycle Activities of Hhp1 Kinase

As explained earlier, serine-183 was mutated to an alanine residue because it resembles a potential Chk1 phosphorylation site. The *chk1* deletion was also found to be epistatic with the *hhp1* deletion (Chapter 3). Interestingly, cells expressing Hhp1 with the S183A mutation displayed a significant longer G2 delay in the presence of CPT (Figure: 5.2.1) compared to wild type cells (Figure: 4.1.10). *Hhp1.S183A* also showed an extended G2 phase in the absence of the topoisomerase 1 poison (Figure: 5.2.1). In contrast to wild type cells, the two G1/S peaks were clearly further apart (approx. 80 min). This indicates that the S183A mutation affects cell cycle progression independently of DNA damage.

Under heat stress conditions, *hhp1.S183A-HA* cells entered a G2 arrest which was however shorter compared to *hhp1-HA* wild type cells (Figure: 5.2.2). Taken together, both experiments indicate a defect in cell cycle regulation when serine-183 has been replaced by an alanine residue. Whether this is caused by an impact on the kinase activity or caused by the loss of a possible phosphorylation event by Chk1 kinase is not yet clear.

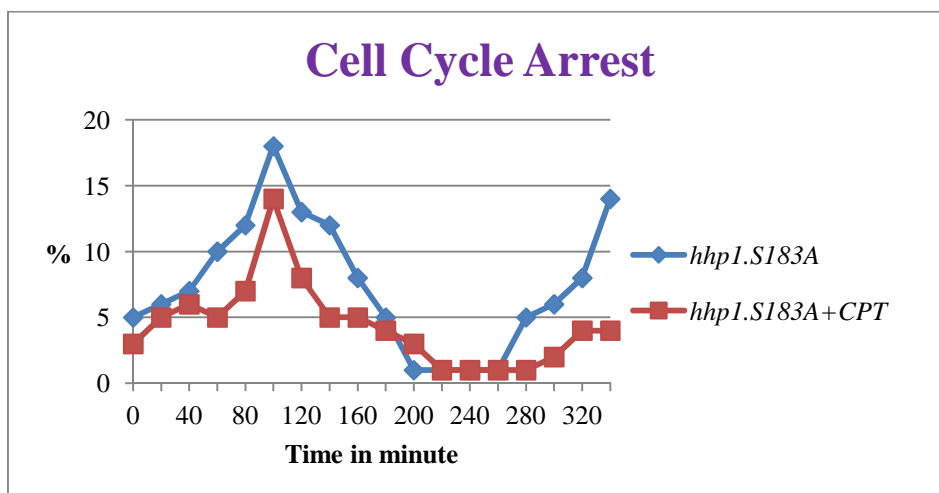


Figure 5.2.1: Cell Cycle Arrest for the *hhp1.S183A* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Treatment of the *hhp1.S183A* strain with 40 μ M CPT resulted in an extended G2 arrest.

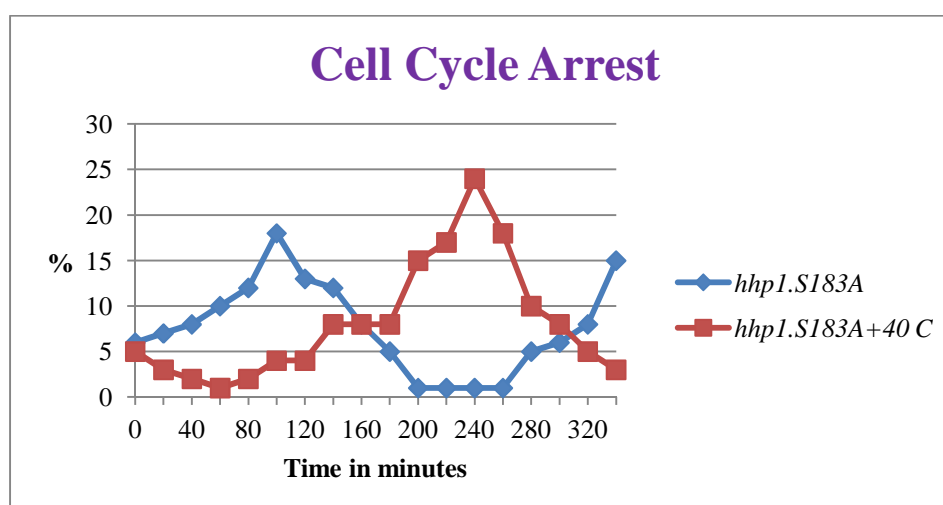


Figure 5.2.2: Heat stress and cell cycle arrest of the *hhp1.S183A* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without introducing heat stress at 40 $^{\circ}$ C. Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). *Hhp1.S183A* cells initiate the G2 arrest but do not maintain it as well as wild type cells.

To test whether the *hhp1.S183A* mutation acts in the same pathway as the deletion of *chk1*, a *hhp1.S183A-HA Δ chk1* double mutant was constructed. The loss of Chk1 had no impact on the protein levels of the mutated Hhp1 kinase (Figure 5.2.3).

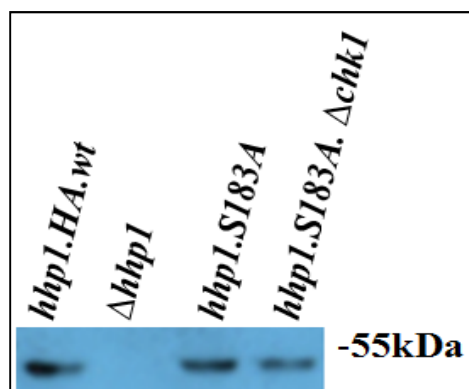


Figure: 5.2.3: Protein levels of the *hhp1.S183AΔchk1* strain. Total protein was isolated, 15μl of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody. Positive control is the *hhp1HA.wild type strain*, and the negative control is the *Δhhp1* deletion strain. *hhp1.S183A* expresses full-length of *hhp1*.

The combination of the S183A mutation with the deletion of *chk1* had a small but interesting impact on the CPT and heat sensitivity (Figure: 5.2.4). Both, the *hhp1.S183A-HA Δchk1* and *Δhhp1 Δchk1* strains grew better at 37°C and at higher CPT concentrations (2-5μM) (Figure: 5.2.4). This rescue effect could indicate a closer functional relationship between both kinases, which is supported by their epigenetic relationship discussed in Chapter 3. It may also imply that S183 is a genuine Chk1 phosphorylation site.

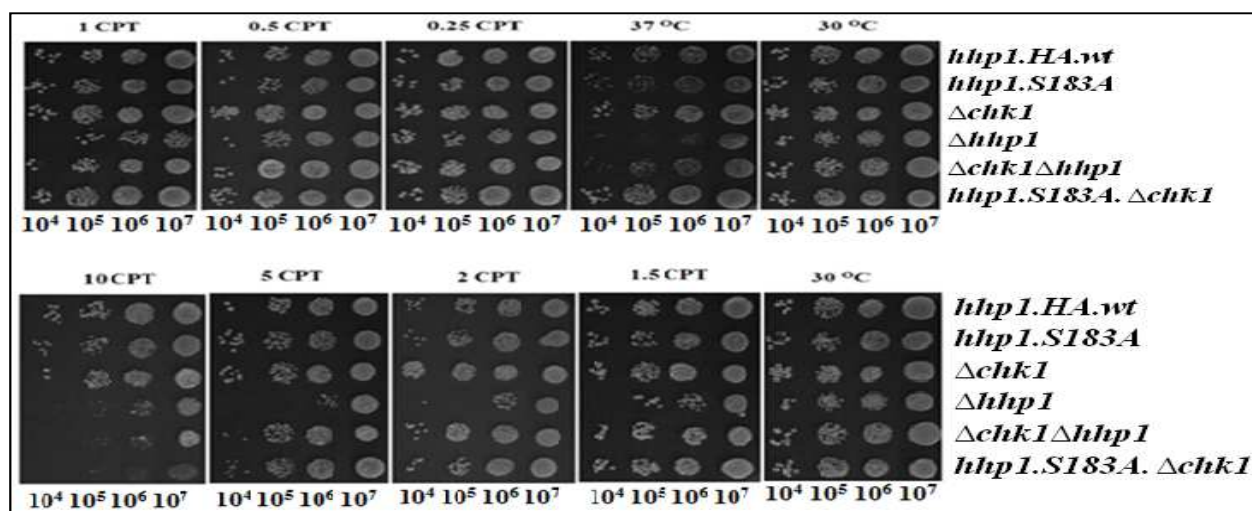


Figure: 5.2.4: CPT (camptothecin) drop test for the *hhp1.S183AΔchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

The analysis of the cell cycle arrest of G2 synchronised *Δchk1 hhp1.S183A-HA* cells revealed also an interesting observation. While the S183A mutant delayed for approximately 60 min in the presence of 40μM CPT (Figure: 5.2.1) loss of Chk1 kinase reduced this extended delay to 20

min (Figure: 5.2.5). It also reduced the extended gap between the G1/S peaks in the absence of CPT.

This implies that Chk1 may become hyper-activated in the S183A mutant thereby enforcing a prolonged inhibitory phosphorylation on the cell cycle regulator Cdc2 either during the normal cell cycle or in the response to CPT. Again this supports the idea that Chk1 and Hhp1 share a close functional link.

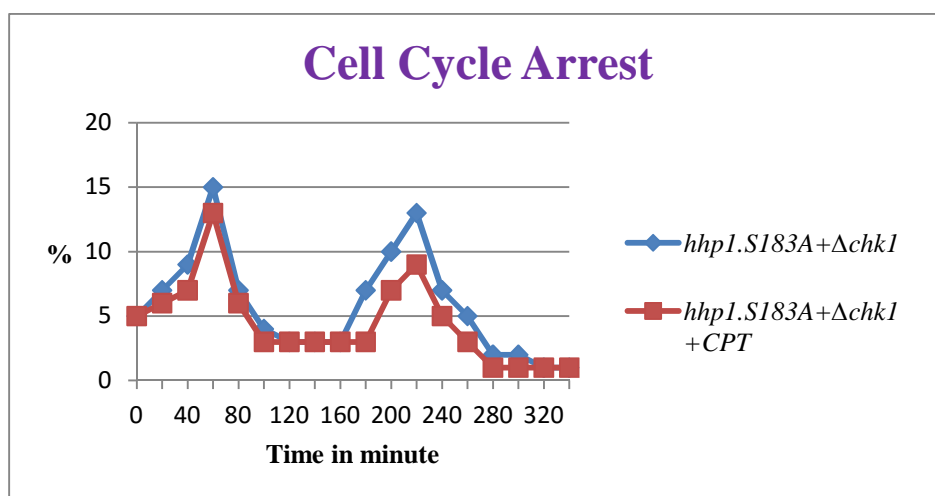


Figure: 5.2.5: Cell Cycle Arrest for the *hhp1.S183A.Δchk1* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Deletion of *chk1* reduces the delayed cell cycle progression of the *hhp1.S183A* mutant.

5.3. Mutated Hhp1 tyrosine-169-phenylalanine change Hhp1 kinase activities

CK1 homologous in yeast differ from their human counterparts as they are dual specific kinases which phosphorylate tyrosine as well as serine and threonine residues (12). Hhp1 contains two tyrosine residues with an interesting locations on the kinase. As shown in Figure: 5.1.1, tyrosine 169 (Y169) is exposed at the surface of the larger regulatory domain and Y227 is located at the bottom of a small groove which forms the nuclear localisation domain. While Y227 has a phosphorylation probability of 90% according to the Netphos 2.0 analysis tool, Y169 scores only 2.5%. To test whether both tyrosine residues are important for the DNA damage response, they were individually mutated to a phenylalanine residue which shares a benzene ring with tyrosine but lacks the hydroxyl group which could undergo phosphorylation. Both mutants

were generated in the analogue-sensitive M84G background which itself has no impact on the cell survival (381) (Figure: 5.1.5, Figure: 5.1.6). To test the significance of the nuclear localisation sequence (222-T KKQKY-227), all six amino acids were deleted in frame. All mutant alleles were integrated at the endogenous *hhp1* locus and re-amplified for DNA sequencing.

Like the M84G single mutant (Figure: 5.1.5), the *hhp1.M84G.Y227F-HA* strain grows better at 37° which implies that the higher (Figure: 5.3.1) resistance to temperature stress is dependent on the M84G mutation in the ATP binding site and not on the mutation of Y227. Rather unexpectedly, removal of the hydroxyl group at position 227 had a strong impact on the MMS and CPT sensitivity of the M84G mutant (Figure: 5.3.1, Figure: 5.3.2). While the single M84G mutant is as resistant as wild type cells (Figure: 5.1.5, Figure: 5.1.6), the *hhp1.M84G.Y227F-HA* strain was significantly more sensitive to both drugs (Figure: 5.3.1, Figure: 5.3.2, Figure: 5.3.3). While it is difficult to exclude the possibility that this sensitivity is a combination of both mutations (M84G and Y227F), it indicates an important role of Y227 in the DNA damage response.

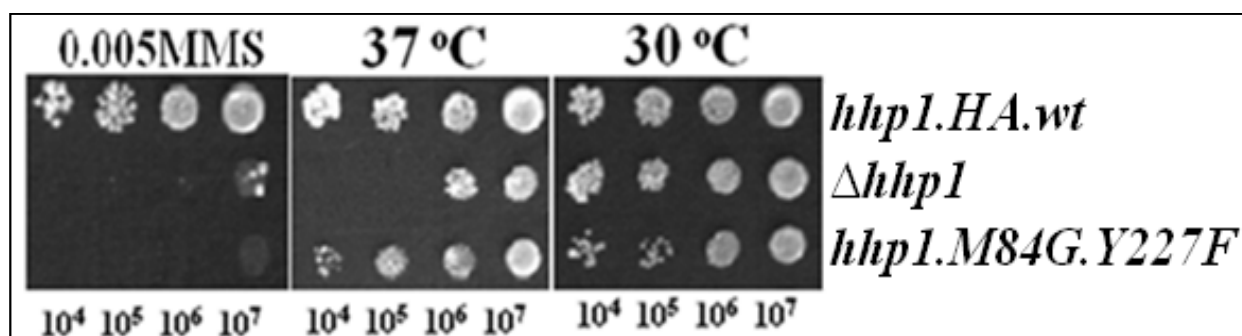


Figure: 5.3.1: MMS (methyl-methanesulfonate) drop test for the *hhp1.M84G.Y227F* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

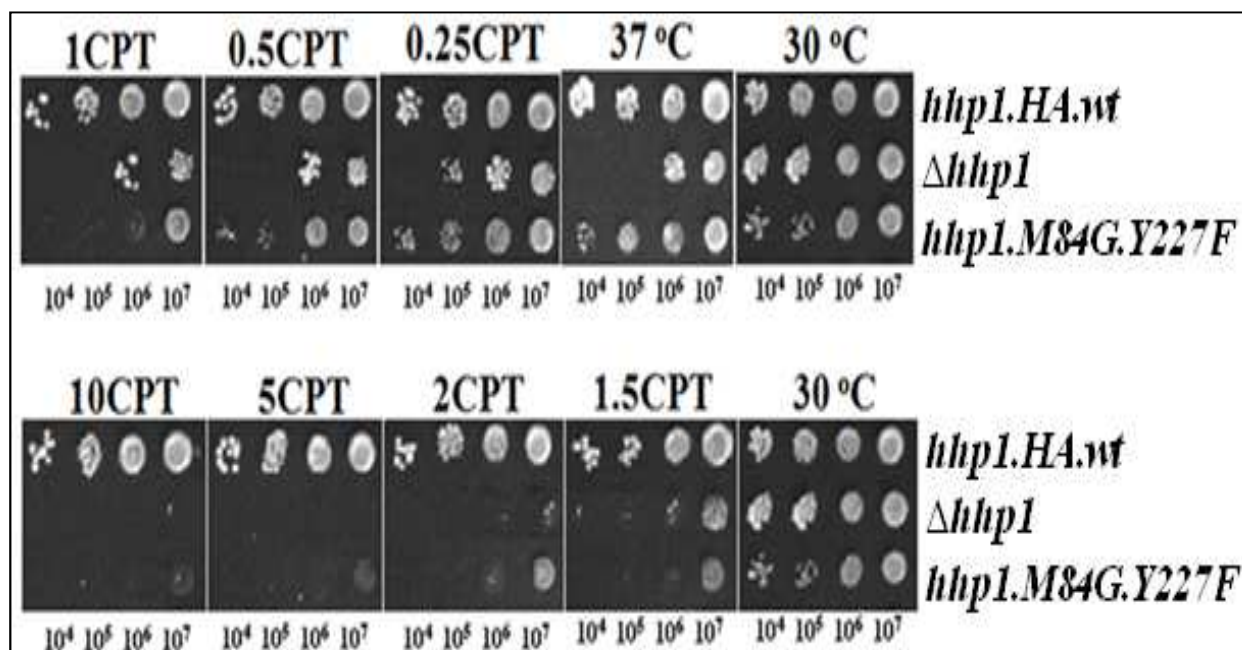


Figure: 5.3.2: CPT (camptothecin) drop test for the *hhp1.M84G.Y227F* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

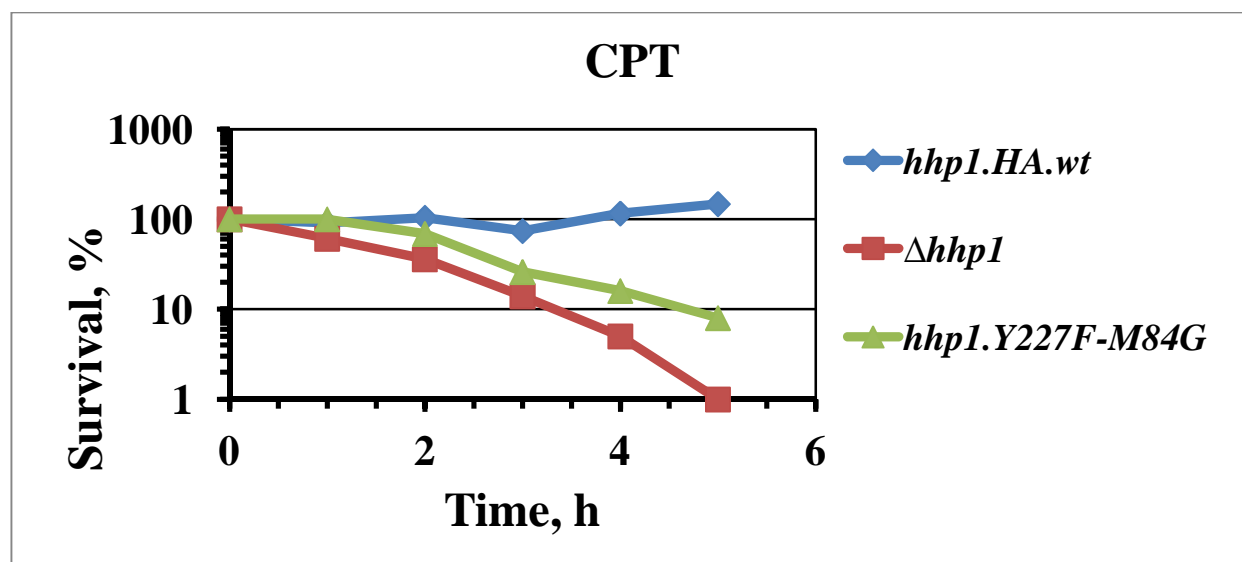


Figure: 5.3.3: CPT (camptothecin) survival assay for the *hhp1.M84G.Y227F* strain. Yeast strains cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40μM CPT. Samples (75μl) were withdrawn at the indicated time points and plated on one YEA plate. The surviving colonies were scored after 3-4 days at 30°C.

As shown in [Figure: 5.3.3](#), the *hhp1.M84G.Y227F-HA* strain was only slightly less sensitive than the *hhp1* deletion strain under acute conditions. While the mutation of Y227 had a clear impact

on the cell survival in the presence of DNA damage, it had little impact on the cell cycle delay. Like the M84G single mutant, the *hhp1.M84G.Y227F-HA* strain delayed significantly longer in the presence of DSBs (Figure: 5.3.4). Although this shows that the main change to the G2 delay in the presence of replication fork damage originates from the M84G mutation and not from the Y227F replacement, the mutation may have a small impact on the G2 delay under heat stress conditions. As shown in Figure: 5.3.5, *hhp1.M84G.Y227F-HA* cells postpone entry into mitosis for 160 min while the M84G strain re-entered the cell cycle already after 60 min (Figure: 5.1.7). The main conclusion from these experiments is the requirement of the hydroxyl group at position 227 for the survival of MMS and CPT induced DNA damage rather than for the regulation of the cell cycle.

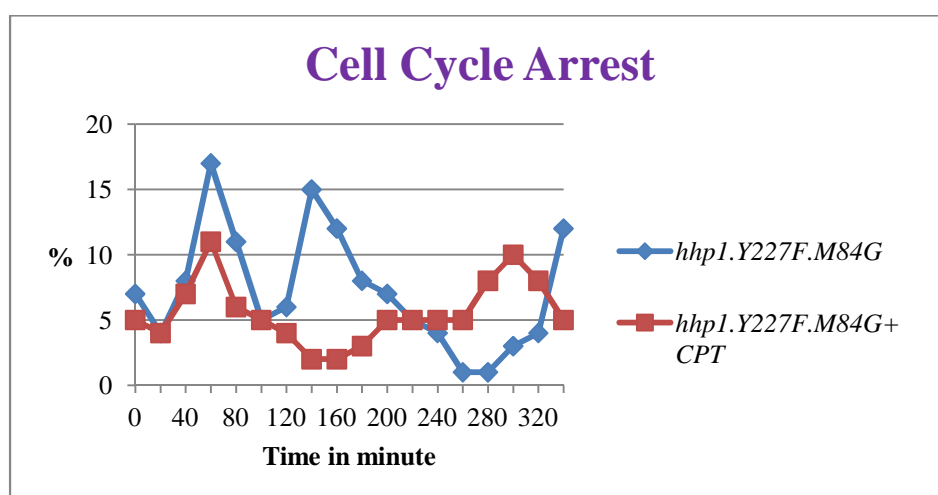


Figure: 5.3.4: Cell Cycle Arrest for the *hhp1.M84G.Y227F* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

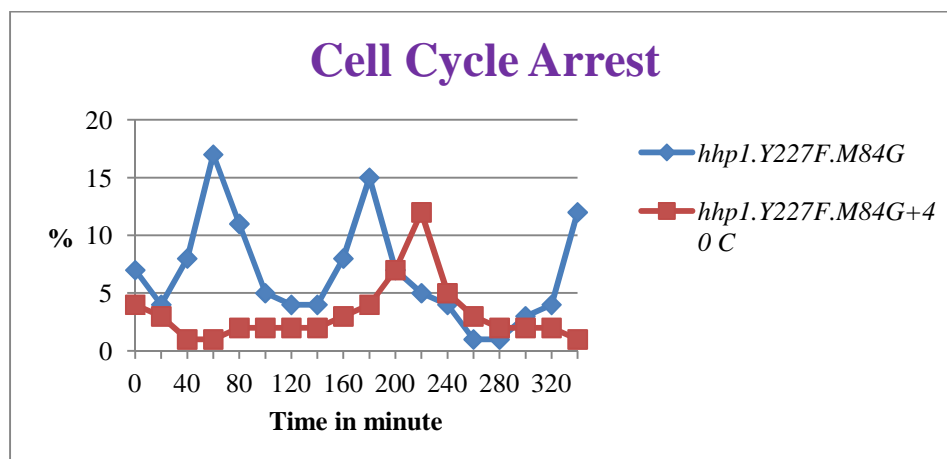


Figure: 5.3.5: Heat stress induced cell cycle arrest of the *hhp1.M84G.Y227F* strain. Indicated cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without (30°C) heat stress at 40 °C. Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

Given that Y227 sits at the bottom of the groove which forms the nuclear localisation domain, the mutation of Y227 may affect the nuclear shuttling of the kinase thereby causing a deletion-like phenotype with regard to cell survival. Consistent with this idea, the *hhp1.M84G. Δ NLS-HA* strain resembles closely the *hhp1.M84G.Y227F-HA* strain. Deletion of the NLS in frame, rendered the M84G mutant CPT (Figure: 5.3.6) and MMS (Figure: 5.3.7) sensitivity, and resulted in cell cycle delays which are reminiscent of the M84G mutant although the G2 arrest in CPT medium was shorter (Figure: 5.3.9). Taken together these findings imply a role for the nuclear localisation domain in the cell survival when DNA becomes damaged by alkylation (MMS) or by replication fork breakage (CPT).

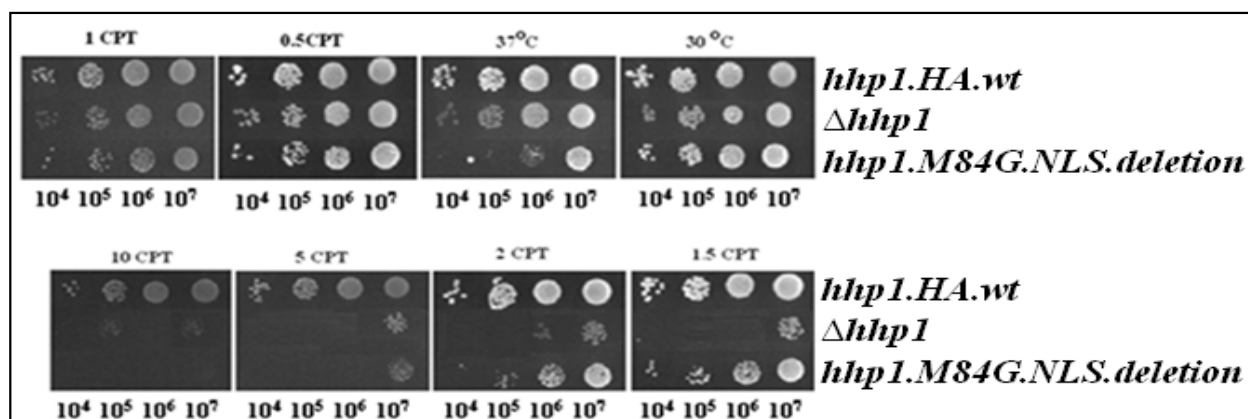


Figure: 5.3.6: CPT (camptothecin) drop test for the *hhp1.M84G.NLS.deletion* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

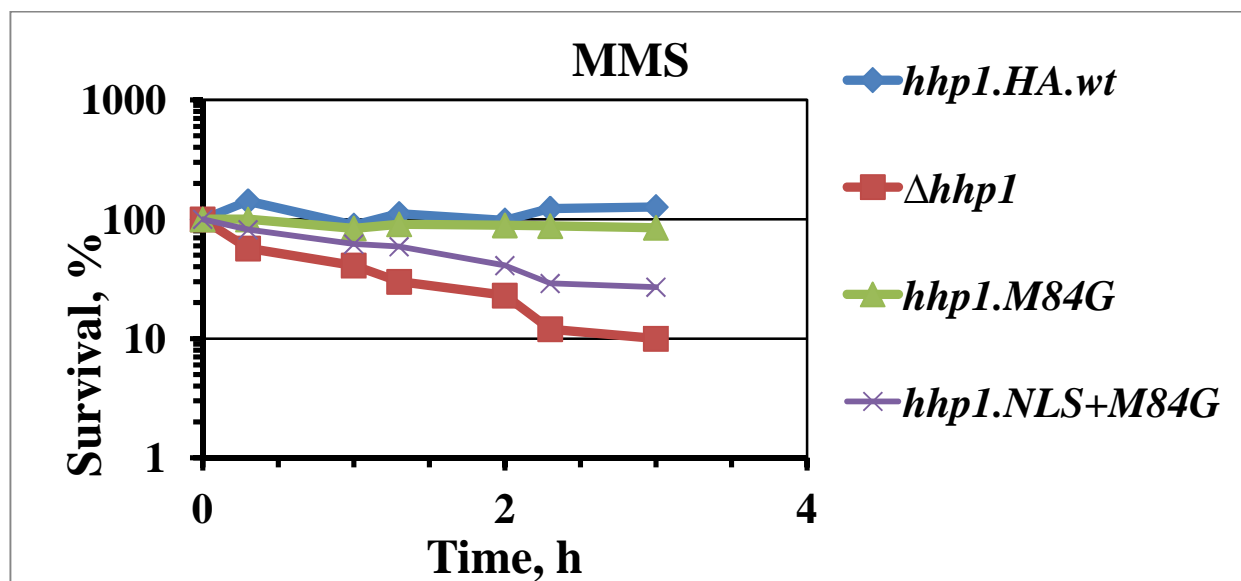


Figure: 5.3.7: MMS (methyl-methanesulfonate) survival assay for the *hhp1.M84G.NLS.deletion* strain. Yeast strains cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 0.05% MMS. Samples (75μl) were withdrawn at the indicated time points and plated on one YEA plate. The surviving colonies were scored after 3-4 days at 30°C.

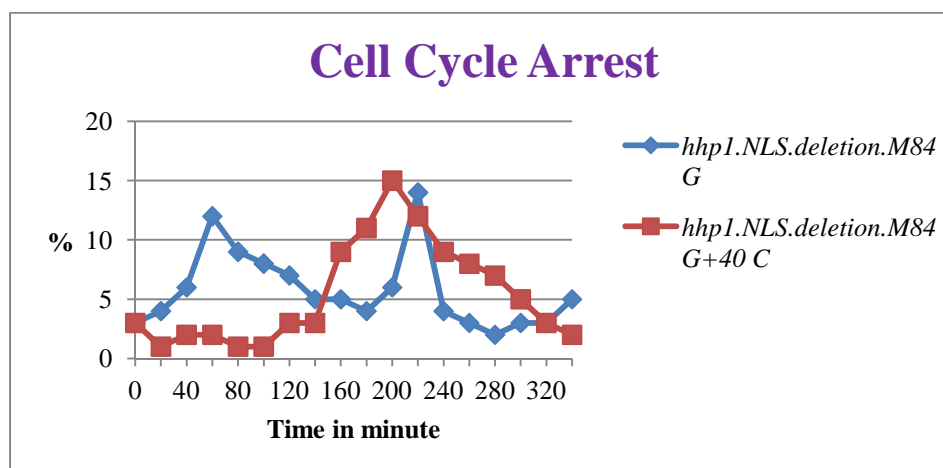


Figure: 5.3.8: Heat stress induced cell cycle arrest for the *hhp1.M84G.NLS.deletion*. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with 30 °C or 40 °C heat. Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

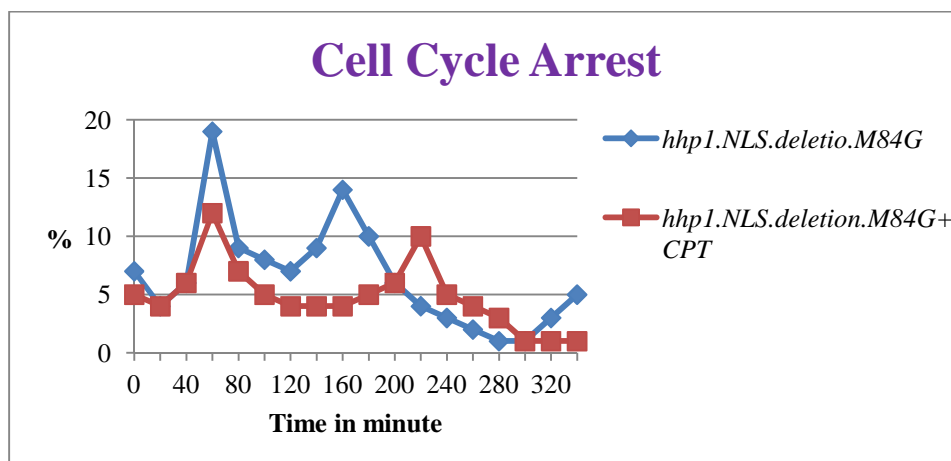


Figure: 5.3.9: Cell cycle arrest for *hhp1.M84G.NLS.deletion* strain in the presence of CPT. Indicated cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

In contrast to the mutation of Y227, the replacement of Y169 by a phenylalanine residue had no impact on the DNA damage sensitivity. The *hhp1.M84G.Y169F-HA* strain survived as well as the M84G single mutant and wild type cells in the presence of CPT (Figure: 5.3.10) and MMS (Figure: 5.3.11). This shows that the high sensitivity of the *hhp1.M84G.Y227F-HA* strain is most likely due to the mutation at position 227.

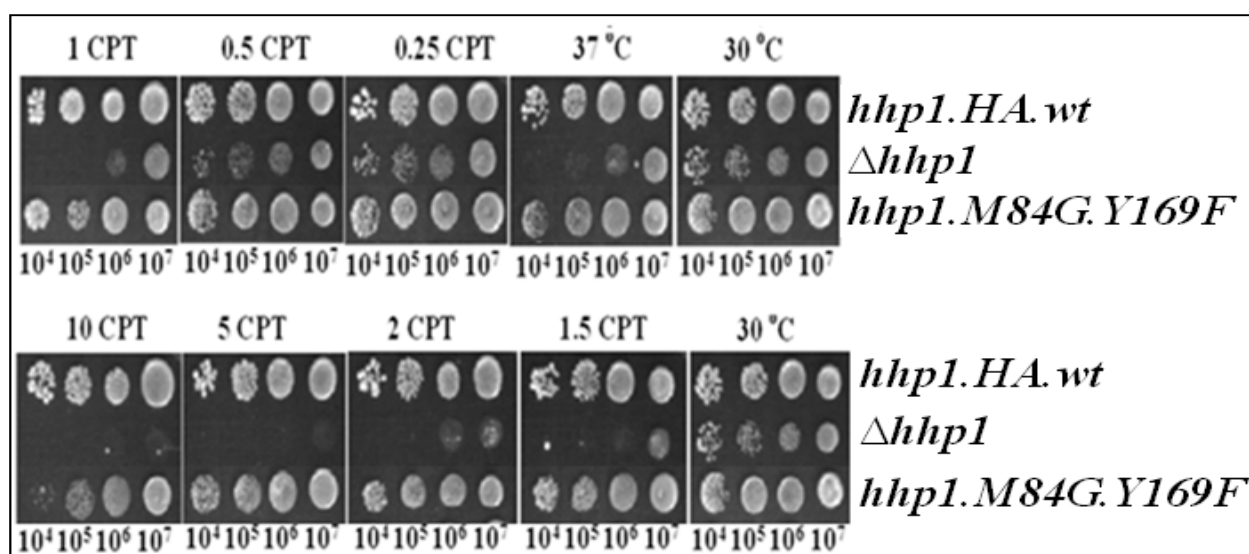


Figure: 5.3.10: CPT (methyl-methanesulfonate) drop test for the *hhp1.M84G.Y169F* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

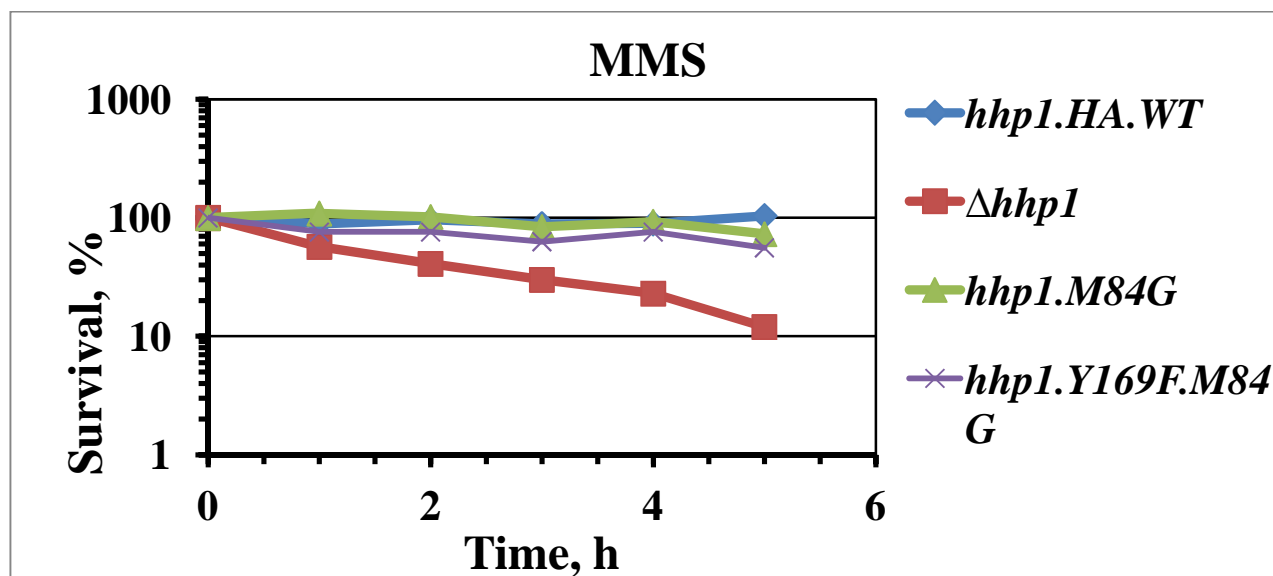


Figure: 5.3.11: MMS (methyl-methanesulfonate) survival assay for the *hhp1.M84G.Y169F* strain. Yeast strains cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 0.05% MMS. Samples (75μl) were withdrawn at the indicated time points and plated on one YEA plate. The surviving colonies were scored after 3-4 days at 30°C.

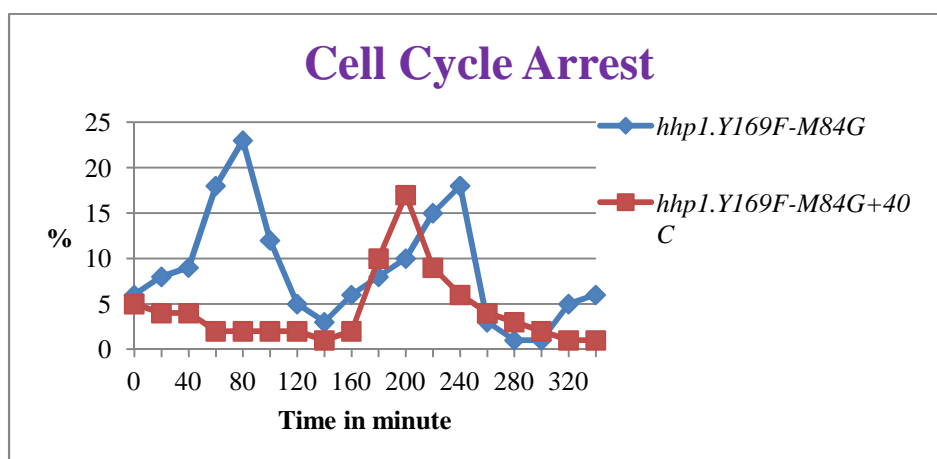


Figure: 5.3.12: Heat stress induced cell cycle arrest for the *hhp1.M84G.Y169F* strain. Indicated cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30°C or at 40 °C as heat stress. Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

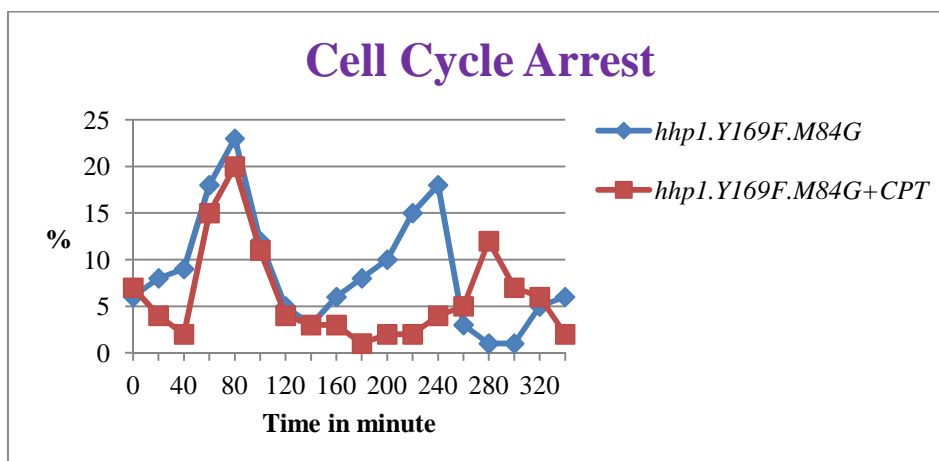


Figure: 5.3.13: Cell cycle arrest for the *hhp1.M84G.Y169F* strain in the presence of CPT. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

As in the case of the Y227F mutation, the replacement of Y169 had no real impact on the G2 arrest changes induced by the M84G mutant (Figure: 5.3.12, Figure: 5.3.13).

In summary, the dominant phenotype of the tyrosine mutants (Y169 or Y227 to a phenylalanine) was the strong increase in MMS and CPT sensitivity of the Y227F mutation which affects the nuclear localisation sequence. This is supported by the similar increase in MMS and CPT sensitivity of the NLS deletion strain. Interestingly, neither the Y227F mutation nor the deletion of the NLS had a strong impact on the cell cycle defect caused by the M84G single mutation.

5.4) Mutation of Tyrosine 227 within the the Nuclear Localisation domain (NLS) effects the DNA Repair Activities of Hhp1 kinase

Since deletion of *chk1* reduced the CPT-induced G2 delay of the S183A mutant (Figure: 5.2.5), the *hhp1.M84G.Y227F*-HA double mutation was combined with the deletion of *chk1* (Δ *chk1 hhp1.M84G.Y227F*-HA). Loss of Chk1 kinase had no impact on the expression levels of the mutated Hhp1 protein (Figure: 5.4.1), but restored MMS and CPT resistance (Figure: 5.4.2, Figure: 5.4.3, Figure: 5.4.4). The impact of the loss of Chk1 was stronger for CPT compared to MMS. This suppression phenotype is in line with the more subtle restoration of CPT resistance in the Δ *chk1 hhp1.S183A*-HA strain (Figure: 5.2.4) and strongly suggests that Chk1 may be aberrantly active in the *hhp1* mutant or that Chk1 starts a DNA repair event which requires wild

type Hhp1 kinase at a later stage. In combination with the results summarised in Chapter 3, the rescue effect could be explained if both kinases were to converge on the Mus81-Eme1 endonuclease. Loss of Chk1 may rescue the CPT sensitivity of the *hhp1.M84G.Y227F-HA* (Figure: 5.4.3) and *hhp1.S83A-HA* strain (Figure: 5.2.4) because Mus81-Eme1 may be aberrantly active in these *hhp1* mutants. Chk1 phosphorylates and activates the endonuclease in G2 to help with the repair of broken DNA replication forks (515). As in the case of the Δ *chk1 hhp1.S183A-HA* mutant, loss of Chk1 reduced the extended G2 delay in CPT medium (Figure: 5.4.5). This would be in line with the idea that Mus81 is hyperactive in the *hhp1* mutant cells thereby triggering a repair problem which prolongs the checkpoint signal and re-entry into mitosis (Figure: 5.4.6). Loss of Chk1 may prevent this hyper-activation thereby rendering the inhibition of Mus81-Eme1 by Hhp1 redundant.



Figure: 5.4.1: Protein levels of the *hhp1.M84G.Y227F.Δchk1* strain. Total protein was isolated, 15 μ l of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody.

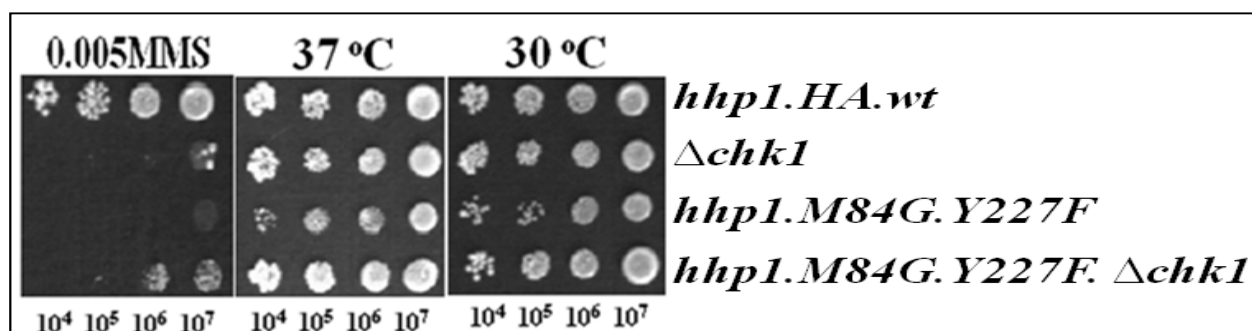


Figure: 5.4.2: MMS (methyl-methanesulfonate) drop test for the *hhp1.M84G.Y227F.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

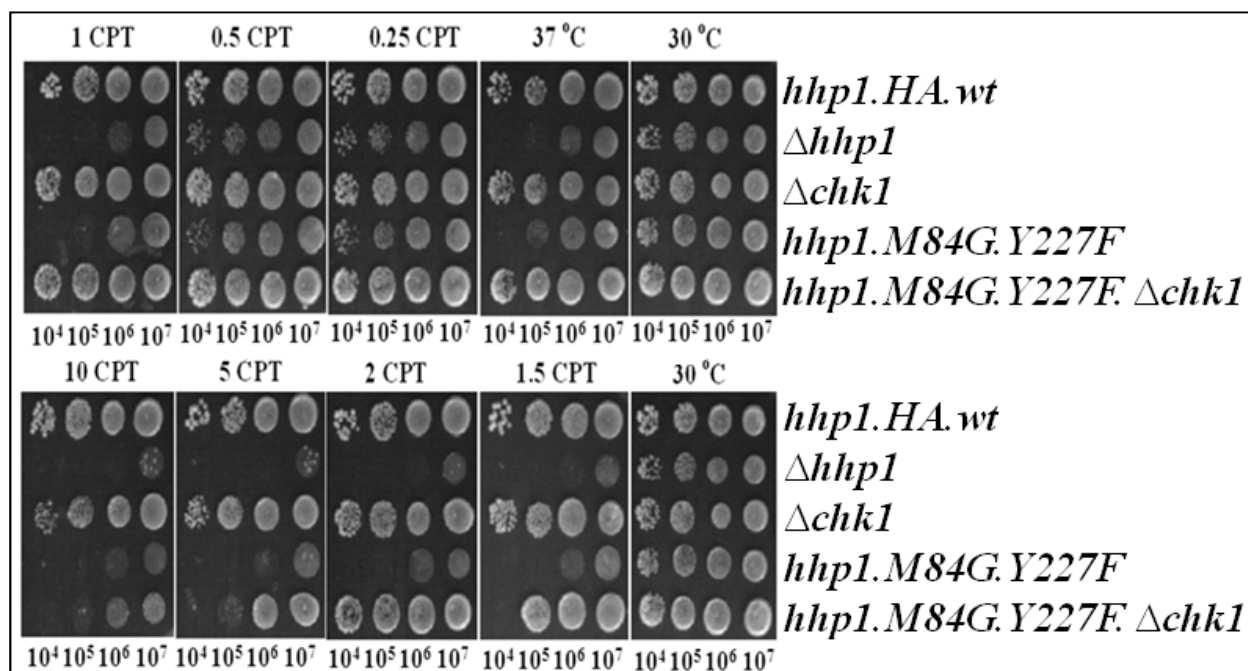


Figure: 5.4.3: CPT (camptothecin) drop test for *hhp1.M84G.Y227F.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

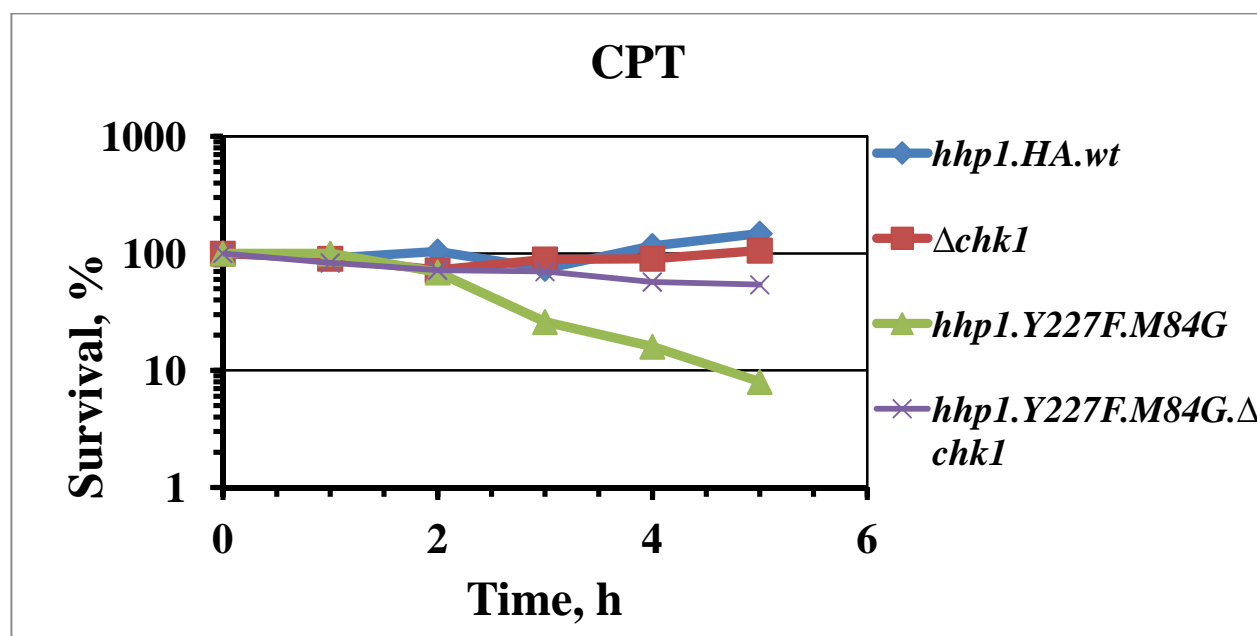


Figure: 5.4.4: Acute CPT (camptothecin) survival assay for the *hhp1.M84G.Y227F.Δchk1* strain. Yeast strains cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40μM CPT. Samples (75μl) were withdrawn at the indicated time points and plated on one YEA plate. The surviving colonies were scored after 3-4 days at 30°C.

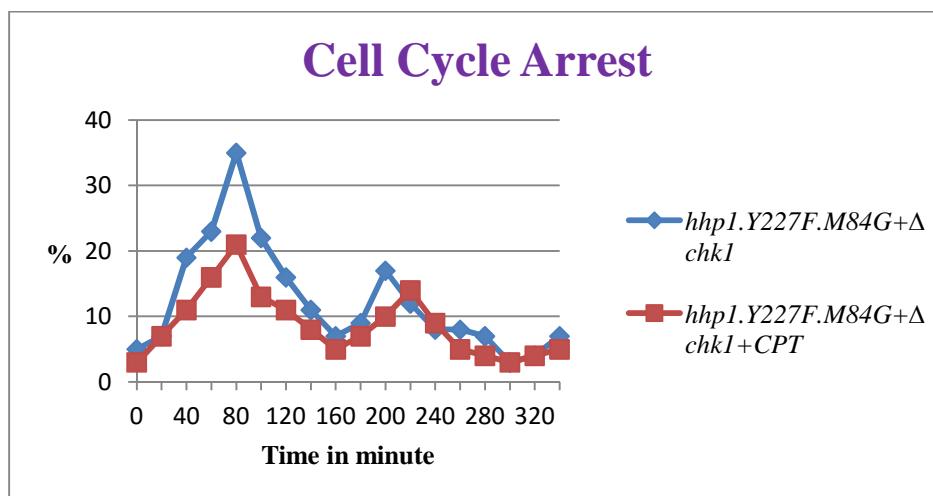


Figure: 5.4.5: Cell cycle arrest for the *hhp1.M84G.Y227F.Δchk1* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

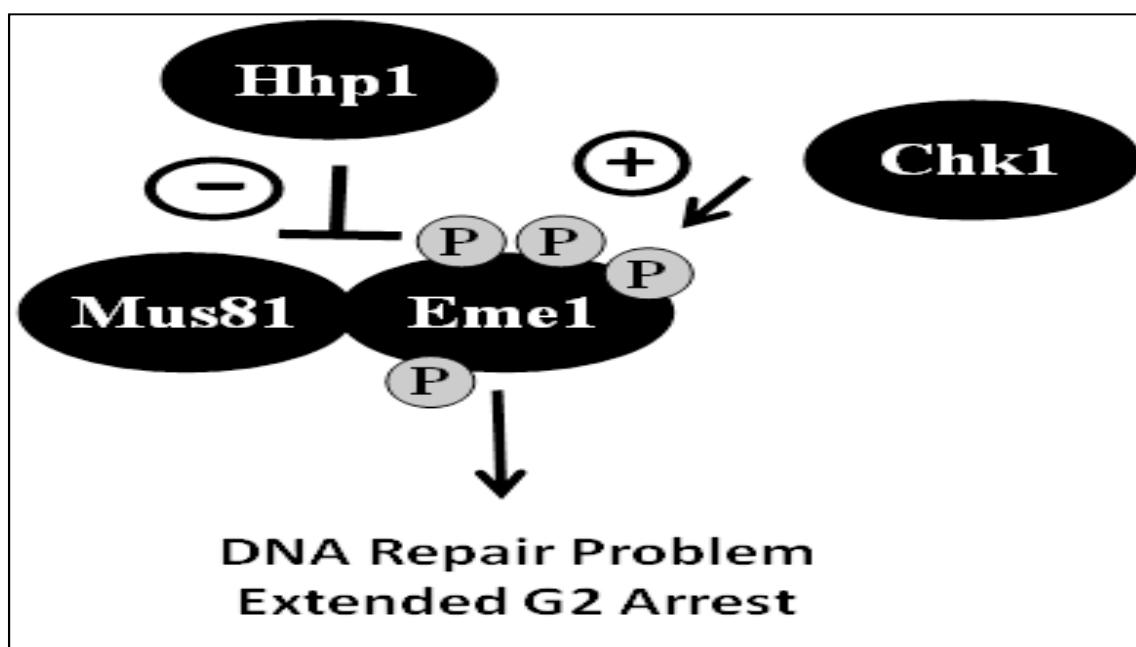


Figure: 5.4.6: Model of Mus81-Eme1 and Hhp1 and Chk1 activities. Mutations in Hhp1 may reduce the inhibitory impact of the kinase on the endonuclease Mus81-Eme1 resulting in its aberrant up-regulation. Since Chk1 phosphorylation of Eme1 is necessary for the activation of the endonuclease in G2 when DNA replication forks break in the presence of the topoisomerase 1 inhibitor camptothecin (CPT), deletion of *chk1* may rescue the CPT sensitivity and reduce the CPT-induced G2 delay because Mus81 would no longer be hyperactive.

To find out whether the rescue of the CPT sensitivity upon loss of Chk1 is specific to the *hhp1.M84G.Y227F-HA* strain, the deletion of *chk1* was also combined with the *hhp1.M84G. Δ NLS-HA* double mutation.



Figure: 5.4.7: Protein levels of the *hhp1.M84G.NLS.deletion.Δchk1* strain. Total protein was isolated, 15 μ l of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody.

As in the case of the Y227A mutation, loss of *chk1* had no impact on the total amount of the *Hhp1.M84G. Δ NLS-HA* protein (Figure: 5.4.7), and it reduced the sensitivity to CPT (Figure: 5.4.8, Figure: 5.4.9). This indicates that it has the inability to shuttle between the nucleus and the cytoplasm which causes the DNA repair defect when DNA replication forks break. Moreover this indicates that the inhibitory activity of Hhp1 on Mus81-Eme1 is executed in the nucleus.

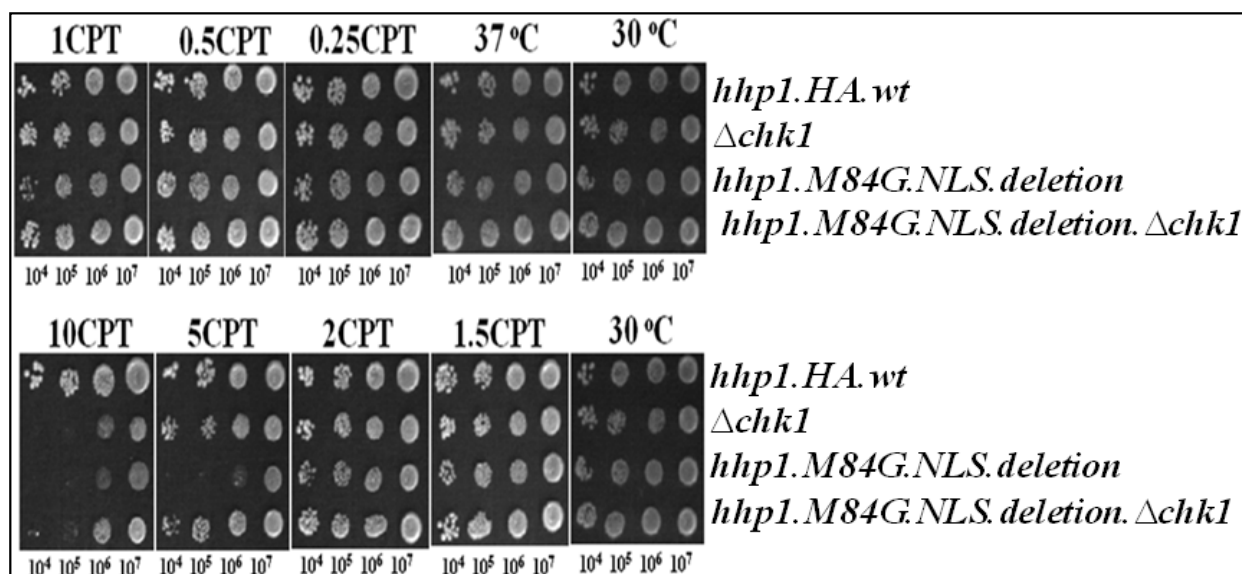


Figure: 5.4.8: CPT (camptothecin) drop test for the *hhp1.M84G.NLS.deletion.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

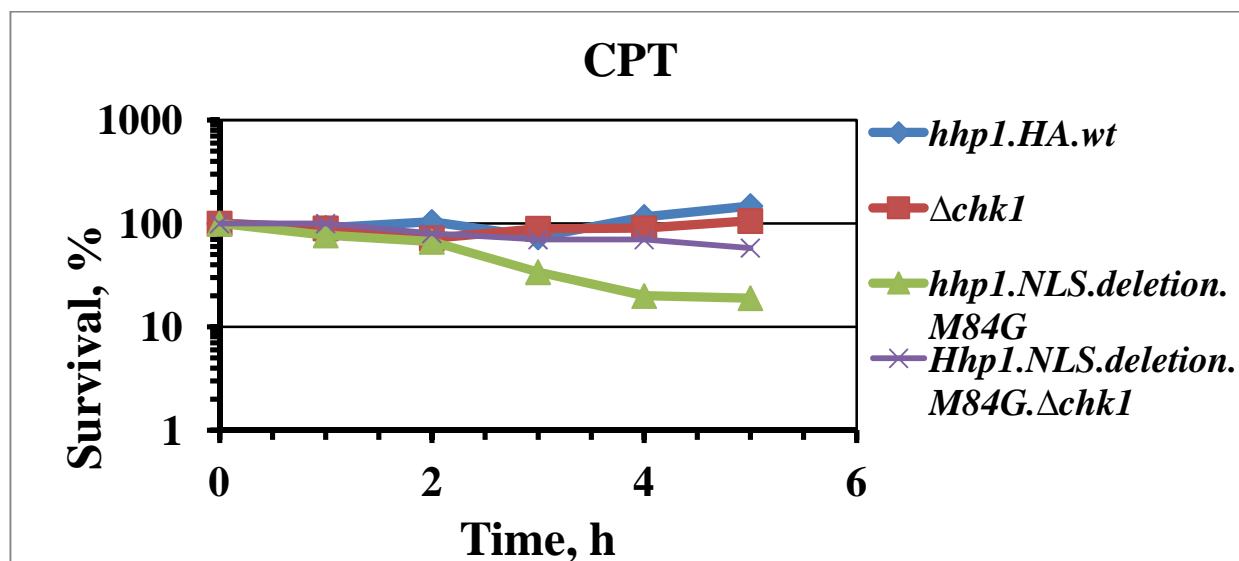


Figure: 5.4.9: Acute CPT (camptothecin) survival for the *hhp1.M84G.NLS.deletion.Δchk1* strain. Yeast strains cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40μM CPT. Samples (75μl) were withdrawn at the indicated time points and plated on one YEA plate. The surviving colonies were scored after 3-4 days at 30°C.

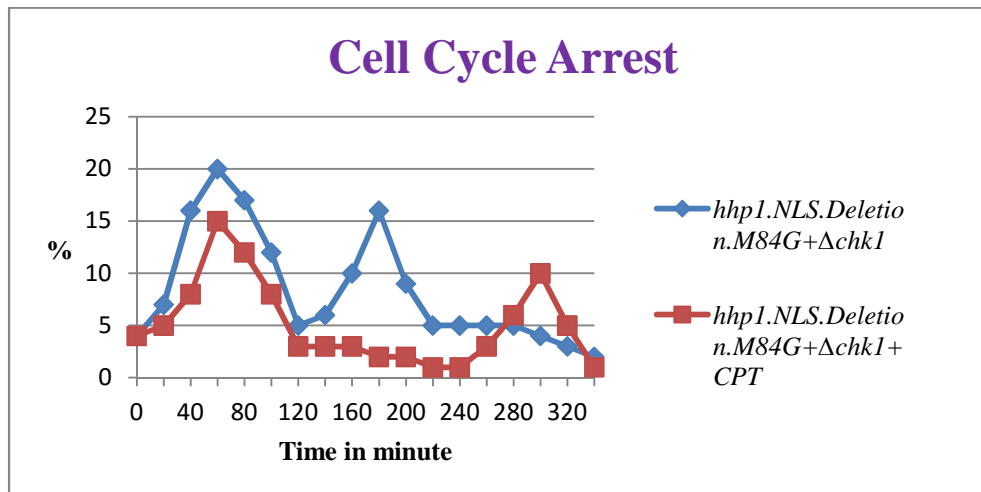


Figure: 5.4.10: Cell cycle arrest for the *hhp1.M84G.NLS.deletion.Δchk1* strain in the presence of CPT. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40μM camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

There was however one important difference between the Y227F and Δ NLS mutants. While deletion of *chk1* reduced the G2 delay in case of the Y227F mutation, it did not result in a similar reduction when Y227 and the rest of the nuclear localisation sequence was deleted

(Figure: 5.4.10). This led to the conclusion that while the NLS and Y227 are both important for the DNA repair response of Hhp1, Y227 may act independently of the NLS in the case of the cell cycle regulation. The latter function may require the phosphorylation of Y227.

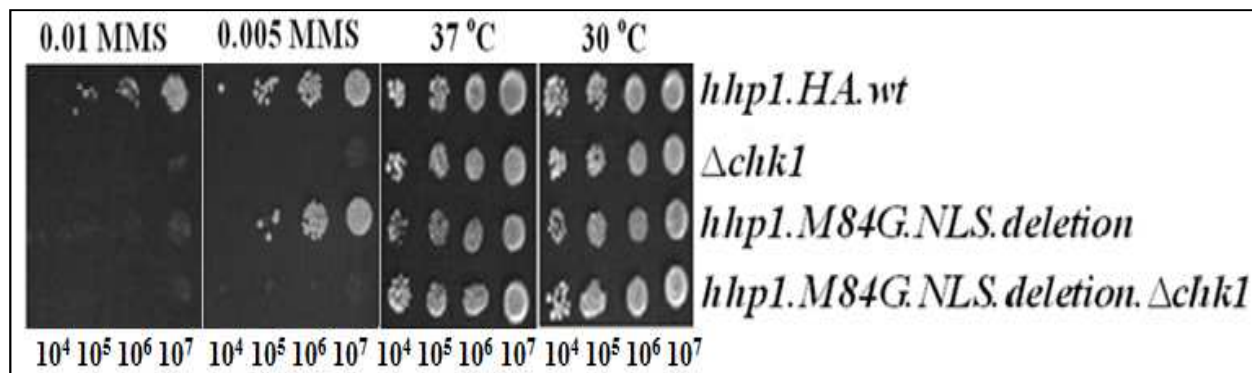


Figure: 5.4.11: MMS drop test for the *hhp1.M84G.NLS.deletion.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

The same explanation may also help to understand why loss of Chk1 does not restore the resistance to MMS (Figure: 5.4.11). How loss of Y227 in the Δ NLS mutant could cause a different phenotype compared to the single Y227F mutation could be explained if the phenylalanine at the bottom of the small groove which forms the NLS domain still allows the domain to function whilst abolishing any phosphorylation at this position. In this sense, the Y227F mutation could be a separation-of-function mutation. If phosphorylation of Y227 were to regulate nuclear shuttling of Hhp1, the un-phosphorylated NLS may cause the kinase to accumulate in one compartment causing the separation of function. Unfortunately, all attempts to visualise the HA-tagged Hhp1 proteins in cells were unsuccessful.

Chapter 6: The Circadian Clock Mutations in Hhp1 affect mainly its Cell Cycle Functions

Chapter Summary

Casein kinase 1 plays key roles in the mammalian circadian clock and most CK1 mutations affecting the clock reduce the kinase activity towards the PERIOD proteins. Three circadian clock mutations in *S.pombe* Hhp1 are the main feature of this chapter. The *tau* mutation found in Syrian Hamster CK1 ϵ .R178C (Hhp1.R180C) and other two mutations found in *Drosophila*: double-time long dbtL.M80I (Hhp1.M82I) and double-time short dbtS.P47S (Hhp1.P49S). All three mutations affect the cell cycle activity of Hhp1 while having only a very limited impact on cell survival in the presence of the topoisomerase 1 inhibitor camptothecin (CPT). Mutant cells expressing the circadian mutations have an extended G2 arrest in the presence of CPT. The main conclusion from these findings is that a drop in kinase activity affects mainly the cell cycle but not the DNA repair functions of Hhp1. The relationship between Hhp1 and the *S.pombe* paralog of TIMELESS (Swi1) was also investigated. Swi1 associates with the DNA replication fork and loads the DNA damage checkpoint protein Mrc1. Deletion of *swi1* renders Δ *hhp1* more CPT sensitive strongly suggesting that both proteins act in parallel pathways when DNA replication forks break.

Given that Wee1 is regulated by the mammalian clock, the genetic link between Wee1 and Hhp1 was studied. A drop in Wee1 activity, which increases activity levels of the main cell cycle regulator Cdc2 kinase, partly reduces the heat and CPT sensitivity of Δ *hhp1* and the double-time short (*hhp1.P49S*) mutation. A similar suppression was observed when Cdc2 activity is increased by the gain-of-function mutation *cdc2.Iw* (G146D). The rescue upon an increase in Cdc2 activity could be explained by the regulation of the endonuclease Mus81-Eme1 since the endonuclease complex is phosphorylated by Cdc2 and most likely also by Hhp1. The chapter also presents a biochemical analysis of Hhp1 and its mutant forms using isoelectric focusing. Wild type Hhp1 separates into four forms on a linear pH gradient from 3 to 10 independently of DNA damage. The *tau* mutation (*hhp1.R180C-HA*) shifts one form to a more positive (alkaline) isoelectric point, while the double-time long mutation (*hhp1.M82I-HA*) increases the number of the negative forms. Two of the forms reside or require the C-terminal domain of Hhp1 as they disappear upon deletion of the tail domain. A kinase-dead mutant of Hhp1 (*hhp1.K40R-HA*) still possesses the four forms strongly implying that auto-phosphorylation is not important.

6.1. Introduction

The circadian clock defines the daily biological cycle of activities based on a 24 hours period of time. The circadian rhythm influences the daily alternation between light and dark and the rhythm between sleep and activity in mammals (361). The circadian system is based on the oscillating expression of key genes including *CLOCK* or *NPAS2*, *BMAL1*, *PERIOD 1-3* and *CRYPTOCHROME 1-2* (360). Casein kinase 1 (CKI) performs diverse roles in the circadian clock. It promotes the nuclear localisation of Period 1 to down-regulate the dimeric master transcription factor CLOCK-BMAL1 (1). The phosphorylation of the PER proteins by CK1 increases over the course of the circadian day and peaks when the repression of the positive transcription factors CLOCK and BMAL1 is maximal. The role of many CK1 phosphorylation sites is however not known. Some phosphorylation events target the PER proteins for degradation while others are important for their nuclear localisation. The breakdown of the PER proteins can reset the clock because it removes the inhibition of the CLOCK-BMAL1 heterodimer. BMAL1 is also phosphorylated by CK1 which is linked with an increase in its transcriptional activity (545). Two important outputs of the clock are cell cycle regulation and DNA repair. Bjarnason Georg, and Richard Jordan (366) stated that the mammalian clock regulates cell cycle progression by influencing the transcription of *Wee1*, *c-myc*, and *cyclin D1*. Wee1 kinase inhibits cell cycle progression by phosphorylating Cdc2 kinase at tyrosine 15, the transcription factor c-myc promotes G1-S progression whereas the cyclin D1-CDK4 complex keeps cells in G1 by blocking members of the retinoblastoma (RB) protein family. Interestingly, the mammalian Timeless (Tim) protein promotes the circadian rhythm (101, 364, 372, 379) and is required for the activity of the intra-S DNA damage checkpoint by directly regulating ATR-Chk1 signalling (101, 111, 546). Proteins closely related to the human Tim protein exist in *Drosophila* (dTim-2/dTimeout) (101, 365), in the budding yeast *S. cerevisiae* (Tof1) (269, 378) and the fission yeast *S. pombe* (Swi1) (118, 373). Both, Swi1 and Tof1 associate directly with DNA replication forks and are mediators of the DNA damage checkpoint. Swi1 prevents replication fork collapse (101, 118) and Tof1 forms a complex with Csm3 and Mrc1 at the DNA replication fork (547).

6.2. The Circadian Clock Mutations analysed in Hhp1

Three mutants were created in the *hhp1* gene of the fission yeast *Schizosaccharomyces pombe* (*S. pombe*): *hhp1.R180C* which equals the mammalian *tau* mutation R178C in *CKIε* of the Syrian Hamster (367), *hhp1.M82I* which is identical to the Double-time long mutation (*dbt^L.M80I*) in *Drosophila* and *Hhp1.P49S* which is similar to Double-time short (*dbt^S.P47S*) in *Drosophila* (142) (Figure: 6.2.1, Figure: 6.2.2).

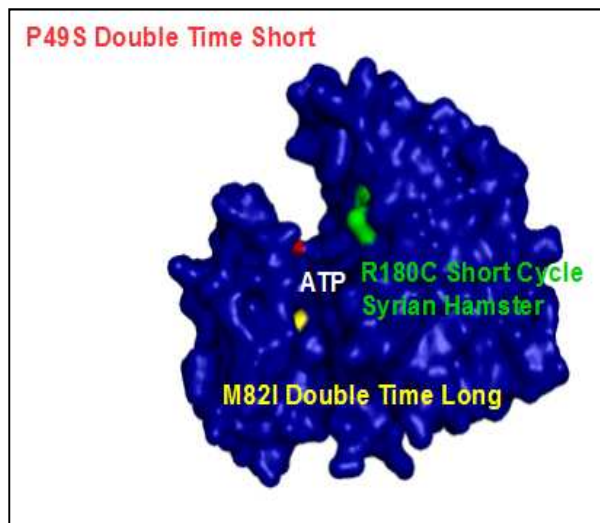


Figure: 6.2.1: Position of the three circadian clock mutations in *S.pombe* Hhp1. Interestingly, all three mutations face the ATP binding site of the kinase. The model is based on the highly related structure of *S.pombe* casein Kinase 1 (Csk1) (582) While proline 49 (proline 50: Csk1) and arginine 180 (arginine 183 in Csk1) are conserved, methionine 82 is replaced by a leucine residue at position 83 in Csk1. The image was generated with Polyview3D (PDB ID: 1CSN).

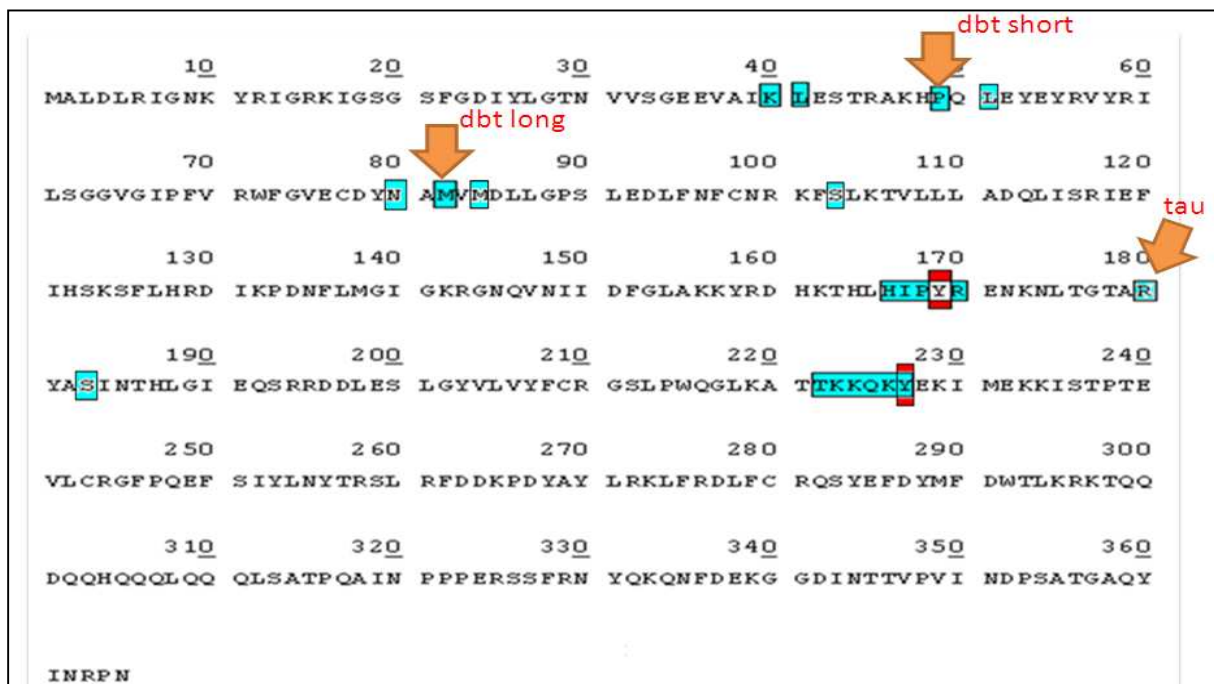


Figure: 6.2.2: Location of the mutations in the amino acid sequence of *S.pombe* Hhp1.

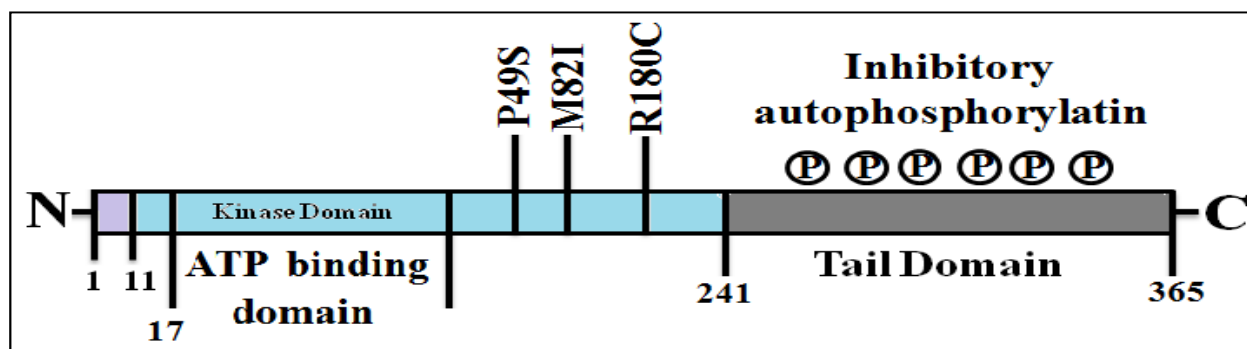


Figure: 6.2.3: Domain structure of *S.pombe Hhp1* kinase. The C-terminal tail domain (tail domain) contains the inhibitory autophosphorylation sites. The three circadian mutations are located within the kinase domain close to the ATP binding site.

The first mutation, *hhp1.R180C* is called the *tau* mutation in the Syrian hamster which shortens the period of the circadian clock period (363, 367). Meng Qing-Jun et. al. (368) published that the *tau* mutation reduces the period of the clock from 24 hours to 20 hours which correlates with the destabilisation of the Period proteins as their turnover increases.

The second mutation, *hhp1.M82I* (in *Drosophila* named *dbt^L.M80I*) extends the period of the circadian clock (62, 142), while the third mutation, *hhp1.P49S* (in *Drosophila*, named *dbt^S.P47S*), shortens the circadian clock (62, 142). Interestingly, all three mutations face the ATP binding site of the kinase (Figure: 6.2.1) which suggests that they may either increase or decrease the kinase activity thereby affecting the regulatory network between the PERIOD inhibitors and the activating CLOCK-BMAL1 complex.

All mutant alleles were generated by fusion PCR and integrated at the endogenous *hhp1-HA* locus using the Cre-lox integration system (258). The integrated alleles were amplified from genomic DNA and sequenced to confirm the mutations and to exclude the presence of additional mutations.

6.2.1. The *tau* mutation Hhp1.R180C

The *tau* mutation was the first circadian clock mutation which was identified in 1988 (*CKI ϵ .R178C*) by Ralph and Menaker (363). The mammalian *tau* mutation leads to the aberrant autophosphorylation of CK1 which may increase its catalytic activity.

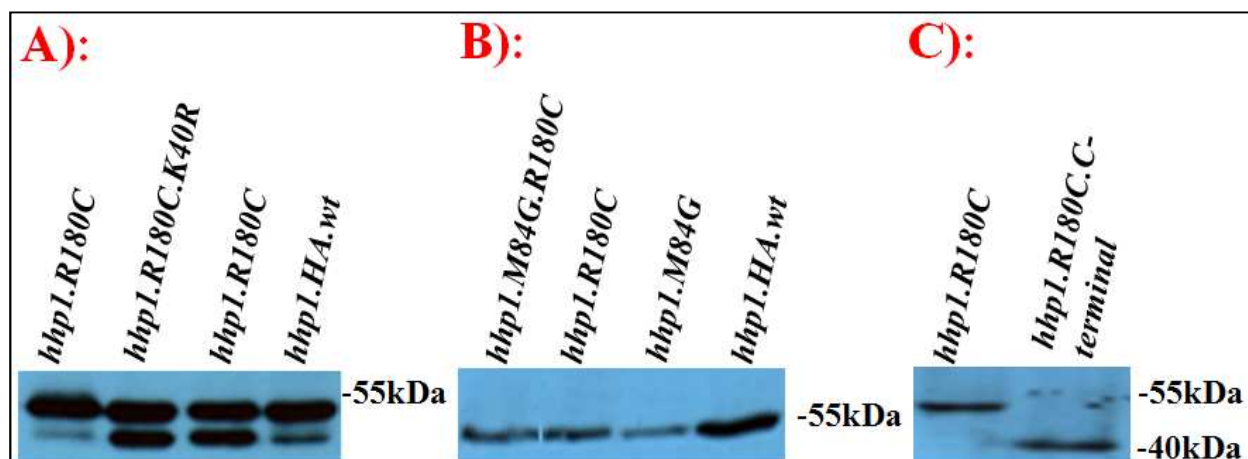


Figure: 6.2.1.1: Protein levels of the Hhp1 mutant proteins. Total protein was isolated, 15 μ l of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody. A): all three bands for *Hhp1.HA.wt*, *Hhp1.K40R*, and *Hhp1.K40R.R180C* run in same level and gave protein size around 55 kDa. B): *Hhp1.HA.wt*, *Hhp1.M84G*, and *Hhp1.M84G.R180C* expressed same proteins size level. C): *Hhp1.R180C.C-terminal.deletion* expressed a smaller sized protein of around 35 kDa

The introduction of a cysteine residue at position 180 (R180C) had no impact on the expression levels of Hhp1 (Figure: 6.2.1.1). Neither did the mutation affect cell survival in the presence of CPT or MMS (Figure: 6.2.1.2, Figure: 6.2.1.3).

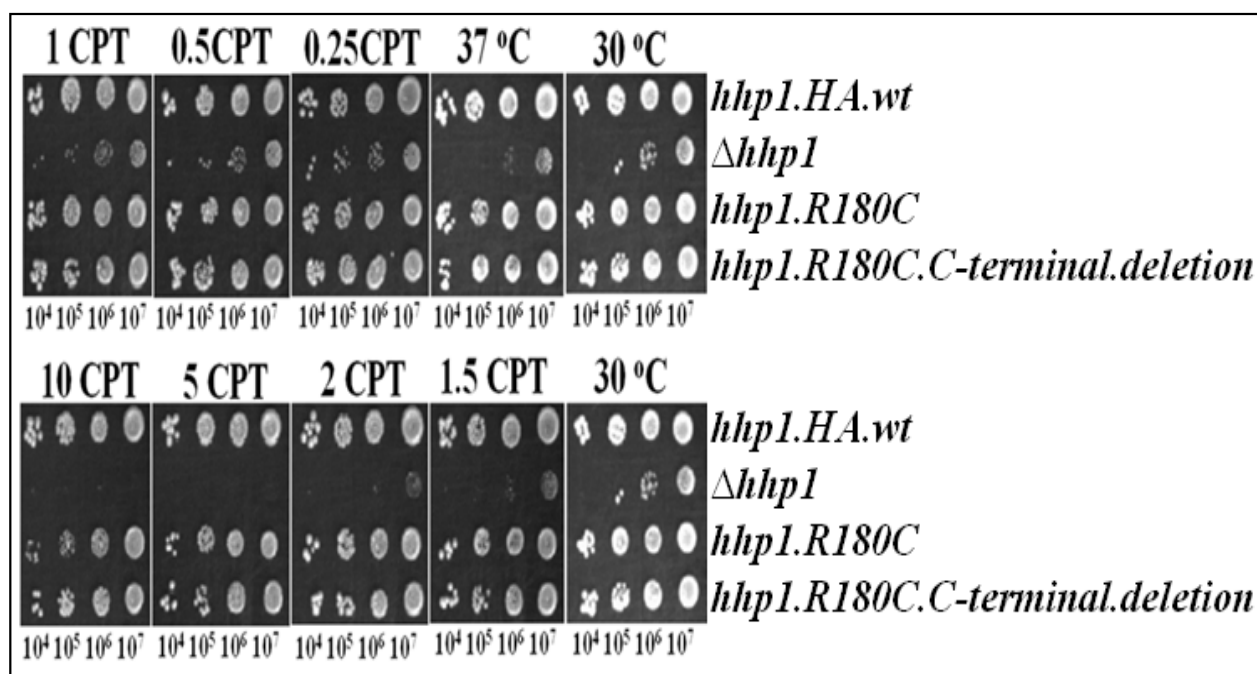


Figure: 6.2.1.2: CPT (camptothecin) drop test for the *tau* mutant and the C-terminal.deletion mutant. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

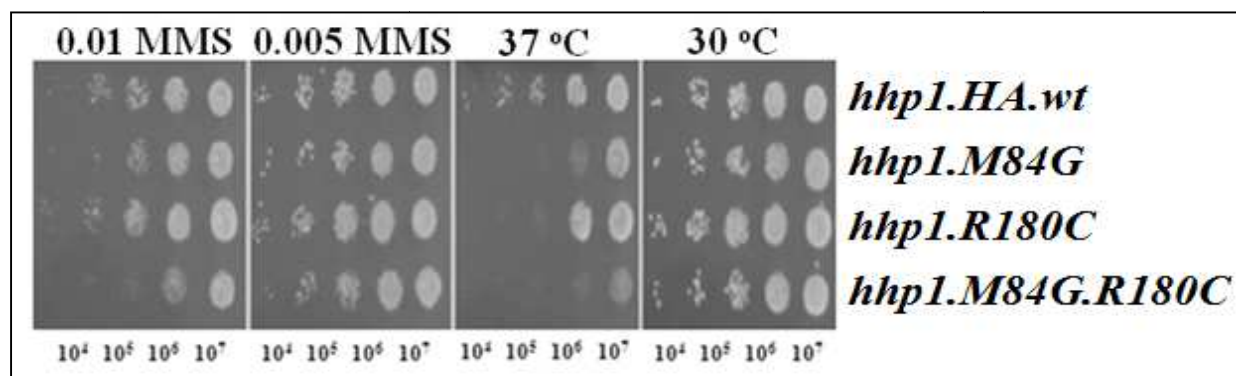


Figure: 6.2.1.3: MMS (methyl-methanesulfonate) drop test for the *tau* and *hhp1.M84G* mutants. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

What was however unexpected is the impact of the *tau* mutation on the duration of the G2 arrest in the presence of CPT. While wild type cells delay transition into G2 only briefly for 20-40 min (Figure: 6.2.1.4), the *hhp1.R180C-HA* mutant arrested for much longer (~140 min) and the cells returned slowly into the cell cycle (Figure: 6.2.1.5). This indicates that the *tau* mutation may be a separation of function mutation which affects the cell cycle regulation of Hhp1 but not the DNA damage sensitivity.

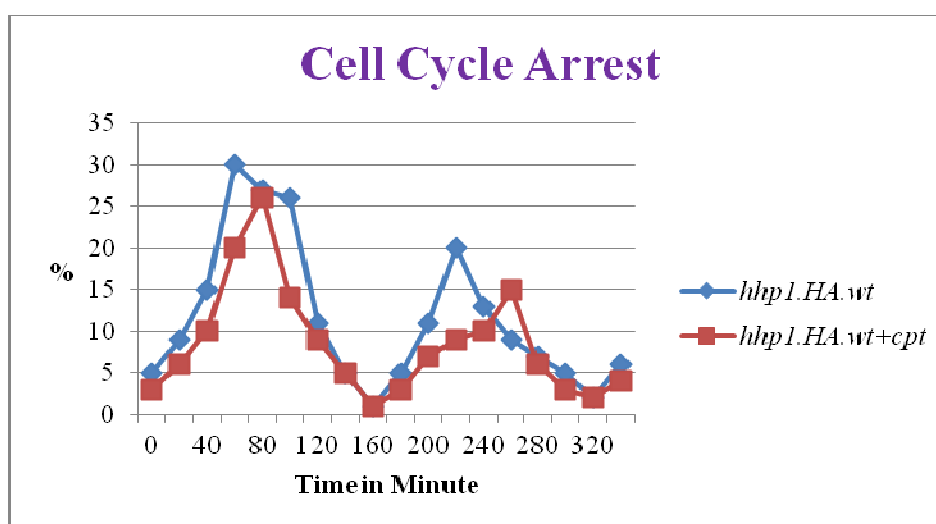


Figure: 6.2.1.4: CPT-induced (Camptothecin-induced) cell cycle arrest of *hhp1.HA.wild type* cells. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT), then cells incubated at 30°C shaker. 75 μ l samples were collected and kept in methanol over night. The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

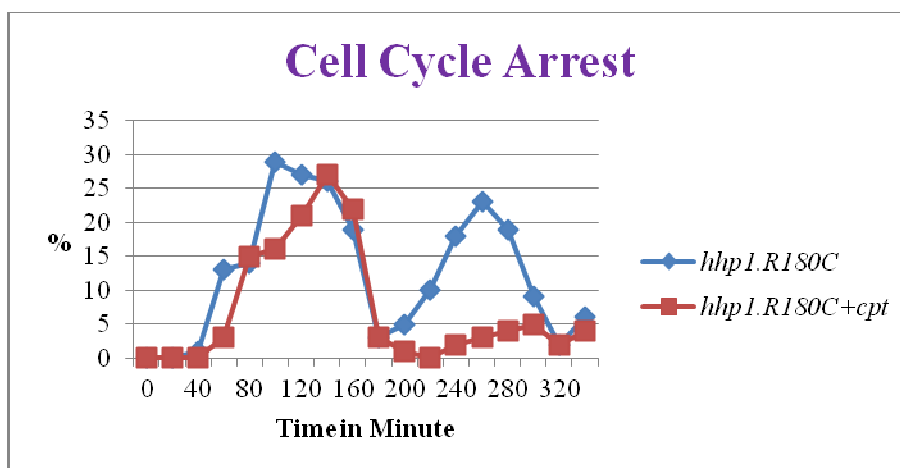


Figure: 6.2.1.5: CPT-induced (Camptothecin-induced) cell cycle arrest of *hhp1.R180C* cells. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M camptothecin (CPT), then cells incubated at 30 °C shaker. 75 μ l samples were collected and kept in methanol over night. The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

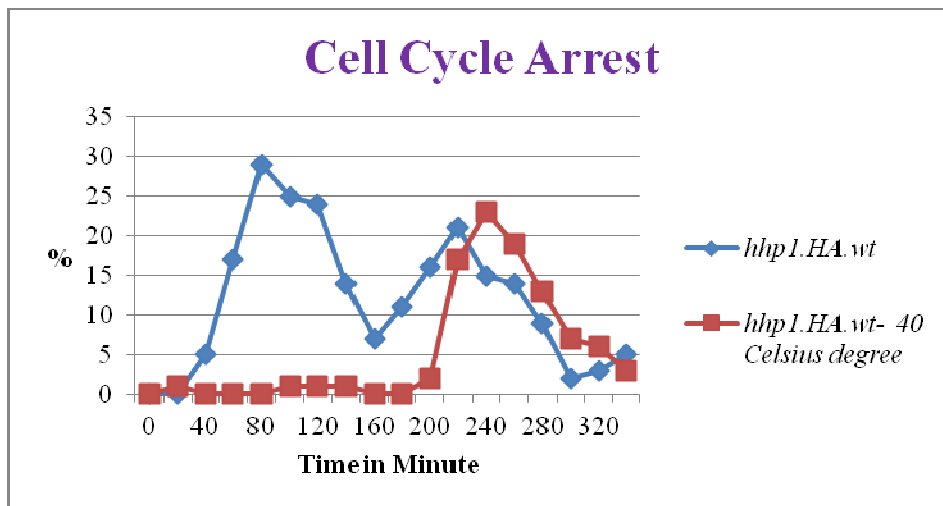


Figure: 6.2.1.6: Heat-induced cell cycle arrest of *hhp1.HA.wild type* cells. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with 30 °C or 40 °C treatment, then cells were incubated at 40 °C shaker. 75 μ l samples were collected and kept in methanol over night. The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

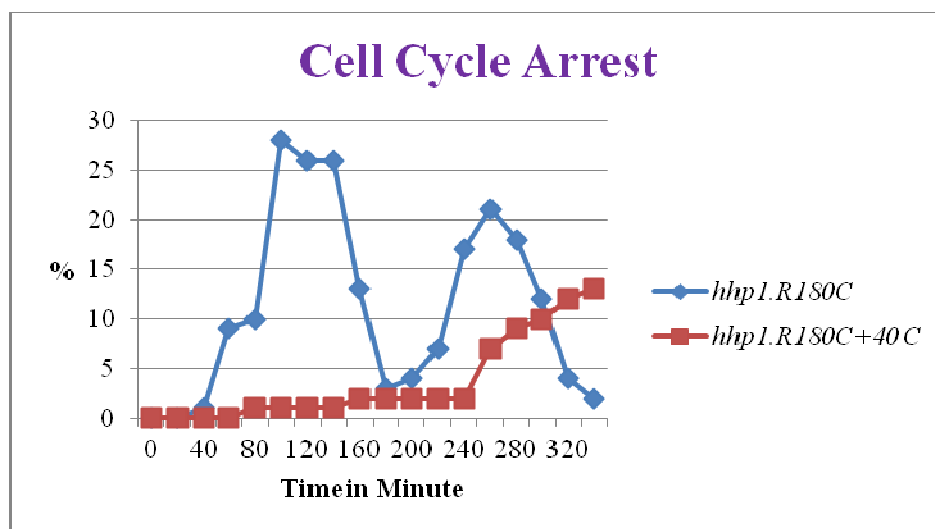


Figure: 6.2.1.7: Heat-induced cell cycle arrest of the *hhp1.R180C* mutant. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C or 40 °C. 75 μ l samples were collected and kept in methanol over night. The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

To test this idea, wild type cells (*hhp1-HA*) and the *hhp1.R180C-HA* mutant were synchronised in G2 and released into rich medium at 30°C or 40°C. Consistent with the earlier experiments (Figure: 6.2.1.6, Figure: 6.2.1.7), wild type cells arrested in the first G2 phase for up to 180 min (Figure: 6.2.1.6). The G2 delay of the *hhp1.R180C-HA* mutant was 60 min longer and cells exited the arrest more slowly (Figure: 6.2.1.7). This prolonged arrest supports the idea that the *tau* mutation affects mainly the cell cycle regulation by Hhp1 kinase.

Since the *tau* mutation results in the aberrant phosphorylation of the mammalian CK1 protein (548), total protein extracts were prepared from untreated wild type cells (*hhp1-HA*) and the *hhp1.R180C-HA* mutant and subjected to isoelectric focusing on a linear strip (pH3-10). As shown in Figure: 6.2.1.8, the wild type Hhp1 protein separates into four forms with different isoelectric points. While three forms (numbers 1-3 in Figure: 6.2.1.8) migrate in the alkaline area of the strip closer to the negative end, one form (number 4 in Figure: 6.2.1.8) is more negatively charged and migrates closer to the positive end of the strip. The *hhp1.R180C-HA* mutant produced also four forms but the position of form 4 moved away from the positive anode closer to the negative cathode (number 5 in Figure: 6.2.1.8). This implies that one form is less negatively charged as a result of the *tau* mutation. This was unexpected since the R180C mutation removes one positive charge from the surface of the protein (Figure: 6.2.1), which

should move all forms closer to the positively charged anode. As this was not the case, the *tau* mutation may affect the post-translational in a more complex manner. One conclusion which can be drawn from this experiment is that the change in form 4 correlates with the cell cycle defect of the R180C mutation. Which may identify form number 4 as being involved in cell cycle regulation.

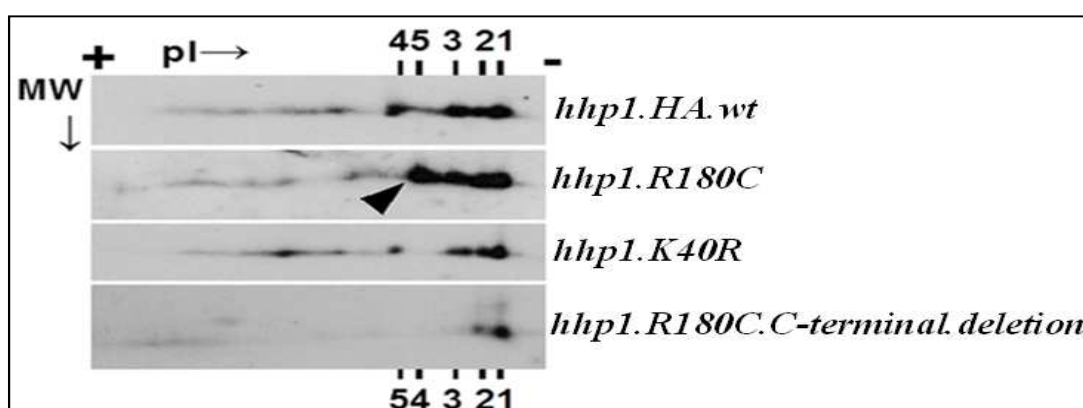


Figure: 6.2.1.8: Isoelectric focusing of Hhp1. Total protein extracts were prepared from the indicated strains without drug treatment. Aliquots were then loaded on a linear pH gradient strip (range 3-10) and separated by charge (isoelectric points). After the run, the strips were placed on a 10% SDS PAGE and the proteins were separated by size. The protein was visualised after the Western blot with the anti-HA antibody.

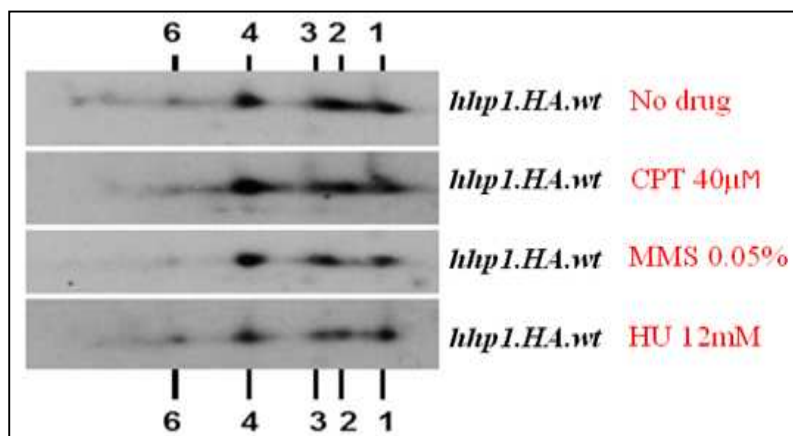


Figure: 6.2.1.9: Isoelectric focusing of wild type Hhp1. Total protein extracts were prepared from *hhp1-HA* wild type cells in the presence or absence of DNA damage. Cells were exposed to 40µM camptothecin (CPT) or 12mM hydroxyurea (HU) for 4 hours at 30°C. While CPT breaks DNA replication forks, HU only arrests forks. Cells were also exposed to 0.05% methyl-methanesulfonate (MMS) for 1 hour at 30°C. MMS causes single-stranded breaks by methylating adenine residues in the DNA.

To test whether the post-translational modification pattern changes in the response to DNA damage, *hhp1-HA* wild type cells were exposed to 40 μ M camptothecin (CPT) or 12mM hydroxyurea (HU) for 4 hours at 30°C. While CPT breaks DNA replication forks, HU only arrests forks. Cells were also exposed to 0.05% methyl-methanesulfonate (MMS) for 1 hour at 30°C. MMS causes single- stranded breaks by methylating adenine residues in the DNA. As shown in [Figure: 6.2.1.9](#) the overall pattern was very similar under all conditions which implies that Hhp1 does not undergo significant changes in its modification pattern in the response to DNA damage. In addition to the four main forms (numbers 1-4), one low abundant form which migrated closer to the anode was detected in all experiments (number 6 in [Figure 6.2.1.9](#)).

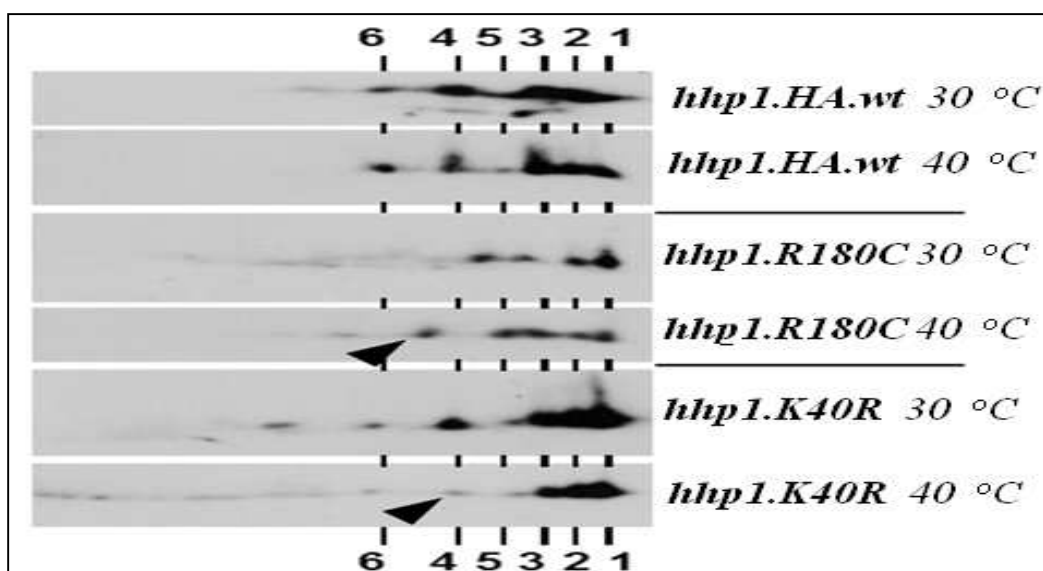


Figure: 6.2.1.10: Isoelectric focusing of Hhp1. Total protein extracts were prepared from the indicated strains either grown at 30°C or after having been exposed to 40°C for 1 hour. The arrow heads highlight temperature induced changes in the post-translational modification pattern.

It was also tested whether the pattern changes under heat stress conditions. As shown in [Figure: 6.2.1.10](#), there was no change in the case of the wild type (*hhp1-HA*). There was however a small change in the case of the R180C mutant. Form number 6 was not very abundant at 30°C but increased in its amount at 40°C at a position slightly closer to the negative cathode. A kinase-dead (*hhp1.K40R-HA*) strain was also tested. While the pattern of signals resembled WT at 30°C, the intensity of form number 4 strongly declined at 40°C ([Figure: 6.2.1.10](#)). It is also noteworthy that the kinase-dead protein shares a very similar post-translational modification pattern with the wild type protein ([Figures: 6.2.1.8, Figure: 6.2.1.10](#)). This implies that the modifications are not caused by auto-phosphorylation of the kinase or that the K40R mutation still enables the kinase

to under-go autophosphorylation. The latter is less likely as the kinase-dead strain resembles the *hhp1* gene deletion.

Since mammalian Hhp1 kinase undergoes autophosphorylation in its C-terminal domain (1), most of this domain (298aa to 356aa) was deleted in frame (Figure: 6.2.1.11) in the *hhp1.R180C-HA* mutant. The last 9 amino acids were left in place as they are highly conserved.

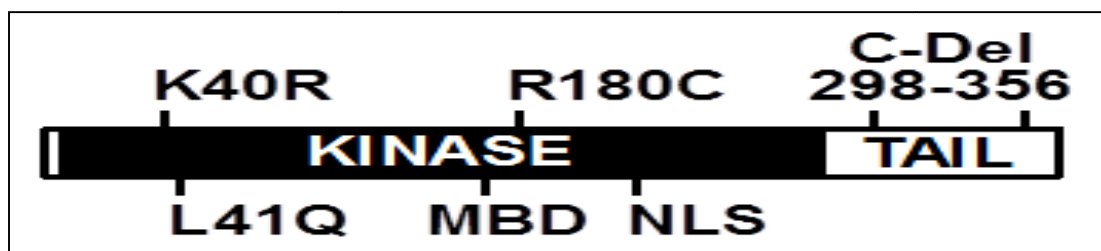


Figure: 6.2.1.11: Domain organisation of *S.pombe* Hhp1. The kinase domain and the C-terminal tail domain are highlighted. The in-frame deletion of the tail domain (298aa to 356aa) is shown.

As shown in Figure: 6.2.1.8, the truncated protein (*hhp1.R180C. Δ C-HA*) migrated at a smaller molecular weight compared to the wild type protein. Very unexpectedly, loss of this large section of the C-terminal domain had no impact on the DNA damage sensitivity (Figure: 6.2.1.2, Figure: 6.2.1.12). It had also only a small impact on the CPT-induced cell cycle arrest as the delay was only slightly longer compared to wild type (Figure: 6.2.1.13).

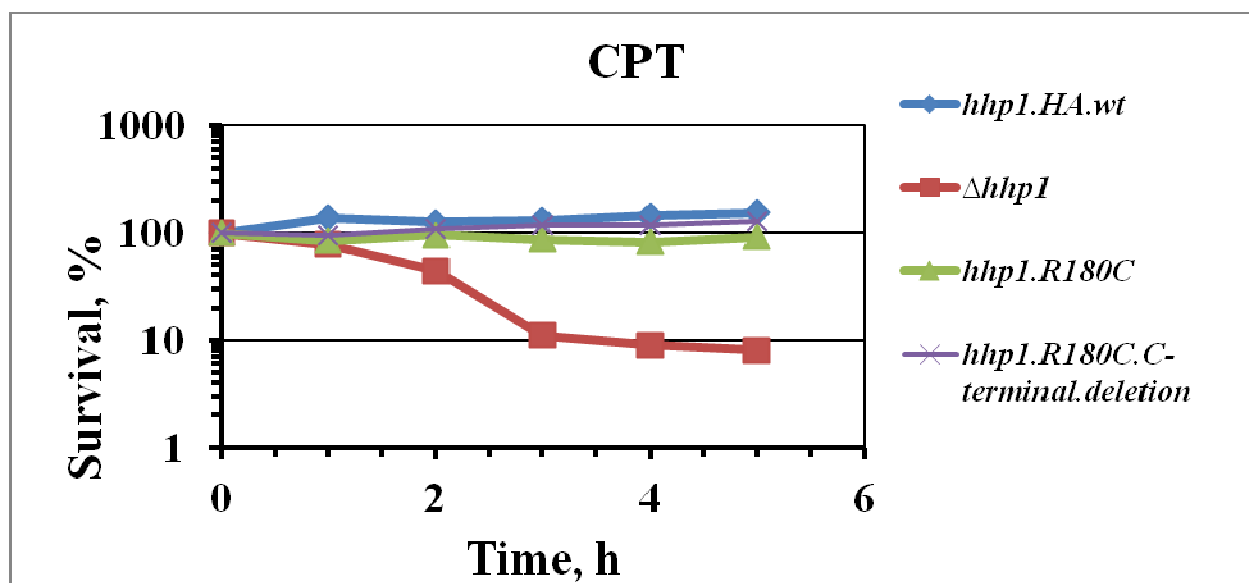


Figure: 6.2.1.12: Acute CPT (camptothecin) survival assay for *tau* mutant and the C-terminal.deletion mutant. The indicated *S.pombe* strains cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40 μ M CPT for five hours period at 30 °C. 75 μ l samples were collected every hour for the duration of five hours.

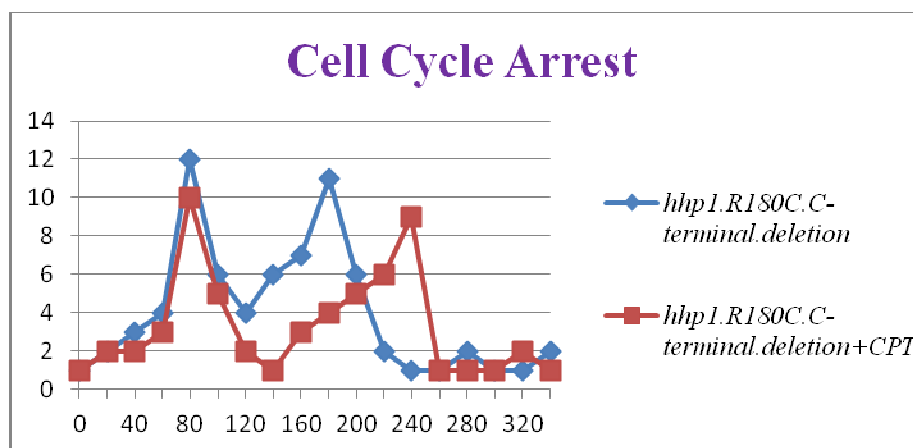


Figure: 6.2.1.13: CPT-induced (Camptothecin-induced) cell cycle arrest for the *hhp1.R180C.C-terminal.deletion* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30°C. 75 μ l samples were collected and kept in methanol over night. The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

The truncated Hhp1 protein was also subjected to isoelectric focusing. As shown in [Figure: 6.2.1.8](#), the loss of the C-terminal domain resulted in the disappearance of the two, more negative forms of Hhp1 as the *hhp1.R180C. Δ C-HA* strain produced only two forms. This implies that the two more negative forms require post-translational modifications within the deleted C-terminal domain. The lack of CPT sensitivity and the small impact on the CPT-induced cell cycle delay are also consistent with the earlier statement that autophosphorylation does not play an important role for *S.pombe* Hhp1.

Given the genetic linkage between Hhp1 and Chk1 ([Chapter 3](#)), the *chk1* gene was deleted in the *hhp1.R180C-HA* strain. Loss of the checkpoint kinase had no impact on the MMS sensitivity of the Δ *chk1 hhp1.R180C. Δ C-HA* strain ([Figure: 6.2.1.14](#)) but increased slightly the CPT sensitivity ([Figure: 6.2.1.15](#)). This increase suggests that Chk1 and Hhp1.R180C act in parallel pathways when DNA replication forks break in the presence of the topoisomerase 1 inhibitor. This could be explained if Hhp1.R180C affects mainly the cell cycle response whereas Chk1 affects the repair of broken forks by the Mus81-Eme1 endonuclease (see [Chapter 3](#), and [Chapter 5](#)).

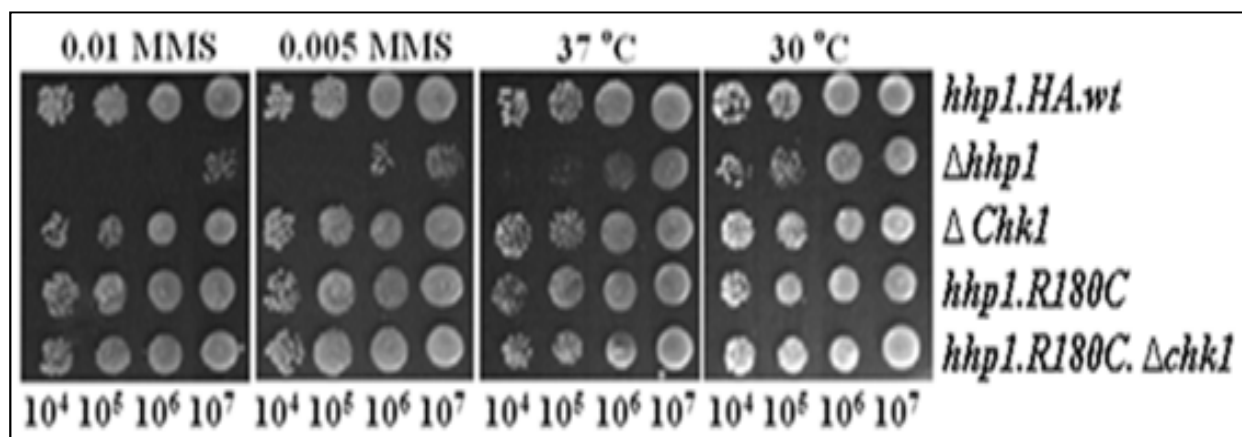


Figure: 6.2.1.14: MMS (methyl-methanesulfonate) drop test for the *hhp1.R180C.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

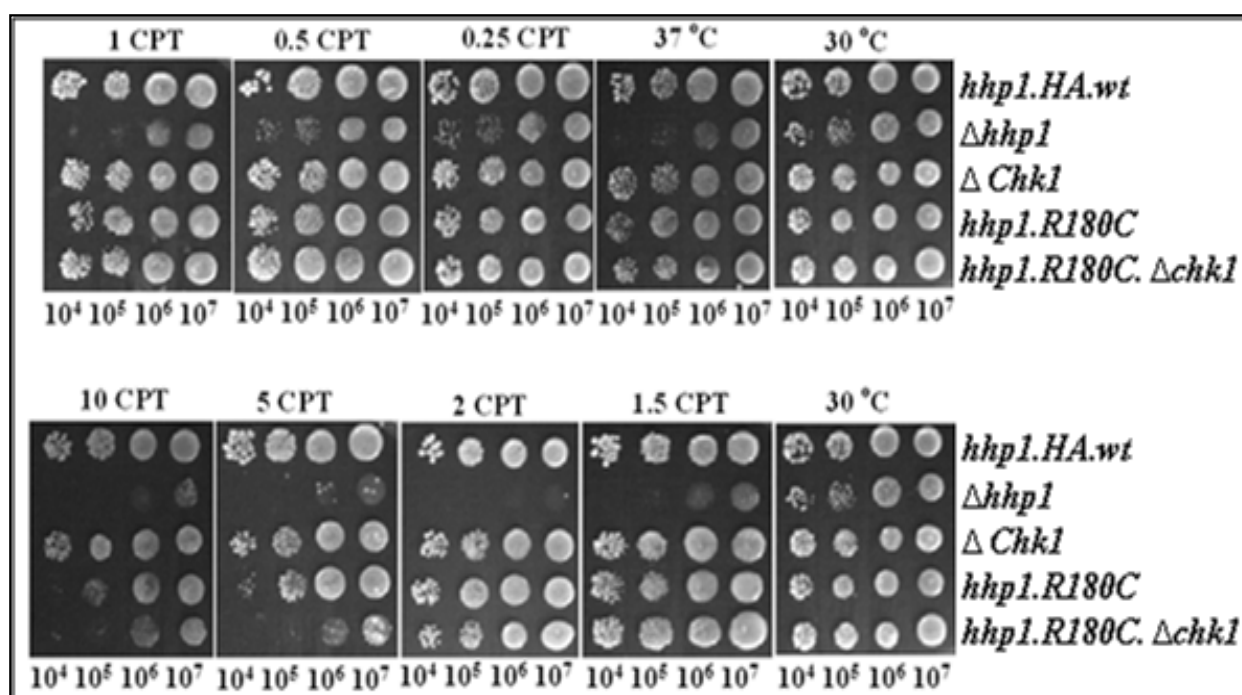


Figure: 6.2.1.15: CPT (camptothecin) drop test for the *hhp1.R180C.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C. sensitivity CPT pattern higher than Hhp1.R180C strain as a result of deleting *Chk1* kinase from Hhp1.R180C cells.

6.2.2. The Hhp1.M82I double-time long Mutant

The *S. pombe* *Hhp1.M82I* mutant is homologous to *dbt^L.M80I* (double-time long M80I) in *Drosophila* (142). The *dbt^L* (M80I) mutation alters the oscillation pattern of the PERIOD

proteins as it may reduce the kinase activity (549) which leads to a delayed degradation of the PERIOD proteins (550).

The equivalent mutation in *S.pombe* Hhp1 (M82I) had neither an impact on the temperature sensitivity nor on the growth of cells in the presence of MMS (Figure: 6.2.2.1). The *hhp1.M82I-HA* strain displayed however a low sensitivity to high doses of CPT (Figure: 6.2.2.2) and the protein level of the mutant may be reduced (Figure: 6.2.2.3). What was however interesting is the observation that the second, faster migrating Hhp1 band may be absent in the mutant strain. A change in the post-translational modification pattern by the M82I mutation within the ATP binding domain (Figure: 6.2.1) is supported by the aberrant isoelectric focusing pattern (Figure: 6.2.2.4). While wild type cells (*hhp1.HA*) contain the four forms of the kinase, *hhp1.M82I-HA* cells appear to express more forms which are all low abundant. Some forms appear to be more negative which indicates a higher degree of phosphorylation. Since the equivalent mutation in *Drosophila* lowers the kinase activity, the break up in more forms with diverse post-translational modifications may have a negative impact on the kinase. If this were to be case, the M82I mutant should have an extended G2 arrest in the presence of CPT as the reduction of Hhp1 kinase activity, for example upon the deletion of the gene, extends the normally short delay (Figure: 3.1.5B). Indeed this was the case, as shown in Figure: 6.2.2.5, *hhp1.M82I-HA* cells delay significantly longer than wild type cells when DNA replication forks break. This interesting finding strongly suggests that the response to DNA damage and the cell cycle regulation by Hhp1 require either different forms of the kinase or different levels of kinase activity. While a reduction in kinase activity has only a minor impact on the survival in the presence of MMS or CPT, the cell cycle delay in the presence of CPT is significantly longer.

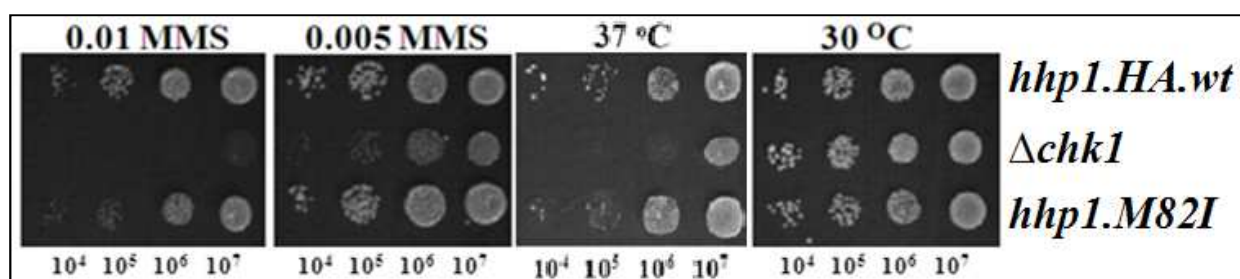


Figure: 6.2.2.1: MMS (methyl-methanesulfonate) drop test for *hhp1.M82I* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

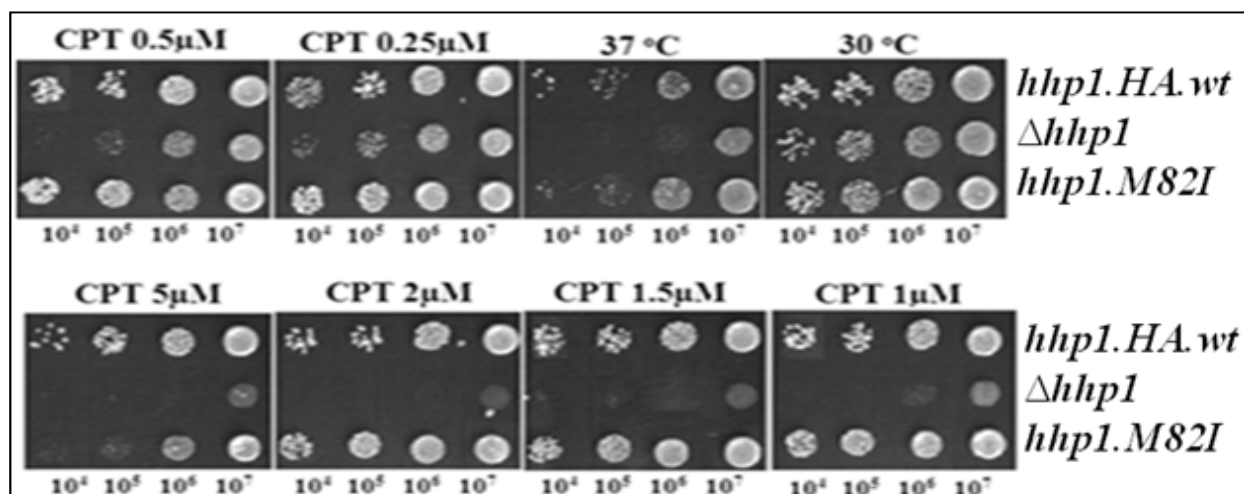


Figure: 6.2.2.2: CPT (camptothecin) drop test for *hhp1.M82I* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

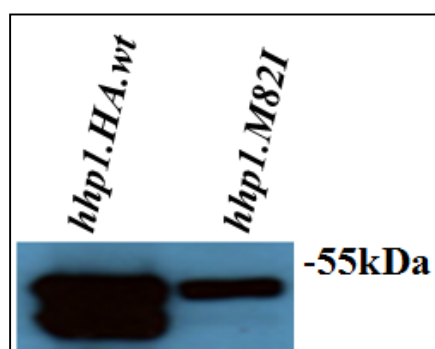


Figure: 6.2.2.3: Western blot for *hhp1.M82I*. Total protein was isolated, 15μl of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody.

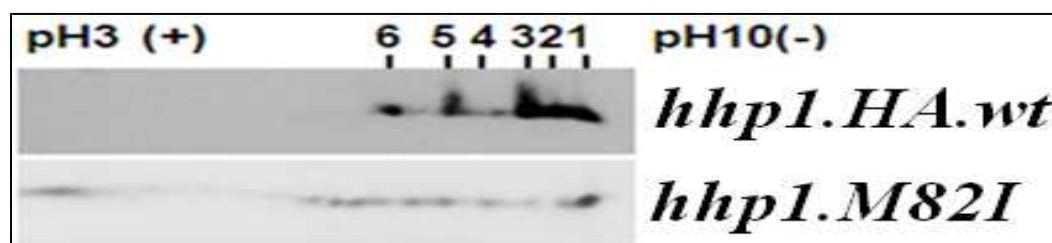


Figure: 6.2.2.4: Isoelectric focusing of the Hhp1.M82I protein. Total protein extracts were prepared from *hhp1-HA* wild type cells and *hhp1.M82I* mutant cells, and separated by charge on a linear 3-10 stop prior to running the samples on a 10% SDS page. Hhp1-HA was visualised with an anti-HA antibody.

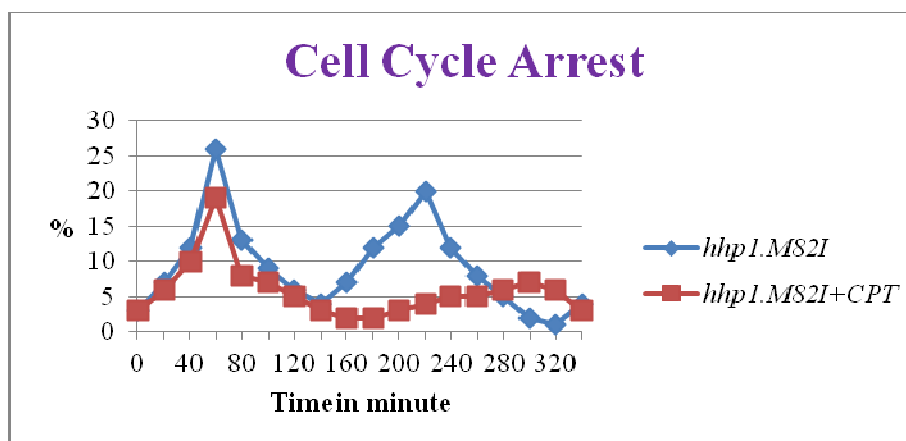


Figure: 6.2.2.5: CPT-induced (Camptothecin-induced) cell cycle arrest of the *Hhp1.M82I* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT, then cells incubated at 30 $^{\circ}$ C shaker. Samples were collected and cells were fixed in methanol over night, and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

6.2.3. The *Hhp1.P49S* double-time short Mutation:

The *dbt^s.P47S* (double-time short P47S) mutation in *Drosophila* has only a very minor negative impact on the kinase activity and does not change the degradation pattern of the PERIOD proteins (142, 550). In cell culture experiments, the PERIOD proteins become hyperphosphorylated in this mutant background (549). Like the double-time long mutation (M82I), the double-time short mutation (P49S) in *S.pombe* *Hhp1* (*hhp1.P49S-HA*) reduces the sensitivity to MMS and CPT only very slightly (Figure: 6.2.3.1, Figure: 6.2.3.2).

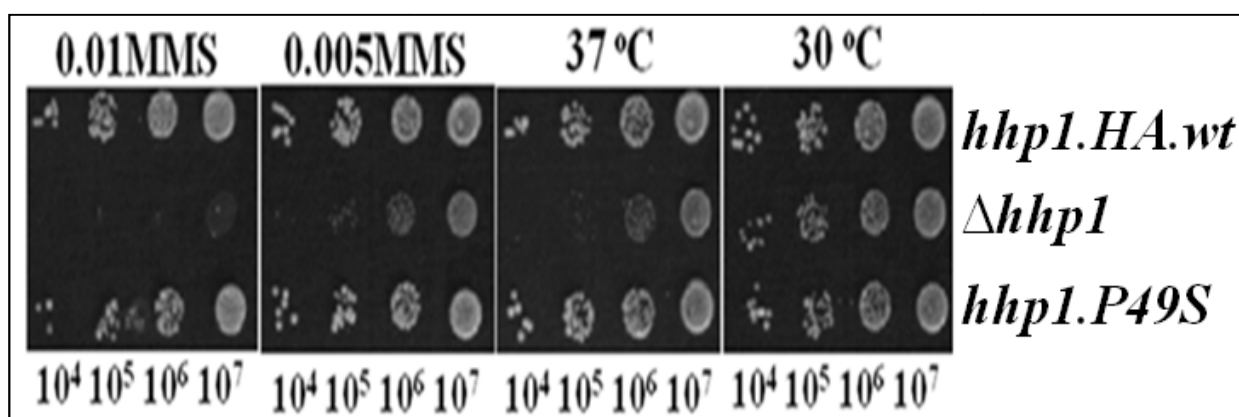


Figure: 6.2.3.1: MMS (methyl-methanesulfonate) drop test for the *hhp1.P49S* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30 $^{\circ}$ C. One plate was incubated at 37 $^{\circ}$ C.

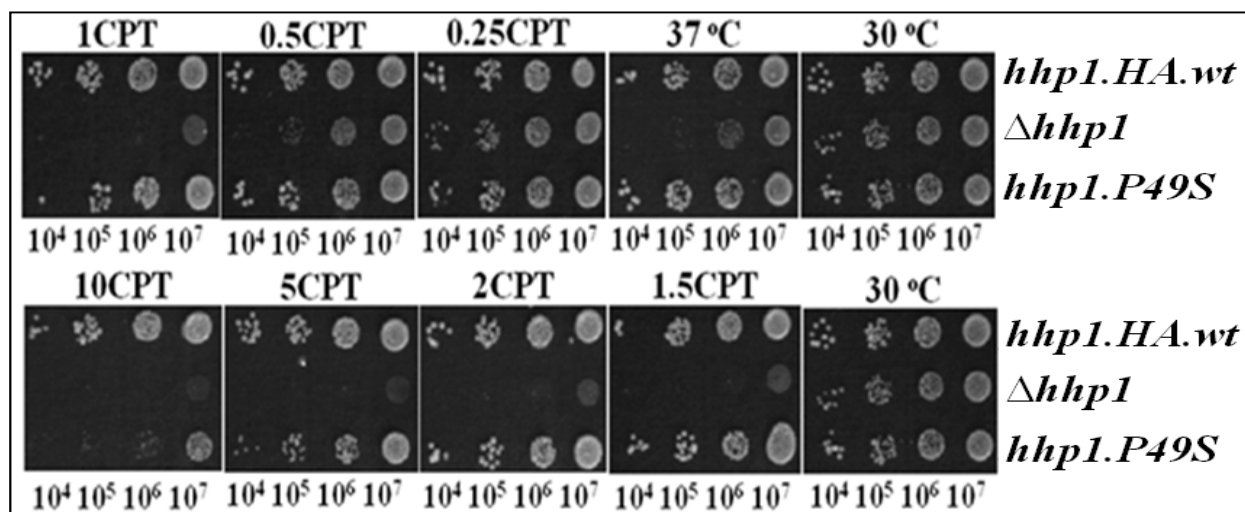


Figure: 6.2.3.2: CPT (camptothecin) drop test for the *hhp1.P49S* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

The isoelectric focusing assay revealed however a striking difference between both mutants. While the M82I (*dbt-L*) increased the number of Hhp1 forms, the P49S (*dbt-S*) decreased the number of forms with a clear reduction in the more negative variants (Figure: 6.2.3.4). Interestingly, the impact of this change on the CPT-induced G2 delay were similar to the extended delay caused by the M82I mutation (Figure: 6.2.3.5), although the *hhp1.P49S-HA* cells re-entered the cell cycle faster after 100 min. In summary, these observations strengthen the conclusion that a reduction in Hhp1 activity levels affects the recovery from a CPT-induced G2 arrest, but has only a small impact on DNA repair and cell survival (Figure: 6.2.3.6). The two double-time mutations reduce both CK1 activity to a different extend and the behaviour changes in the mutant flies may be linked with the distinct degradation pattern of the PERIOD proteins.

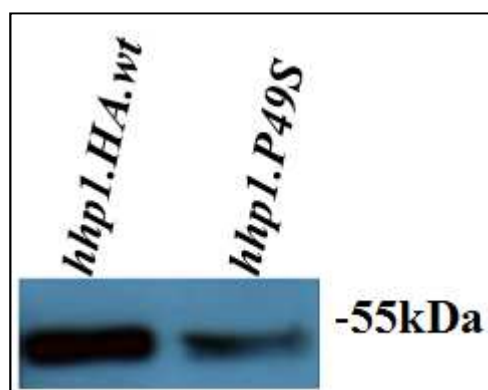


Figure: 6.2.3.3: Western blot for *hhp1.P49S*. Protein was isolated, 15 μ l of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody. *hhp1.P49S* mutant proteins sizes as shown by electrophoresis full-length of *hhp1* proteins.

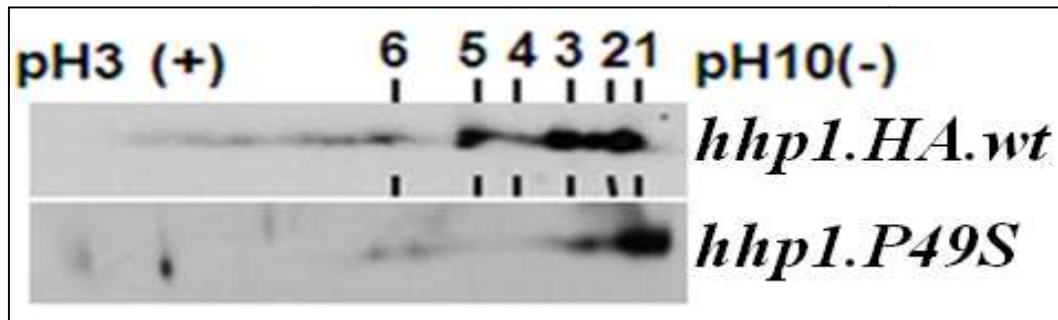


Figure: 6.2.3.4: Isoelectric focusing of the Hhp1.P49S protein. Total protein extracts were prepared from *hhp1-HA* wild type cells and *hhp1.P49S* mutant cells, and separated by charge on a linear 3-10 stop prior to running the samples on a 10% SDS page. Hhp1-HA was visualised with an anti-HA antibody.

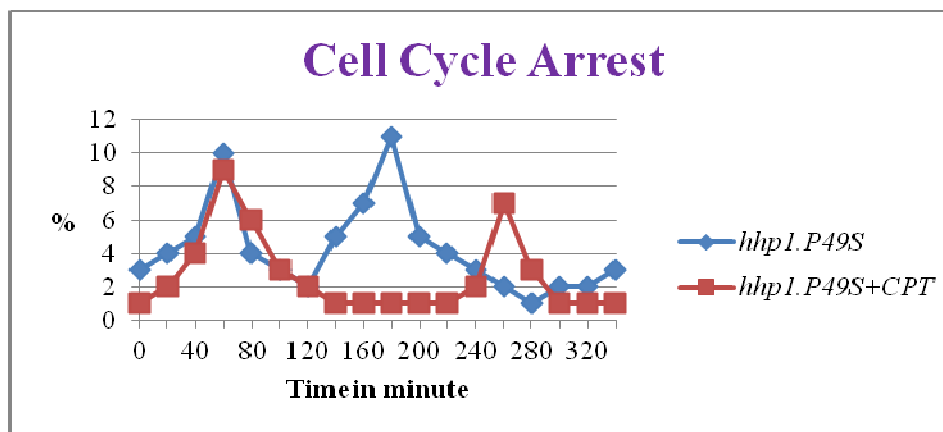


Figure: 6.2.3.5: CPT-induced (Camptothecin-induced) cell cycle arrest of the *hhp1.P49S* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT. Cells samples were collected and fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

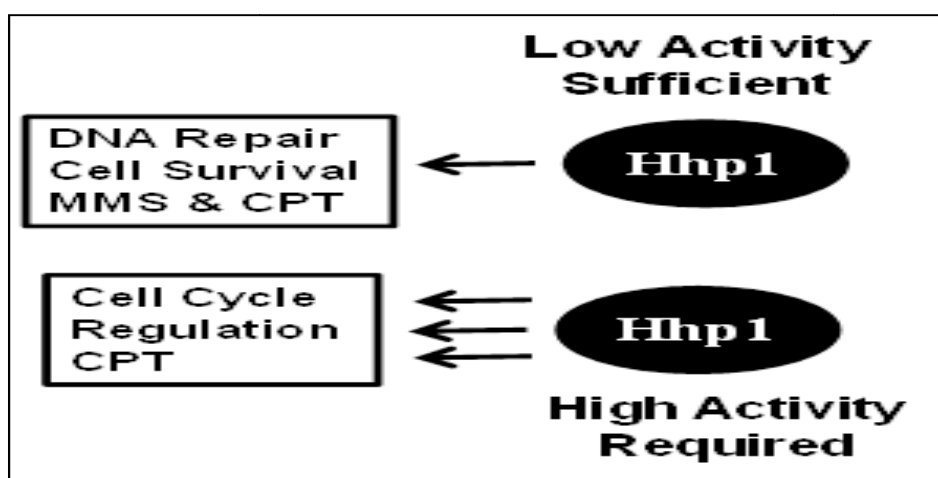


Figure: 6.2.3.6: Different activity levels of the kinase maybe required for DNA repair and cell cycle regulation. A reduction in kinase activity by the circadian mutations delays re-entry into the cell cycle without affecting cell viability in the presence of DNA damage.

6.3. The Relationship between Hhp1 kinase and Timeless (Swi1)

The TIMELESS (TIM) protein acts jointly with the PERIOD proteins in the negative feedback loop which controls the activating of the CLOCK-BMAL1 transcription factor. The degradation of TIM in the early morning promotes the progressive phosphorylation of the PERIOD proteins by CK1 and their degradation (551). Independent of its functional link with the PERIOD proteins, TIM is also important for the progression through DNA replication and the activity of the intra-S ATR-Chk1 DNA damage checkpoint (111, 552). The *S.pombe* protein which may be the paralogue of TIM is Swi1. Swi1 binds to chromatin and facilitates the recruitment of the S phase checkpoint protein Mrc1 (516). Swi1 acts in a DNA replication pathway jointly with Hsk1 (Cdc7) kinase in the response to stalled replication forks (535), and may regulate the recombinational repair of broken forks (553).

To test the relationship between Hhp1 and Swi1 a $\Delta hhp1 \Delta swi1$ double mutant was constructed. As shown in Figure: 6.3.1, the double mutant was as CPT sensitive as the $\Delta hhp1$ single mutant suggesting that Swi1 (TIM) and Hhp1 act in the same pathway when DNA replication forks break. Since the strains are very sensitive to CPT, the test was repeated under acute survival conditions. When cells were exposed to 40 μ M CPT for 5 hours, the $\Delta hhp1 \Delta swi1$ double mutant (Figure: 6.3.2) was more sensitive than the two single deletion strains. This shows that Swi1 and Hhp1 act in two parallel pathways when DNA replication forks break. Cells without Swi1 ($\Delta swi1$) delay progression through G2 briefly for 40 min (Figure: 6.3.3), while the $\Delta hhp1 \Delta swi1$ double mutant displayed a longer delay of approximately 80 min (Figure: 6.3.4) which resembles the extended G2 arrest of the $\Delta hhp1$ single mutant (Figure: 3.1.5B). Taken together, these data do not support a close link between Swi1 and Hhp1 when DNA replication forks break in the presence of CPT.

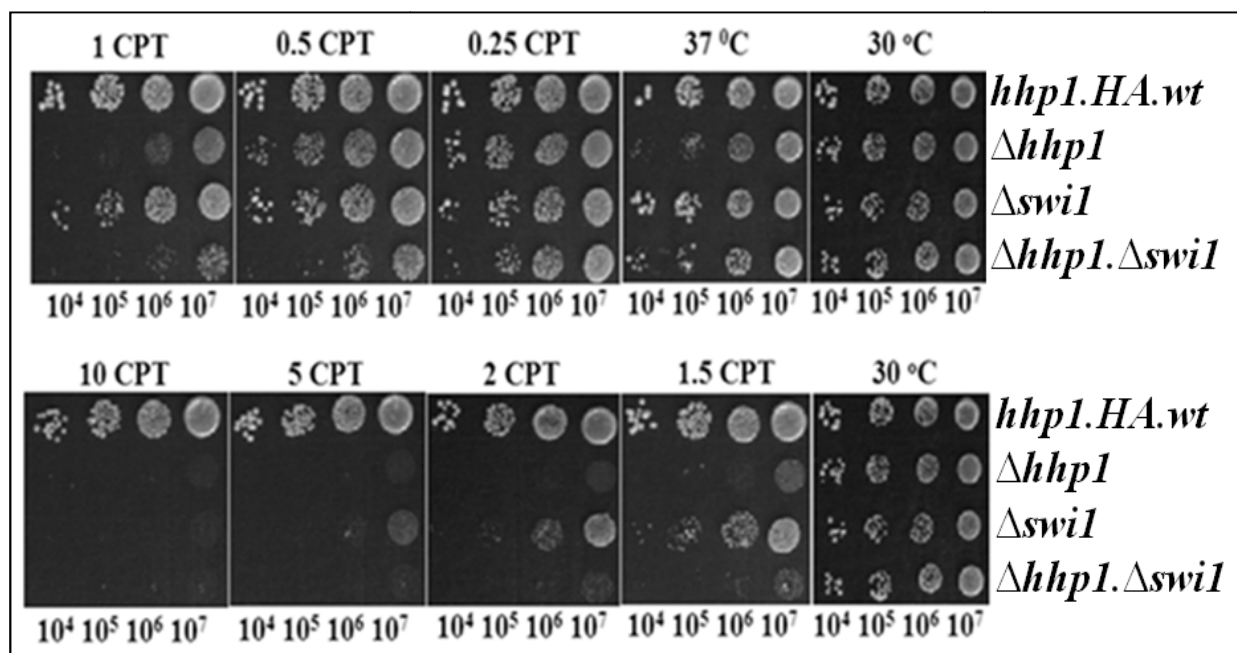


Figure: 6.3.1: CPT (camptothecin) drop test for $\Delta hhp1\Delta swi1$ strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

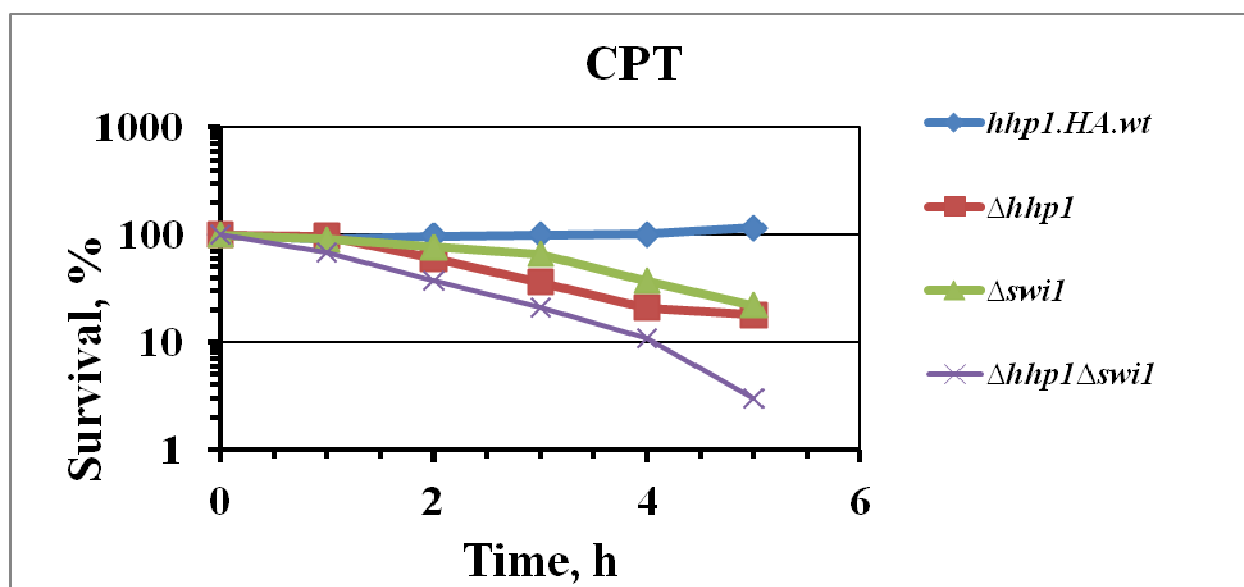


Figure: 6.3.2: Acute CPT (camptothecin) survival assay for the $\Delta hhp1\Delta swi1$ strain. The indicated *S.pombe* strains were cultured in YEA medium overnight at 30°C. Cells were harvested and treated with 40μM CPT and incubated again for five hours at 30°C. 75 μl samples were collected every hour for the duration of five hours.

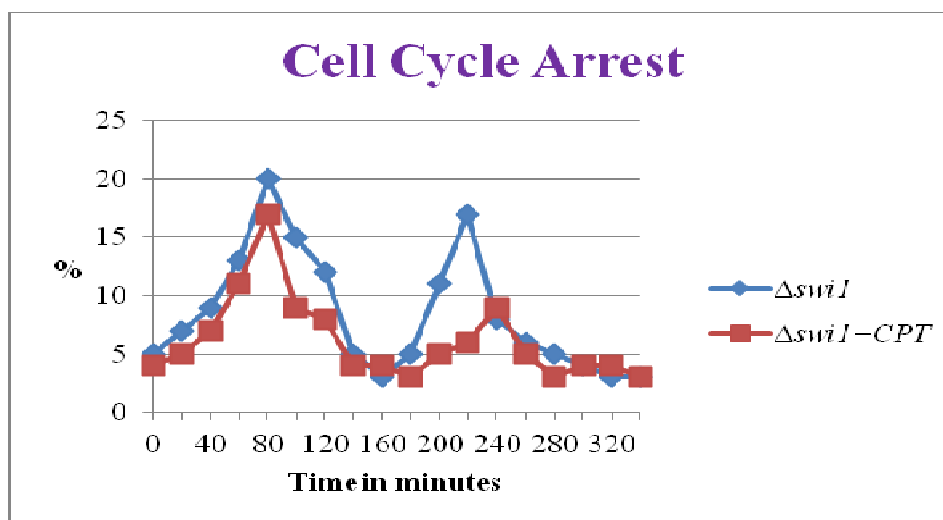


Figure: 6.3.3: CPT-induced (Camptothecin-induced) cell cycle arrest of the $\Delta swi1$ single mutant. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 °C. Samples were collected and cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

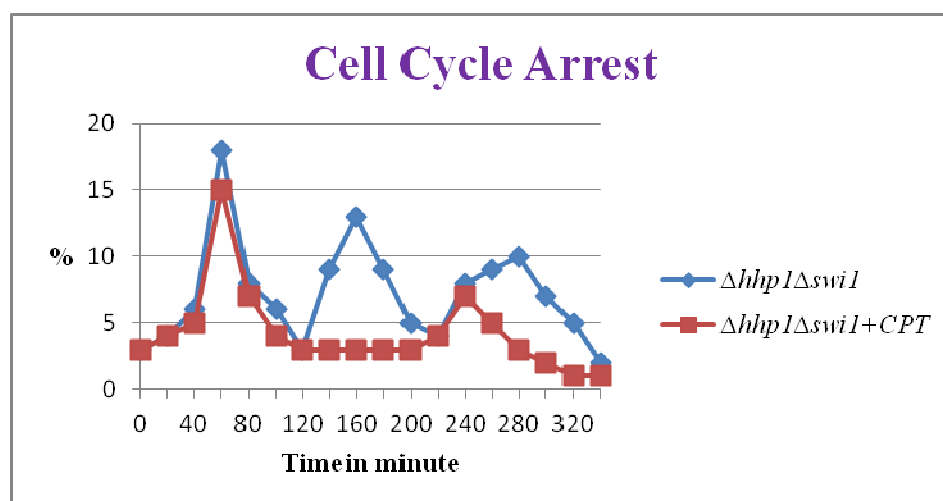


Figure: 6.3.4: CPT-induced (Camptothecin-induced) cell cycle arrest of the $\Delta hhp1\Delta swi1$ double mutant. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40 μ M CPT at 30 oC. Samples were collected and cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum).

Given that the circadian clock directly regulates the expression of Wee1 kinase (375), the link between Hhp1, its circadian mutations and Wee1 was explored. In *S.pombe*, cells without Wee1 kinase ($\Delta wee1$) are alive but DNA damage sensitive (224) (Figure: 6.3.5). This DNA damage sensitivity is not shared by the gain-of-function *cdc2.1w* (G146D) mutation in Cdc2 which is insensitive to the inhibition by Wee1 via its tyrosine 15 phosphorylation (272). Since both strains ($\Delta wee1$ and *cdc2.1w*) enter mitosis earlier than wild type (220), the DNA damage sensitivity of Wee1 is probably unrelated to the cell cycle defect. Indeed recent results indicate that the sensitivity is linked with the accumulation of unphosphorylated Cdc2 in $\Delta wee1$ cells (554). The unphosphorylated pool of Cdc2 associates with the DNA damage checkpoint kinase Chk1 and may directly affect the response to DNA damage. As shown in Figure: 6.3.5, $\Delta wee1$ cells are sensitive to a broad range of drugs whereas the *cdc2.1w* mutant is mainly sensitive to CPT. Cells without Wee1 are also temperature sensitive and resemble in this respect the $\Delta hhp1$ strain (Figure: 4.2.7).

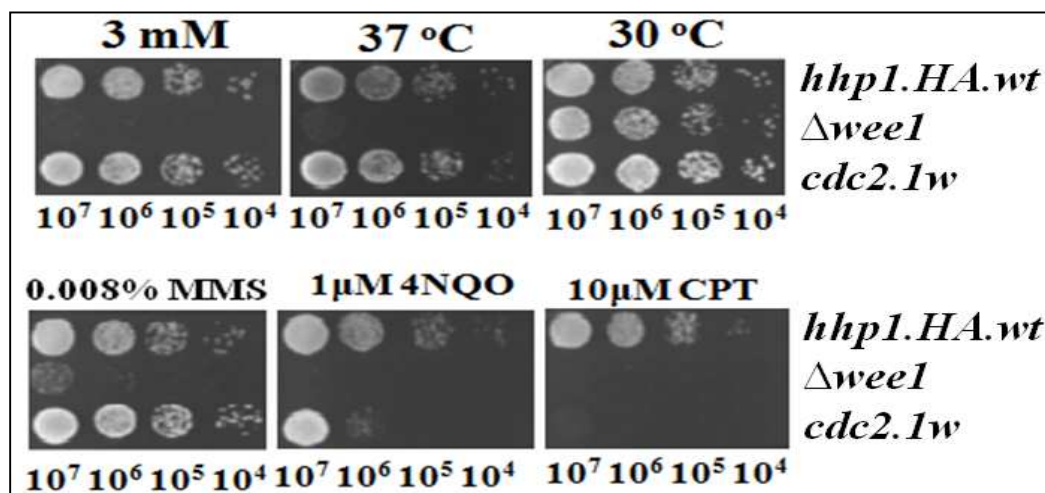


Figure: 6.3.5: DNA damage sensitivity profile of cells without Wee1 or with a hyper-active Cdc2 kinase (insensitive to Wee1 inhibition). Serial dilutions of wild type (WT), $\Delta wee1$ and *cdc2.1w* (G146D) cells were dropped on rich medium plates containing the indicated drug [MMS (Methyl-methanesulfonate), CPT (Camptothecin), HU (Hydroxyurea), and NQO (4-nitroquinoline 1-oxide)] concentrations and incubated at 30°C for 3 days. One plate without a drug was incubated at 37°C.

To test the relationship between the *hhp1* deletion and the *wee1-50* loss-of-function, and the gain-of-function *cdc2.1w* mutation, $\Delta hhp1 wee1-50$ and $\Delta hhp1 cdc2.1w$ strains were constructed. The loss-of-function *wee1-50* allele was used as several attempts to construct a $\Delta hhp1 \Delta wee1$ strain failed. As shown in Figure: 6.3.6, an increase in Cdc2 kinase activity by either lowering the inhibitory Wee1 kinase or by introducing the G146D mutation in *cdc2*, partially reduces the

CPT sensitivity of the *hhp1* deletion. This places Hhp1 kinase in the same DNA damage response pathway to broken replication forks as Wee1 and Cdc2.1w.

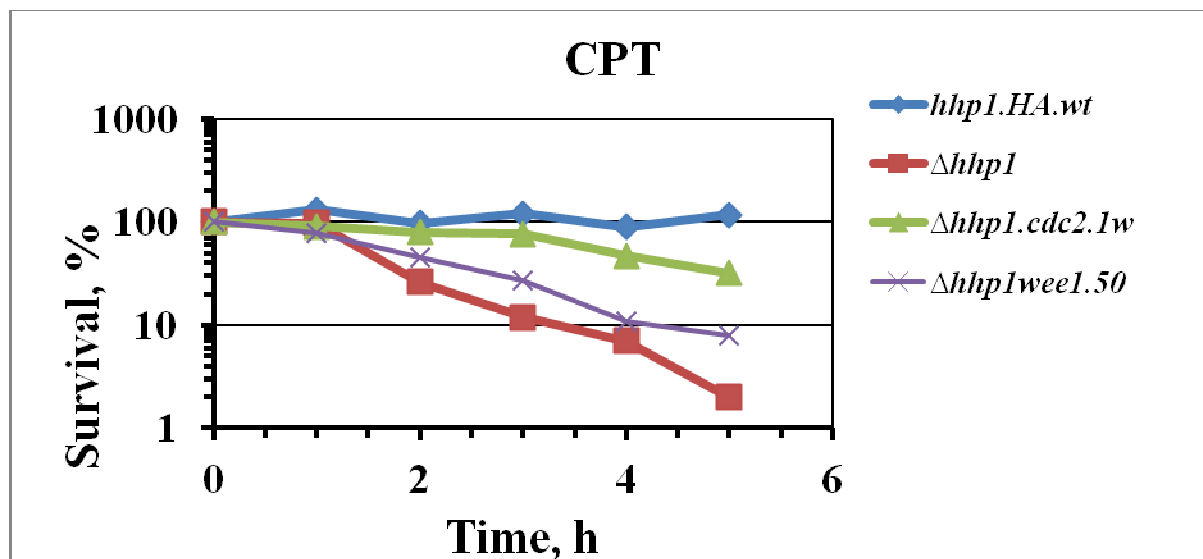


Figure: 6.3.6: A drop in Cdc2 kinase activity suppresses the CPT (camptothecin) sensitivity of *hhp1* deletion cells. The indicated *S.pombe* strains were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40μM CPT and incubated again for five hours at 30 °C. 75 μl samples were collected every hour for duration of five hours.

Consistent with this conclusion, the *hhp1.R180C-HA* strain (*tau* mutation) is as CPT and MMS sensitive as the *wee1* deletion (Δ *wee1 hhp1.R180C-HA) (Figure: 6.3.7, Figure: 6.3.8).*

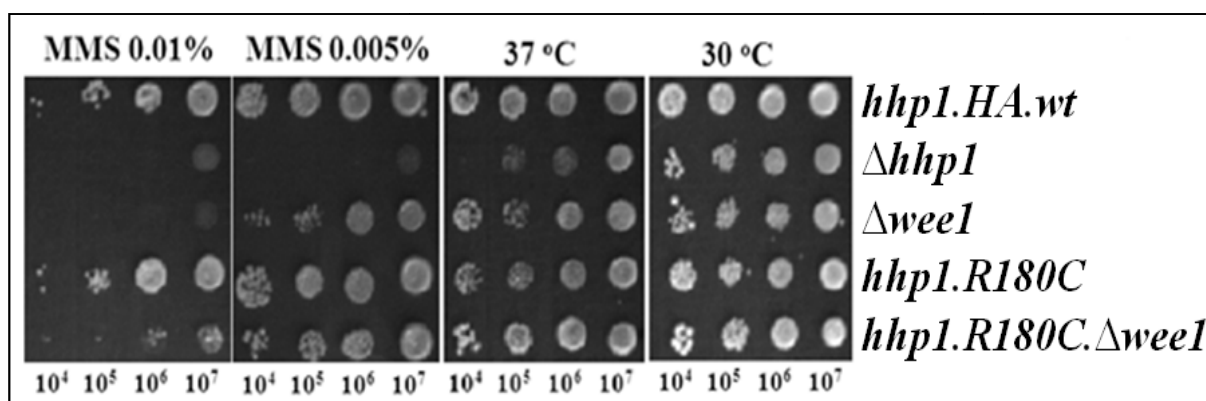


Figure: 6.3.7: MMS (methyl-methanesulfonate) drop test for the *hhp1.R180C.Δwee1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

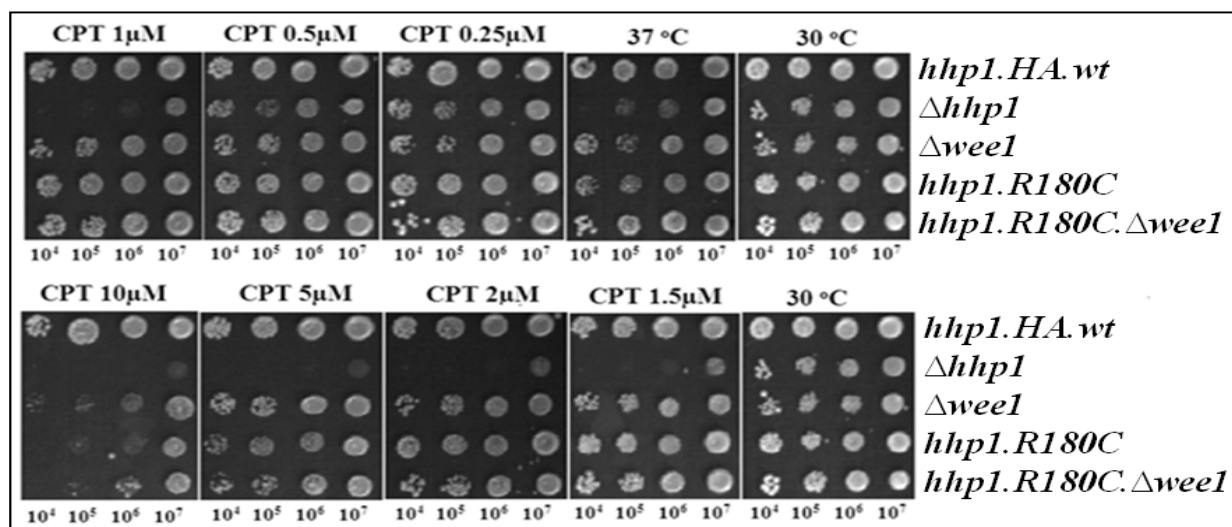


Figure: 6.3.8: CPT (camptothecin) drop test for the *hhp1.R180C.Δwee1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

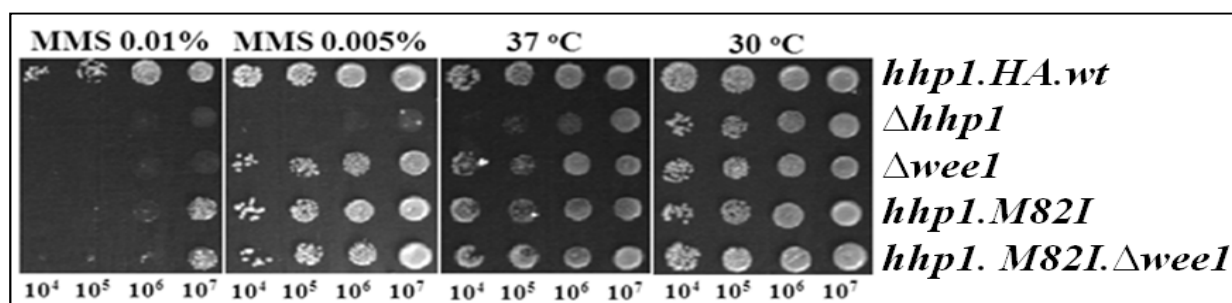


Figure: 6.3.9: MMS (methyl-methanesulfonate) drop test for the *hhp1.M82I.Δwee1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

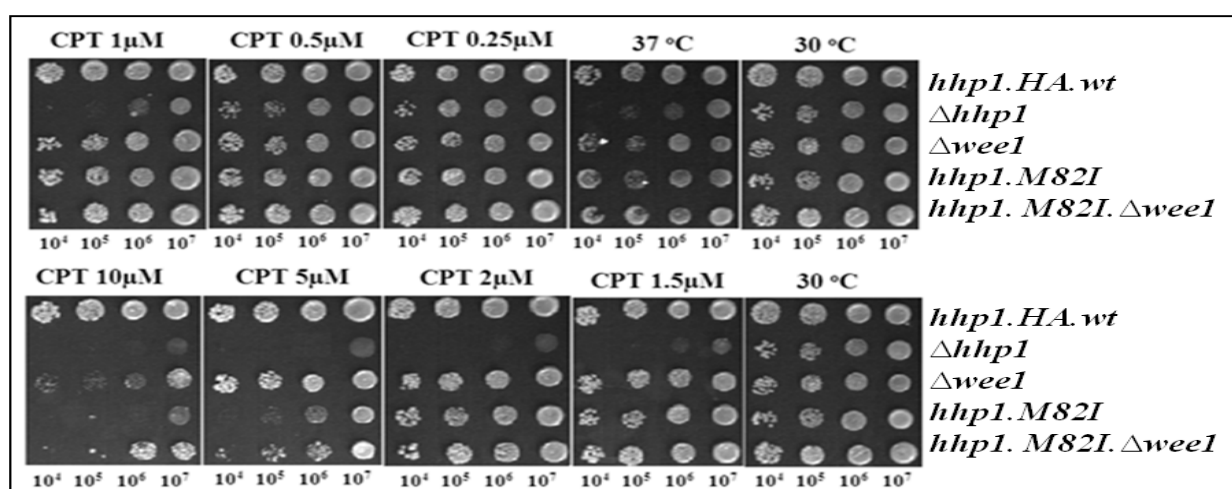


Figure: 6.3.10: CPT (camptothecin) drop test for the *hhp1.M82I.Δwee1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

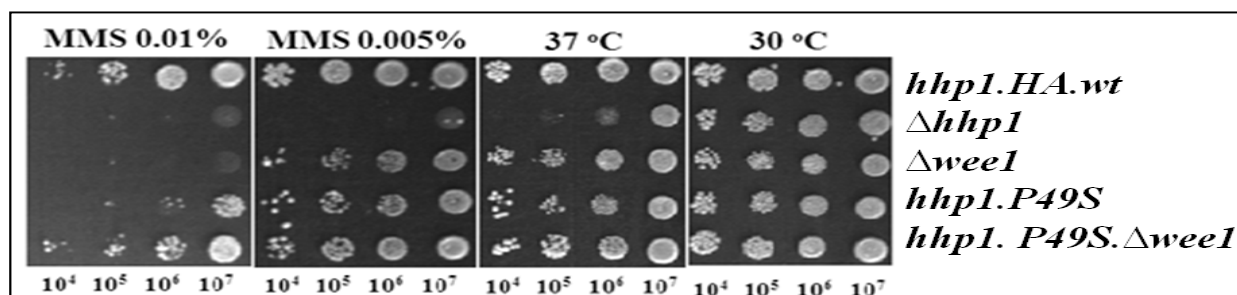


Figure: 6.3.11: MMS (methyl-methanesulfonate) drop test for the *hhp1.P49S.Δwee1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

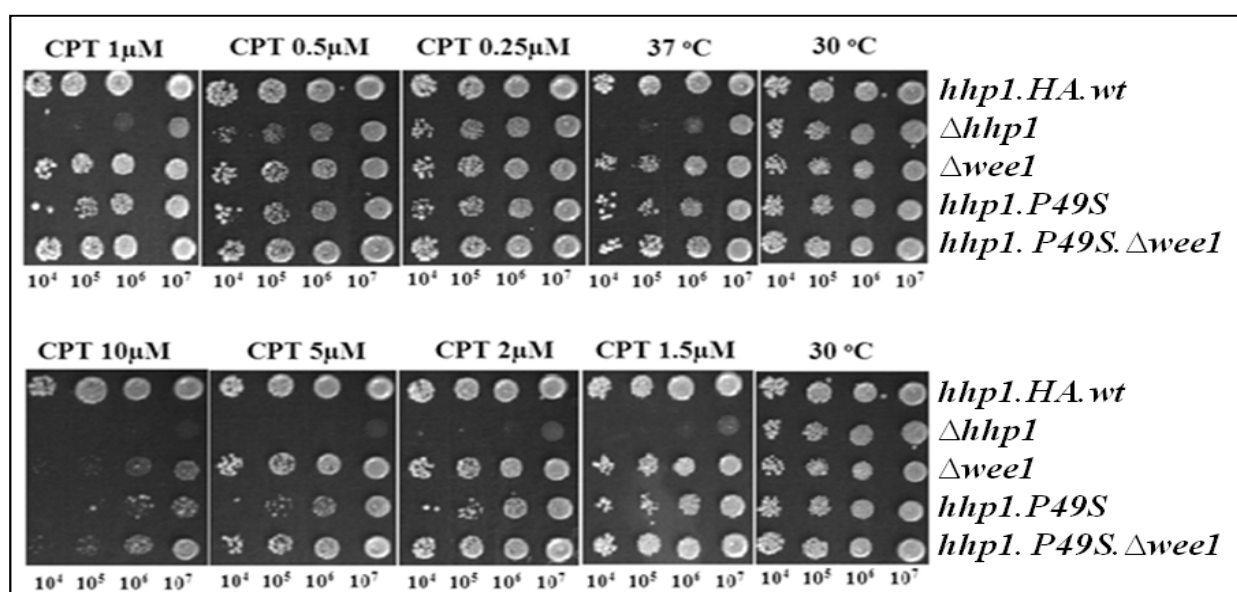


Figure: 6.3.12: CPT (camptothecin) drop test for the *hhp1.P49S.Δwee1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

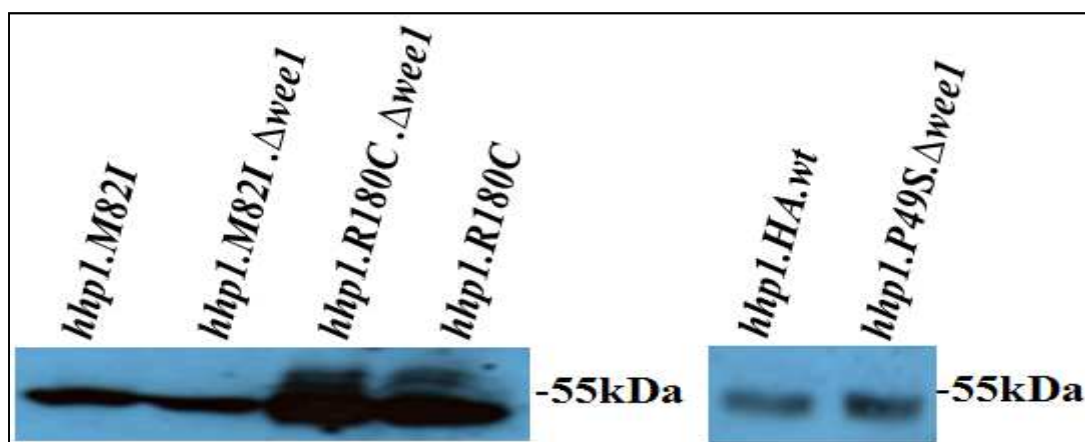


Figure: 6.3.13: Protein levels of the *hhp1* mutants with the *wee1* deletion. Total protein was isolated, 15μl of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody. Proteins bands sizes around 55kDa.

In the case of the *dbt-S* (P49S) and *dbt-L* (M82I) mutations, the deletion of *wee1* slightly reduced the CPT sensitivity (Figure: 6.3.10, Figure: 6.3.12) as observed for the *wee1-50 Δ hhp1* double mutant strain (Figure: 6.3.6). The two circadian clock mutants differed however in the response to MMS, while the *Δ wee1 hhp1.M82I-HA* double mutant was as MMS sensitive as the *wee1* deletion strain (Figure: 6.3.9), the *Δ wee1 hhp1.P46S-HA* strain was more MMS resistant than cells without *wee1* (Figure: 6.3.11). However, the protein levels of the mutated Hhp1 kinase was not affected by the loss of Wee1 (Figure: 6.3.13).

These findings imply that a drop in Hhp1 kinase activity caused by the circadian clock mutations or the deletion of *hhp1* can be partly compensated by loss of Wee1. Since the unphosphorylated pool of Cdc2, which interacts with Chk1 (554), accumulates in the absence of Wee1, it is possible that Hhp1 kinase is required for the formation of this Cdc2 DNA damage response complex. This would also explain why Chk1 is epistatic with *hhp1* mutations. Whether the Mus81-Eme1 complex is also part of this repair network is not yet known, but is very likely since both, Cdc2 and Chk1, need to activate the Mus81-Eme1 endonuclease (515).

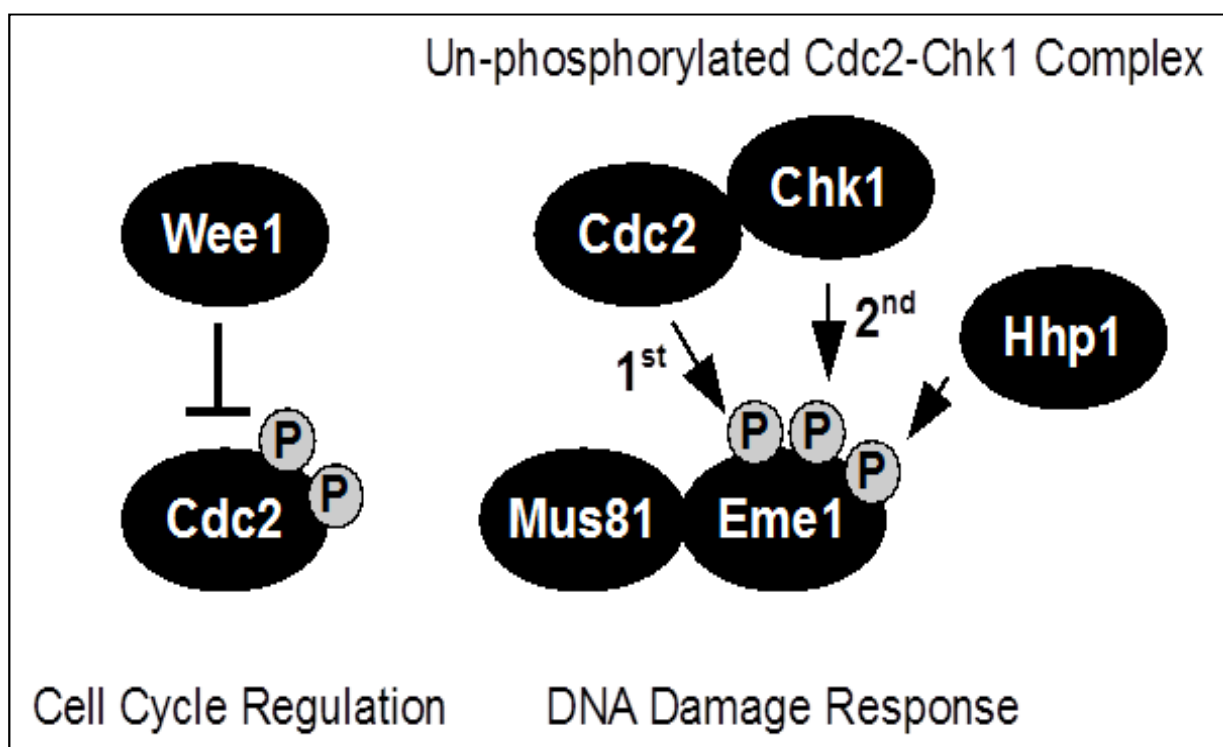


Figure: 6.3.14: Model of the Role of Hhp1 in the regulation of Mus81-Eme1. There is a suggested genetic link between Wee1, Cdc2, and Chk1 which may regulate the Mus81-Eme1 complex. Cdc2 phosphorylates Eme1 to facilitate the phosphorylation event on Eme1 by Chk1 when DNA damage is present. Also Hhp1 kinase is required to phosphorylate Eme1 to activate the Mus81-Eme1 complex.

The genetic links between Wee1, Cdc2 and Chk1 could be explained by a combined regulation of the endonuclease Mus1-Eme1 (Figure: 6.3.14). Eme1 is first phosphorylated by Cdc2 in G2 which primes the endonuclease for the phosphorylation by Chk1 in the presence of DNA damage (515), Hhp1 could also modify Eme1 which could be important for the formation of the Mus81-Eme1 complex. Alternatively Hhp1 could also be functionally linked with Chk1 kinase. Cdc2 in the complex with Chk1 is not phosphorylated (554). Loss of Wee1, which phosphorylates Cdc2 at tyrosine 15 and threonine 14 (233), results in an increase in the unphosphorylated Cdc2 pool which could compensate for a reduction in Hhp1 kinase activity either in response to the circadian clock mutations or upon deletion of the *hhp1* gene.

Chapter 7: The Breast Cancer Mutation *Hhp1.L51Q* affects specifically the DNA Damage but not the Heat Response

Chapter Summary

Common breast cancer mutants in human CK1 are *CK1 ϵ .L39Q* (*Hhp1.L40Q*), beside *CK1 ϵ .L49Q* (*Hhp1.L51Q*), *CK1 ϵ .N78T* (*Hhp1.N80T*), and *CK1 ϵ .S101R* (*Hhp1.S103R*). To find out how these mutations affect DNA repair and cell cycle progression, the beside *CK1 ϵ .L49Q* (*Hhp1.L51Q*) mutation was created in *S.pombe*. Very unexpectedly, the replacement of leucine 51 by a glutamine residue at the transition point between the loop which spans across the ATP binding site and the beginning of an alpha-helix, affects only the DNA repair and cell cycle responses to the topoisomerase 1 inhibitor CPT, but not to heat stress. *S.pombe* cells harbouring this mutation are CPT sensitive and delay G2 for longer. The heat sensitivity and cell cycle delay is however normal at 40°C. Both, the CPT sensitivity and the extended G2 arrest are suppressed upon loss of Chk1 kinase. Since Chk1 acts on the endonuclease Mus81-Eme1, the inability to activate this DNA repair enzyme may explain the suppression.

The breast cancer mutation displays also an interesting genetic interaction with the cell cycle regulator Cdc25 phosphatase. Cdc25 removes the inhibitory tyrosine (Y) 15 phosphorylation from Cdc2 which is attached by Wee1 kinase. The L51Q mutation overcomes loss of Cdc25 activity as the *cdc25.22 hhp1.L51Q* mutant strain regains the ability to grow again at the restrictive temperature of 37°C. This rescue could be explained by a re-activation of the mutated Cdc25 enzyme or a inhibition of Wee1 when Hhp1 kinase levels drop.

7.1. Hhp1 Kinase and Cancer

Cancer is one of the most challenging diseases worldwide. Several mutations in human *CK1ε* (*Hhp1*) kinase were suggested to cause breast cancer i.e., *CK1ε.L39Q* (*Hhp1.L40Q*), *CK1ε.L49Q* (*Hhp1.L51Q*), *CK1ε.N78T* (*Hhp1.N80T*), and *CK1ε.S101R* (*Hhp1.S103R*) (6, 88). The *hhp1* mutation at L51Q is commonly diagnosed in breast cancer. The leucine-51 to glutamine mutation leads to aberrant cells growth which is recognized as cancer (88). The circadian clock was also linked to breast cancer and colorectal cancers as both increase amongst night shift workers (370, 420). This link between the circadian clock and the cell cycle may well be linked to mutations in CK1 (421). Filipinski E, et al., (424) stated that changes in the expression pattern of mouse *Per2*, *Bmal1*, and *Rev-erbα* genes effected cancer proliferation. Another possible link between the molecular clock and the cell cycle machinery could be regulation of Wee1 kinase expression (375). Given that the transcription factor c-myc is an oncogene, its regulation by the BMAL1-CLOCK complex may also result in uncontrolled cell growth (423). Mammalian *Per1* and *Per2* may directly regulate the tumour suppressor p53 via their link with Mdm2 which controls the nuclear localisation and stability of p53 (394, 423, 425, 370, 427). *CK1ε* (*Hhp1*) is directly linked with circadian disorders of rest-activity or sleep-wakefulness rhythms in mammals (6, 421, 428). For example, the phosphorylation of *Per2* at serine-662 is required to prevent the familial advanced sleep phase syndrome (6, 68).

7.2. The Hhp1.L51Q Breast Cancer Mutation

Mammary carcinomas (breast cancer) are one of the most common neoplasias in women (88). Interestingly mutations in highly conserved amino acids of human casein kinase 1ε were detected in breast cancer patients. (78). *CK1ε* is a Serine/Threonine kinase which regulates cell proliferation, differentiation, cell migration, and the circadian clock (88). *CK1ε* is involved in p53 regulation and the Wnt signalling pathway, DNA repair and cell cycle regulation (1, 78). The following mutations were detected in the N-terminal kinase domain of human *CK1ε* (*Hhp1*): *CK1ε.L39Q* (*Hhp1.L41Q*), *CK1ε.L49Q* (*Hhp1.L51Q*), and *CK1ε.S101R* (*Hhp1.S103R*) (78, 88) (Figure: 7.2.1).

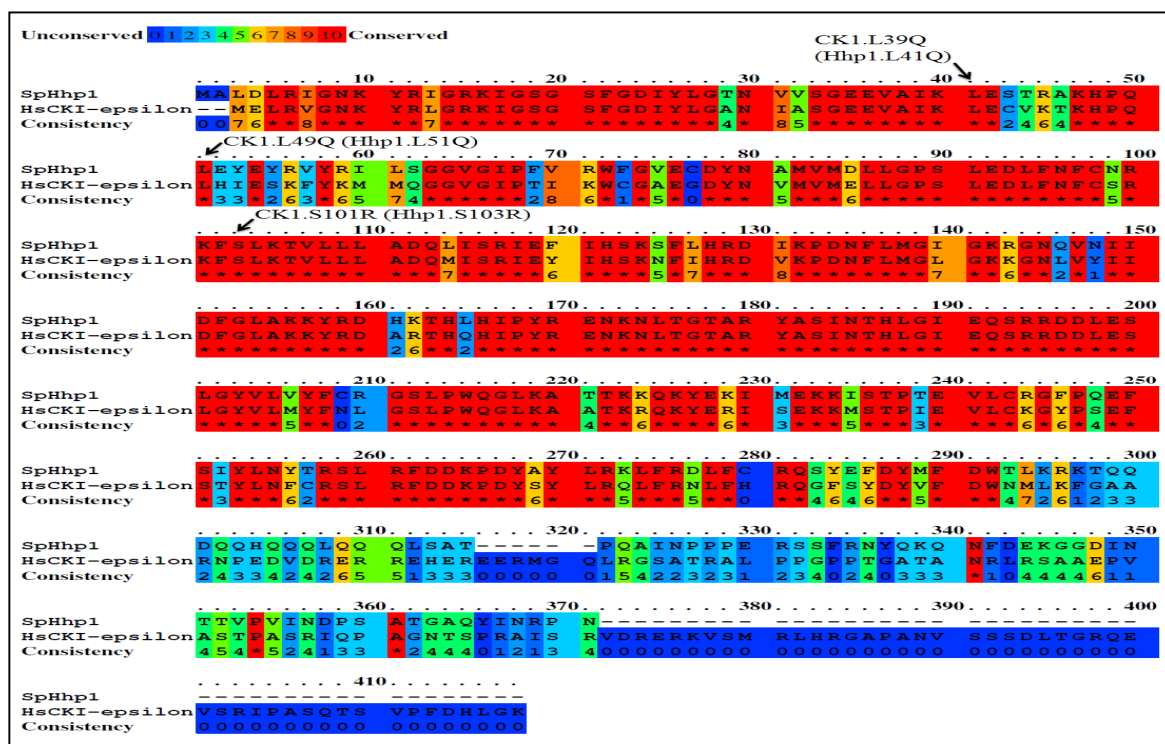


Figure 7.2.1: Location of the human breast cancer mutations L39Q, L49Q and S101R in the Hhp1 protein. (source: PRALINE sequence alignment tool; available at: <http://zeus.few.vu.nl/jobs/5688f2f65cc5ce5d62b41c7c506eebfe/>; accessed 13 October 2015). Human CKI ϵ : NP_689407.1, SPHhp1: CAA20311.1, and SCHrr25: CAA97918.1. Human CKI ϵ : NP_689407.1, and SPHhp1: CAA20311.1.

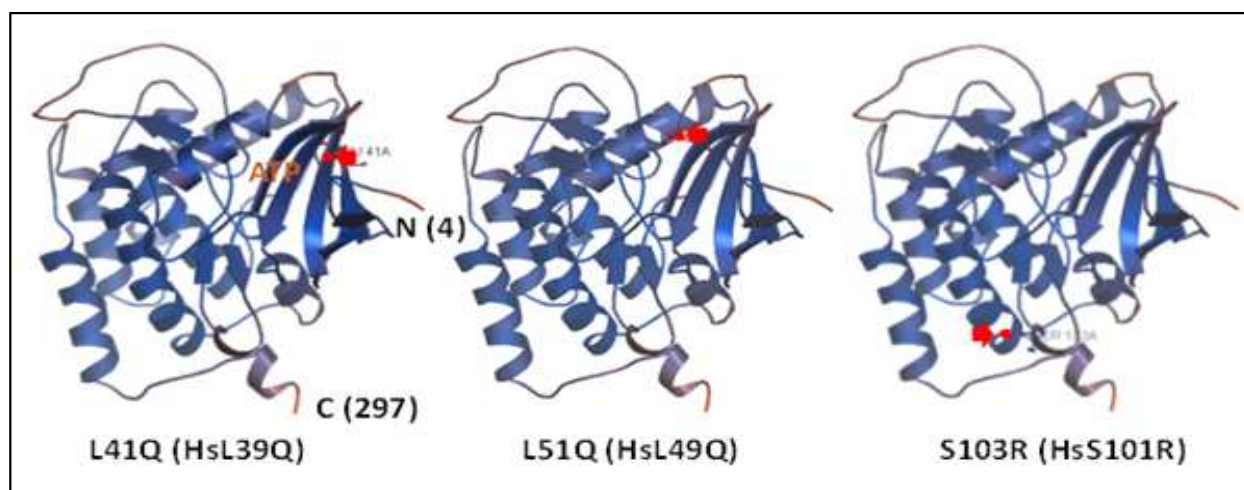


Figure 7.2.2: Location of the breast cancer mutations in Hhp1 kinase. The structure of *S.pombe* Hhp1 was modelled using the tool Swiss Model (protein template: 3sv0.1.A (500), identity: 76.4%, cover: amino acids 4-297 (81%)). L41 is located in a beta-sheet which faces the ATP binding site (active site). L51 and S103 are both located at the end of an alpha helix (red arrows). The N and C-termini are indicated.

Interestingly, leucine 51 and serine 103 are both located at the transition point between an alpha helix and a loop region (Figure: 7.2.2), while L41 is part of a beta-sheet which forms the ATP binding site (active site). These mutations may affect the activity of the kinase as they may have an impact on its structure or conformational changes, and may therefore contribute to tumour progression (88). Characterized *CK1 ϵ* mutants (*CK1 ϵ .L39Q*, *CK1 ϵ .L49Q*, and *CK1 ϵ .S101R*) which were identified in mammary carcinomas have limited kinase activity and that may therefore lead to a reduced phosphorylation of physiological targets, for example in the Wnt/ β -catenin pathway (88, 429) which may decrease cell adhesion and promote cell migration (429). Importantly, there is very little known of how these mutations (*CK1 ϵ .L39Q* (*Hhp1.L40Q*), *CK1 ϵ .L49Q* (*Hhp1.L51Q*), and *CK1 ϵ .S101R* (*Hhp1.S103R*)) affect the kinase and how they impact on the DNA damage response. Work in human cell lines has shown that the mutation L39Q affects the ability of *CK1 ϵ* to phosphorylate the Wnt signalling protein Dishevelled (88). This reduction in wnt signalling leads to the up-regulation of the small G protein Rac1 which reduces cell adhesion.

7.3. Characterisation of *Hhp1.L51Q*

Since no information is available on how the mutation L49Q affects the human kinase, the corresponding amino acid leucine-51 was replaced by a glutamine residue using the Cre-Lox technology (258), and the point-mutated *hhp1.L51Q-HA* gene (C-terminally haemagglutinin tagged) was integrated at its endogenous locus in the *hhp1* base strain. The mutated *hhp1.L51Q.HA* gene was re-amplified from the integration strain by PCR and sequenced to confirm the mutation. As shown in Figure: 7.3.2, the mutation had no impact on the overall amount of the kinase.

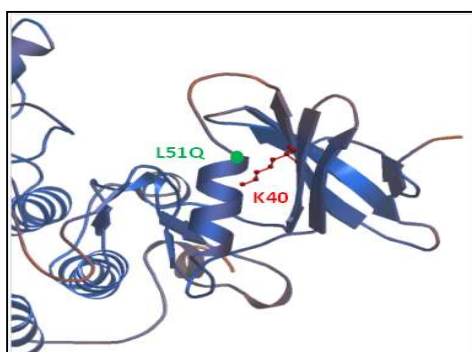


Figure: 7.3.1: Location of the L51Q mutation of *Hhp1*. Leucine 51 is located at the transition from the loop arching over the active site to the alpha helix of the larger second lobe. Lysine 40, which makes contact with the ATP in the active site is also shown.

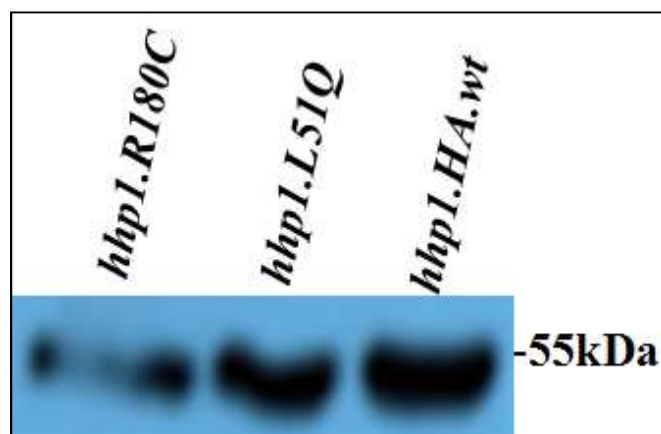


Figure: 7.3.2: Protein levels of Hhp1.L51Q. Total protein was isolated, 15 μ l of the protein separated on a 10% SDS gel, transferred onto nitrocellulose membrane and detected with an anti-HA antibody. The protein size is between 40kDa and 55kDa.

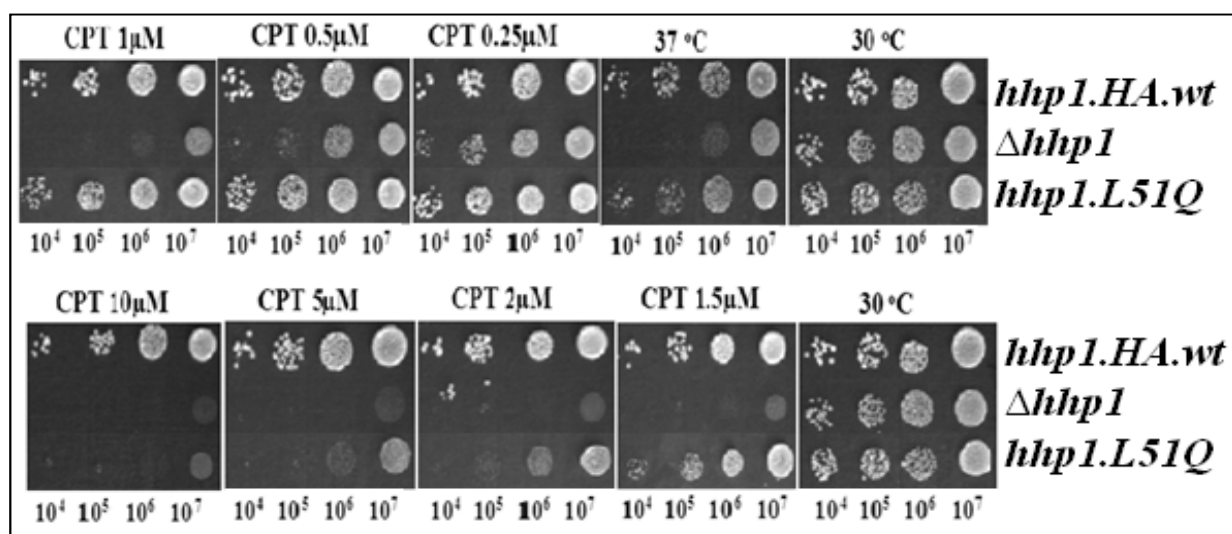


Figure: 7.3.3: CPT-Drop (Camptothecin-Drop) test for the *hhp1.L51Q* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

To find out whether the replacement of leucine 51 by a glutamine residue at the transition from a loop, which connects one of the beta-sheets of the active site with the first alpha helix of the second, larger domain of Hhp1 (Figure: 7.3.1), has an impact on the DNA damage response, the *hhp1* deletion strain was tested against the *hhp1.L51Q.HA* strain and *hhp1.HA* wild type cells (Figure: 7.3.3).

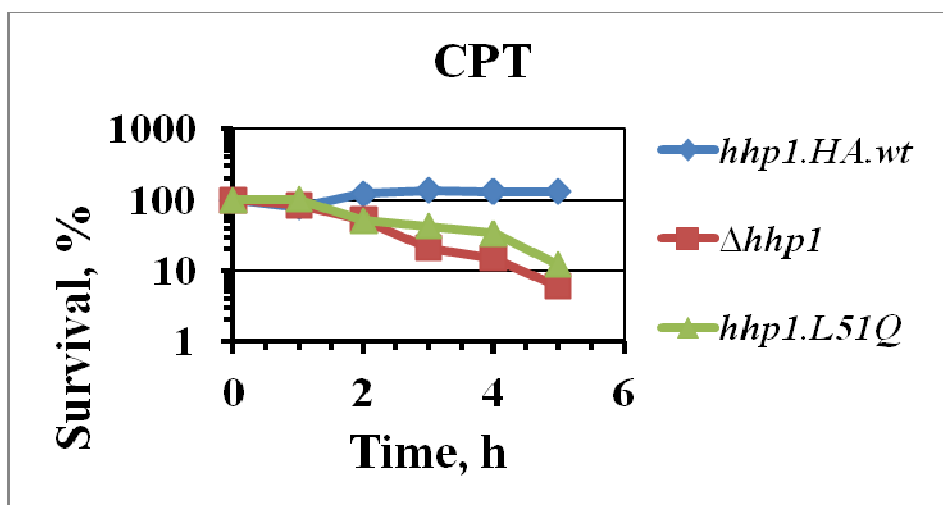


Figure: 7.3.4: Acute CPT (camptothecin) survival assay for the *hhp1.L51Q* strain. The indicated yeast strains were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40 μ M CPT and incubated again for five hours at 30 °C. 75 μ l samples were collected every hour.

While *hhp1.L51Q.HA* cells were clearly CPT sensitive, they were slightly less sensitive than the deletion strain (Figure: 7.3.3, Figure: 7.3.4). Interestingly, the point mutant strain is not temperature sensitive like the gene deletion. This implies that the kinase activity of Hhp1.L51Q is reduced but not completely abolished. Given that the more subtle reduction of the kinase activity in the circadian clock mutant (Chapter 6) only affects the cell cycle but not survival, the breast cancer L51Q mutant may have a much stronger effect on the kinase activity.

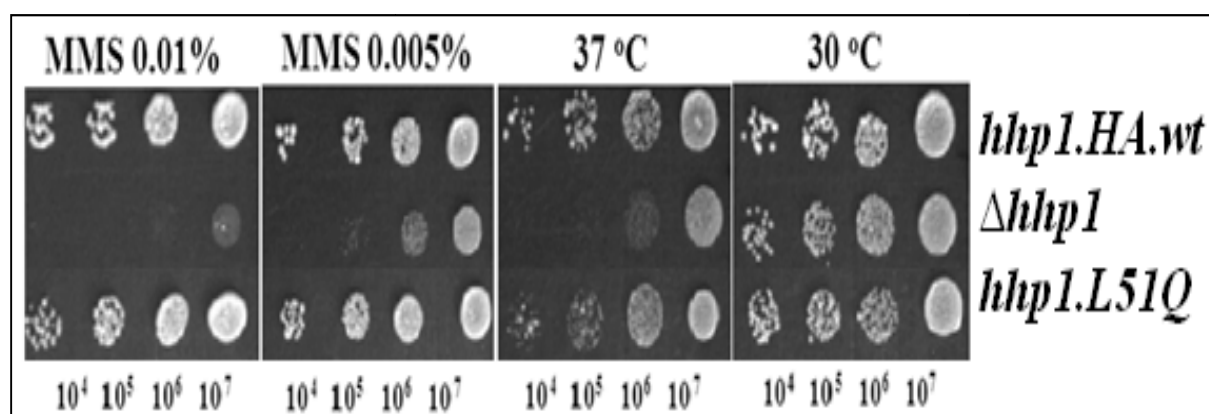


Figure: 7.3.5: MMS-Drop (Methyl-methanesulfonate-Drop) test for the *hhp1.L51Q* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C.

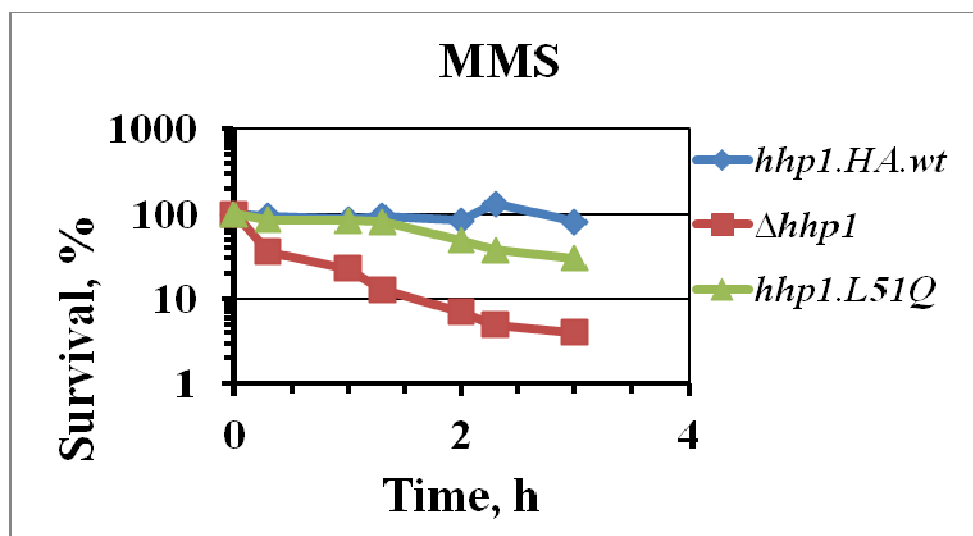


Figure: 7.3.6: Acute MMS (methyl-methanesulfonate) survival assay for the *hhp1.L51Q* strain. The indicated yeast strains were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 0.05% MMS and incubated again for three hours at 30 °C. 75 μ l samples were collected every 30 min.

Compared to CPT, the MMS sensitivity of the *hhp1.L51Q.HA* strain was much lower as cells grew on rich medium plates containing up to 0.01% of the alkylating drug (Figure: 7.3.5). An acute survival test at 0.05% MMS revealed however a subtle sensitivity when cells were exposed to this drug for more than 2 hours (Figure: 7.3.6). This unexpected finding suggests that the threshold of the Hhp1 kinase activity may be different for different types of DNA damage. The response to broken replication forks may require a higher kinase activity than the response to methylated DNA which is repaired by base excision repair. It also shows that the L51Q mutation is a partial loss-of-activity mutation.

To test how this reduction in activity affects the G2 arrest in the presence of CPT, *hhp1.HA* wild type, Δ *hhp1* and *hhp1.L51Q.HA* cells were synchronised in G2 by lactose gradient centrifugation and released in rich medium with or without the topoisomerase 1 inhibitor CPT. As shown in Figure: 7.3.8, Figure: 7.3.9, the L51Q mutation resulted in a similarly extended G2 arrest as the deletion of *hhp1*. While wild type delayed only briefly for 20 min (Figure: 7.3.7), the G2 arrest lasted approximately 80 min in the L51Q mutant (Figure: 7.3.9). This finding is in line with the high CPT sensitivity of the mutant strain and supports the idea that the breast cancer mutation has a strong impact on Hhp1 kinase as both, DNA repair and cell cycle are affected.

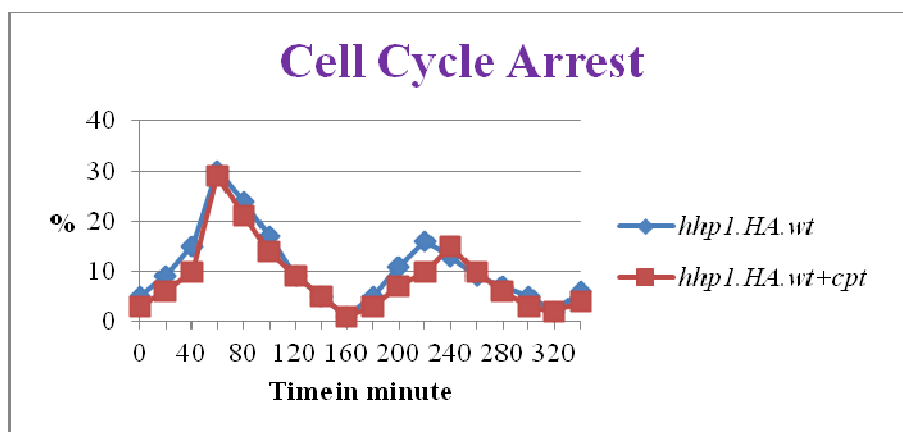


Figure: 7.3.7: Cell Cycle G2 arrest for *hhp1.HA.wild type*. Yeast cells were cultured in YEA medium overnight at 30 °C. Cells were harvested, synchronised in G2 and released into YEA medium at 30°C with or without 40µM CPT (camptothecin). The peak in septation coincides with G1/S.

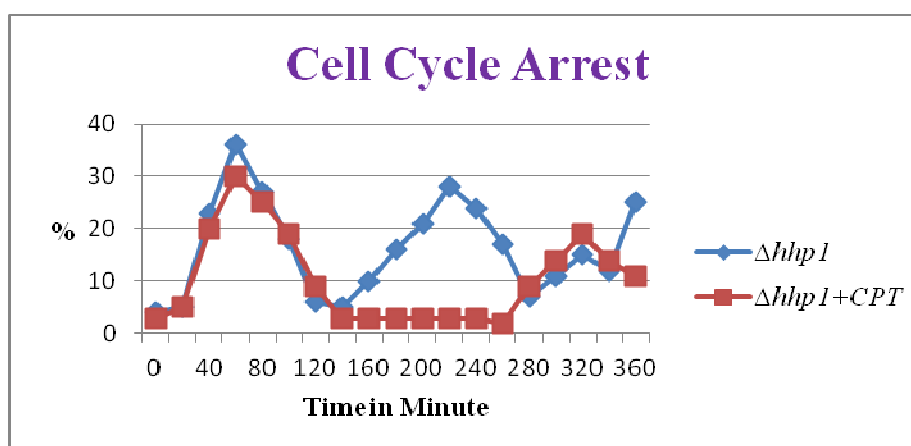


Figure: 7.3.8: Cell Cycle G2 arrest for $\Delta hhp1$. Yeast cells were cultured in YEA medium overnight at 30 °C. Cells were harvested, synchronised in G2 and released into YEA medium at 30°C with or without 40µM CPT (camptothecin). The peak in septation coincides with G1/S.

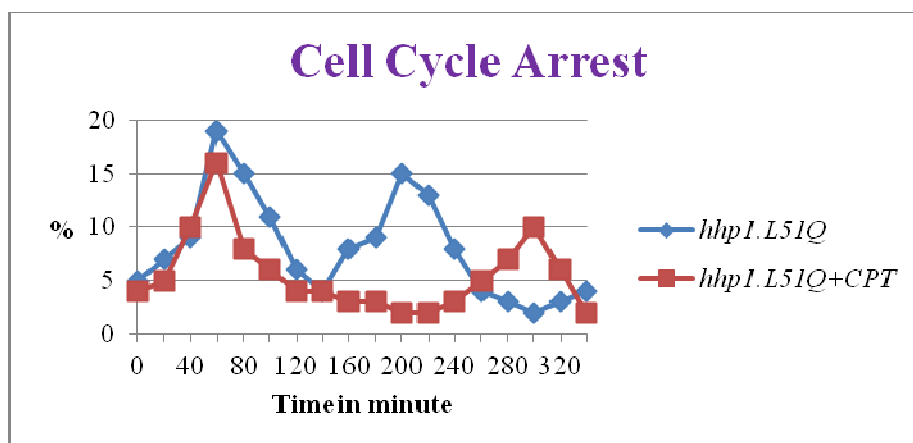


Figure: 7.3.9 : Cell Cycle G2 arrest for *hhp1.L51Q*. Yeast cells were cultured in YEA medium overnight at 30 °C. Cells were harvested, synchronised in G2 and released into YEA medium at 30°C with or without 40 μ M CPT (camptothecin). The peak in septation coincides with G1/S.

Since previous work has shown that elevated temperatures arrest *S.pombe* cells in the first G2 phase after the release from a lactose gradient, the synchronised cells were released in rich medium at 30°C and 40°C. Consistent with the previous findings, *hhp1-HA* wild type cells arrested for approximately 140 min at 40°C before re-entering the cell cycle (Figure: 7.3.10). Loss of Hhp1 (Δ *hhp1*) extended this G2 arrest significantly and cells failed to re-enter the cell cycle during the course of the experiment (up to 340 min) (Figure: 7.3.11). A reduction in the kinase activity by the L51Q mutation had only a very small impact on the G2 arrest (Figure: 7.3.12). This mutant arrested for 160 min before re-entering the cell cycle. The absence of an extended heat arrest is in line with the absence of a temperature sensitivity when cells are grown at 37°C (Figure: 7.3.3). Taken together, this implies that cells can restart the cell cycle after a temperature shock with low Hhp1 activity levels, but not, or only very slowly, without Hhp1 kinase. It also reveals a significant difference between heat and CPT induced G2 arrests. While the breast cancer mutation L51Q prolongs the CPT-induced G2 arrest, it has not the same impact on the heat arrest.

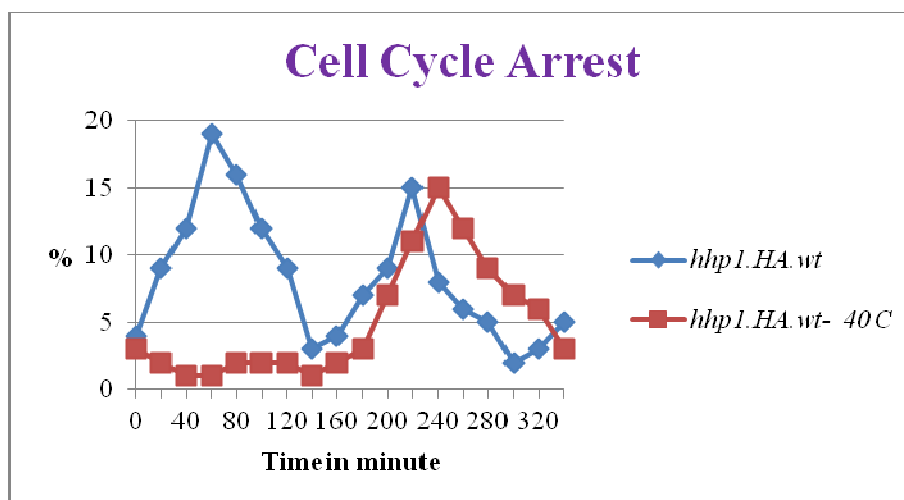


Figure: 7.3.10: Heat-induced cell cycle G2 arrest for *hhp1.HA.wild type*. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C or 40 °C. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

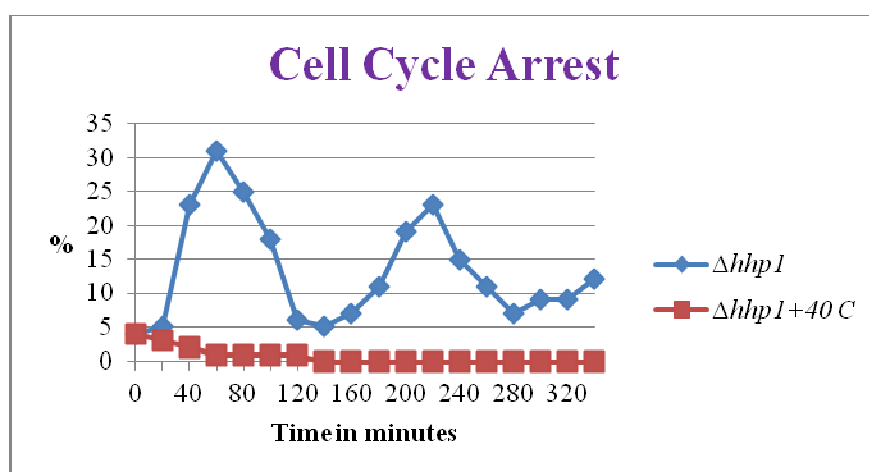


Figure: 7.3.11: Heat-induced cell cycle G2 arrest for Δ *hhp1* deletion cells. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C or 40 °C. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

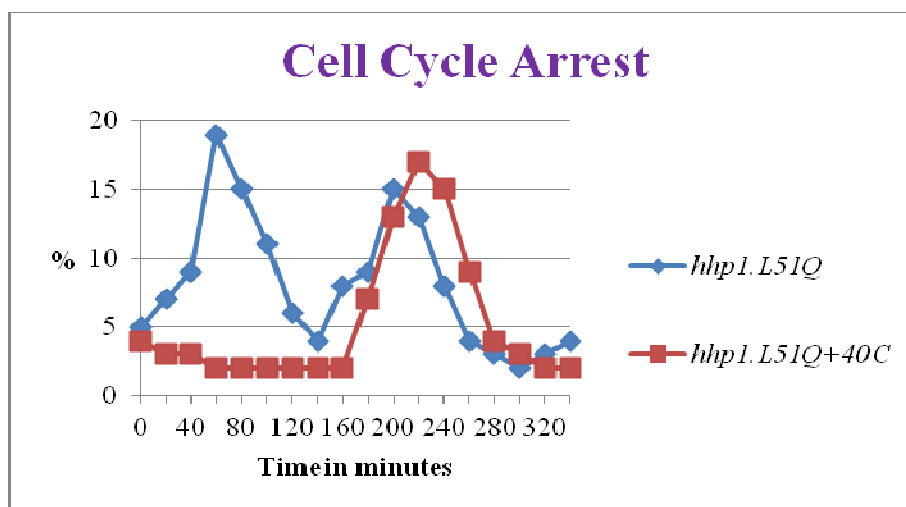


Figure: 7.3.12: Heat-induced cell cycle G2 arrest for the *hhp1.L51Q* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C or 40 °C. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

7.3.1. The Breast Cancer Mutation *Hhp1.L51Q* affects *Chk1* Function

Given the genetic link between Hhp1 and Chk1 kinase (Figure: 3.3.3.1), the *hhp1.L51Q* mutant allele was combined with the deletion of *chk1* ($\Delta chk1.hhp1.L51Q$). As shown in Figure: 7.3.1.1, the *hhp1.L51Q* strain is mildly MMS sensitive whereas cells without Hhp1 or Chk1 are highly MMS sensitive. Interestingly, the reduction in kinase activity due to the L51Q mutation reduces the MMS sensitivity of $\Delta chk1$ cells to the lower levels observed for the *hhp1.L51Q* strain. This suggests a role of Hhp1 upstream of Chk1 as the reduction in Hhp1 function may affect a DNA response activity which is later dependent on Chk1 kinase.

The same set of strains was also tested for the response to CPT. In the acute exposure test (Figure: 7. 3.1.2), *hhp1.L51Q* cells were only mildly sensitive and $\Delta chk1$ cells were resistant. The $\Delta chk1.hhp1.L51Q$ strain was as CPT sensitive as the *hhp1.L51Q* single mutant which supports the conclusion that both kinases act in the same pathway.

Under chronic exposure conditions, the $\Delta chk1 hhp1.L51Q$ double mutant was less sensitive than the *hhp1.L51Q* strain (Figure: 7. 3.1.3) which is in line with its reduced MMS sensitivity (Figure: 7. 3.1.1). To find out whether the reduction in CPT sensitivity is related to the G2 arrest, $\Delta chk1$ and $\Delta chk1 hhp1.L51Q$ cells were synchronised in G2 and released into rich medium with and without 40 μ M CPT. Since both strains showed a G2 delay between 20 min and 40 min in the

presence of the topoisomerase 1 inhibitor, it is unlikely that the increased survival of the $\Delta chk1$ *hhp1.L51Q* double mutant is linked with the G2 arrest (Figure: 7. 3.1.4, Figure: 7. 3.1.5).

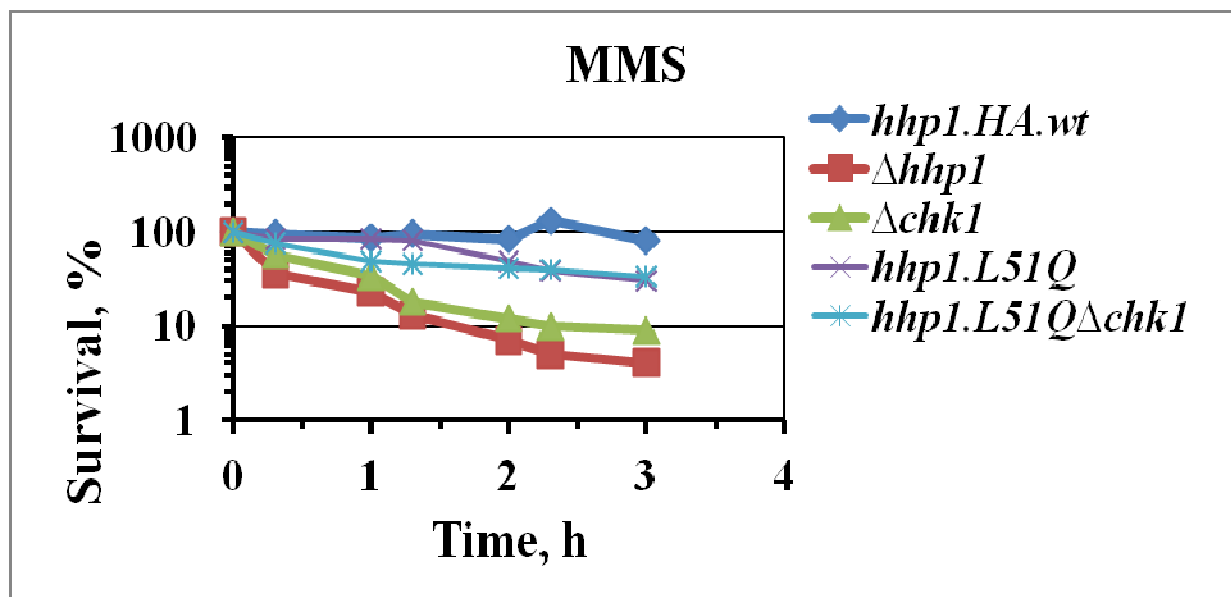


Figure: 7. 3.1.1: Acute MMS (methyl-methanesulfonate) survival assay for *hhp1.L51Q $\Delta chk1$ cells. Yeast strain cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 0.05% MMS and incubated again for three hours at 30 °C. 75 μ l samples were collected every hour. Plates were incubated for 4 days at 30°C and colonies were counted.*

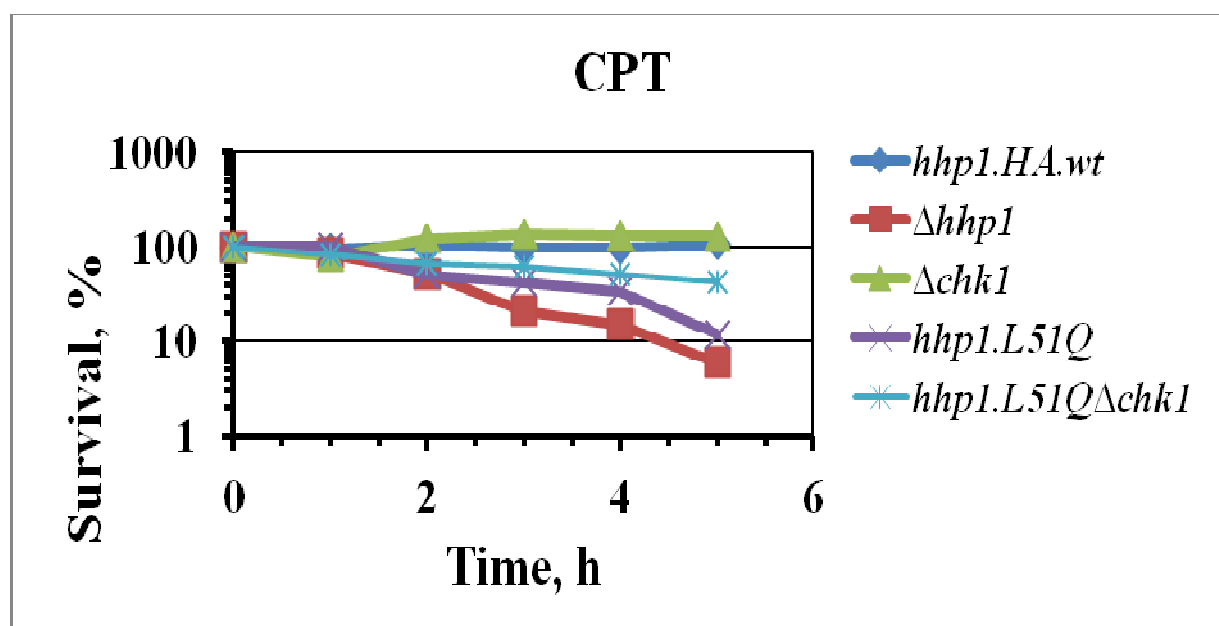


Figure: 7.3.1.2: Acute CPT (Camptothecin) survival assay for the *hhp1.L51Q $\Delta chk1$ strain. Yeast cells were cultured in YEA medium overnight at 30 °C. Cells were harvested and treated with 40 μ M CPT and incubated again for five hours at 30 °C. 75 μ l samples were collected every hour. Plates were incubated for 4 days at 30°C and colonies were counted.*

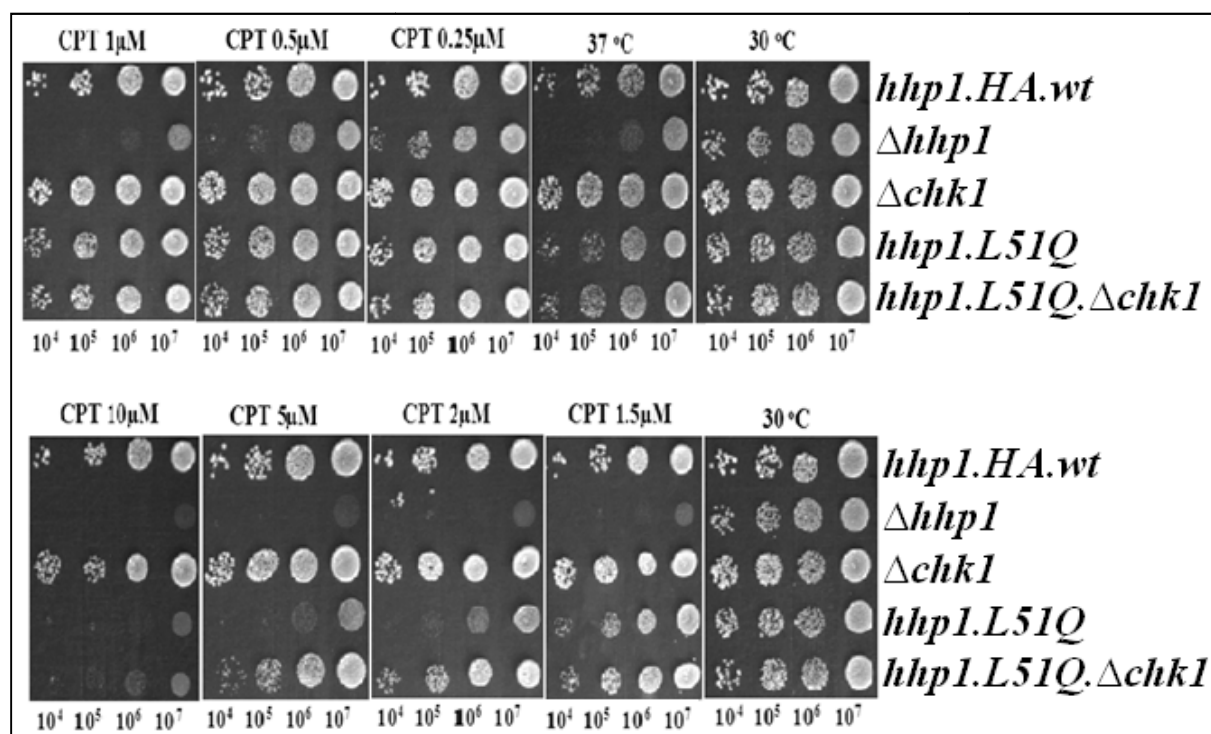


Figure: 7.3.1.3: CPT-Drop (Camptothecin-Drop) test for the *hhp1.L51Q.Δchk1* strain. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C. One plate was incubated at 37°C.

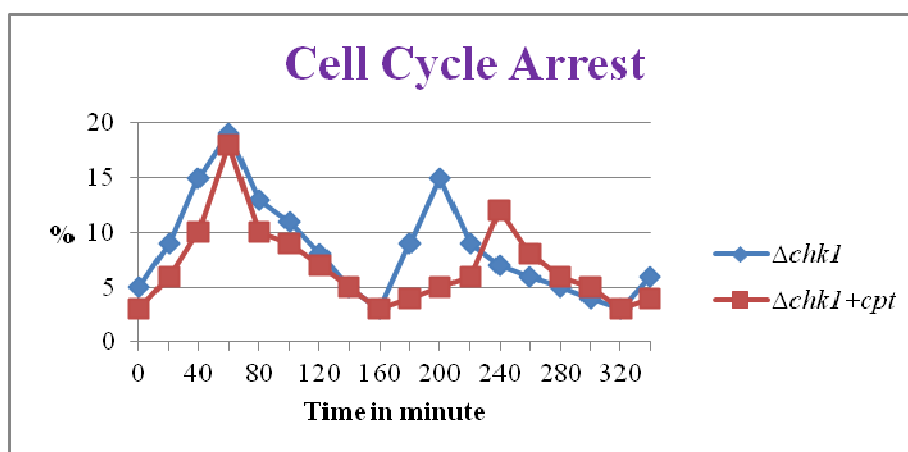


Figure: 7.3.1.4: CPT-induced (Camptothecin-induced) cell cycle G2 arrest for the $\Delta chk1$ strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C with or without 40μM CPT. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). Cell Cycle G2 arrest for $\Delta chk1$. Indicated cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium with or without 40μM camptothecin (CPT). Samples were withdrawn at the indicated time (20 min) points. Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%). $\Delta chk1$ cells were treated without 40μM CPT processed 80 min delay in G2.

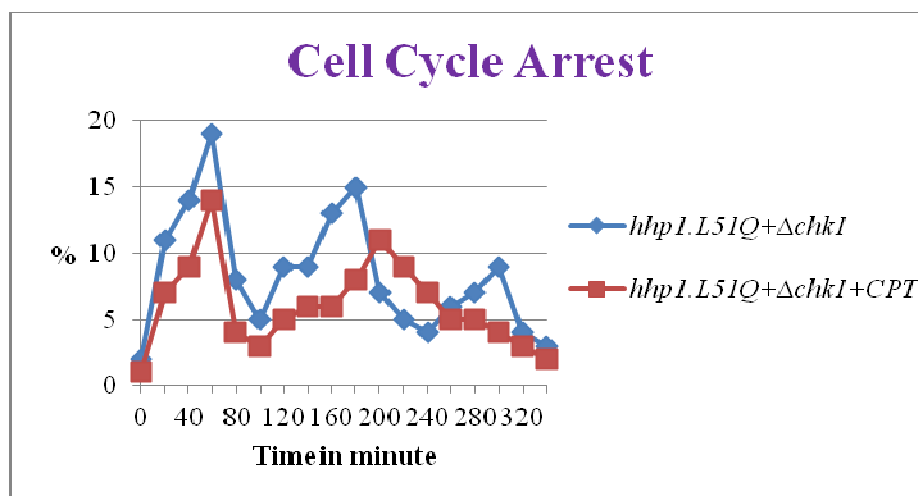


Figure: 7.3.1.5: CPT-induced (Camptothecin-induced) cell cycle G2 arrest for the $\Delta chk1$ *hhp1.L51Q* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C with or without 40 μ M CPT. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

Taken together these findings are consistent with the earlier results for the $\Delta chk1$. $\Delta hhp1$ strain as they place both kinases in the same pathway. What is novel is the conclusion that Hhp1 may act down-stream of Chk1 kinase.

7.3.2. Hhp1.L51Q affects the cell cycle regulator Cdc25 phosphatase

The *cdc25.22* allele is a temperature sensitive loss-of-function mutant of Cdc25 phosphatase which carries a tyrosine residue at position 532 instead of a cysteine (C532Y) (233). Cells with reduced Cdc25 activity remain for longer in G2 since the inhibitory tyrosine 15 phosphorylation of Cdc2 cannot be removed (543). *S. pombe* Cdc25 has multiple phosphorylation sites: S99, S148, S178, S192, S204, S206, T226, S234, S359, T561, S567, and T569. Kinases which are suggested to phosphorylate Cdc25 are Cds1 kinase, Chk1 kinase, Sty1 kinase, and Srk1 kinase (310, 486). *S. pombe* Cdc2 (CDK1) associates with different cyclins dependent on the cell cycle stage. The G1/S cyclins Cig1, Cig2 and Puc1, and the G2/M cyclin Cdc13. Amongst them, Cdc13 is the only essential cyclin as all other cyclins can be deleted without affecting progression through the cell cycle (478). The kinases Wee1 and Mik1 phosphorylate Cdc2 at tyrosine 15 to delay cell cycle progression (479, 480). Mik1 may act specifically in S phase in the response to unrepliated DNA (483), whereas Wee1 is active during the cell cycle. Given the important role of cell cycle regulators in cancer development (542), the *hhp1.L51Q* mutant was combined with the *cdc25.22* allele (*hhp1.L51Q.cdc25.22*).

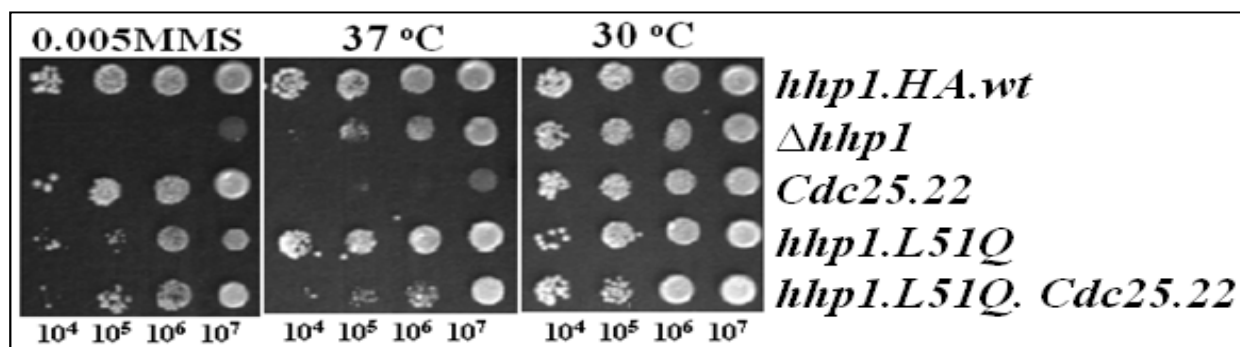


Figure: 7.3.2.1: MMS-Drop (Methyl-methanesulfonate-Drop) test for Hhp1.L51Q.cdc25.22. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentration. Plates were incubated for 4 days at 30°C.

Cells with reduced Cdc25 activity (*cdc25.22*) are unable to grow at 37°C since cells accumulate in G2 with tyrosine 15 phosphorylated Cdc2 (Figure: 7.3.2.1). This cell cycle defect is not associated with DNA damage sensitivity. Very unexpectedly, a reduction in Hhp1 activity by the L51Q mutation restored some growth of the *hhp1.L51Q.cdc25.22* strain at the restrictive temperature. There was no impact of the MMS sensitivity of the *hhp1.L51Q* mutation. This suppression could be explained in two ways. Either a drop in Hhp1 activity reduces the levels of the inhibitory tyrosine 15 phosphorylation or it re-activates the mutated Cdc25 phosphatase.

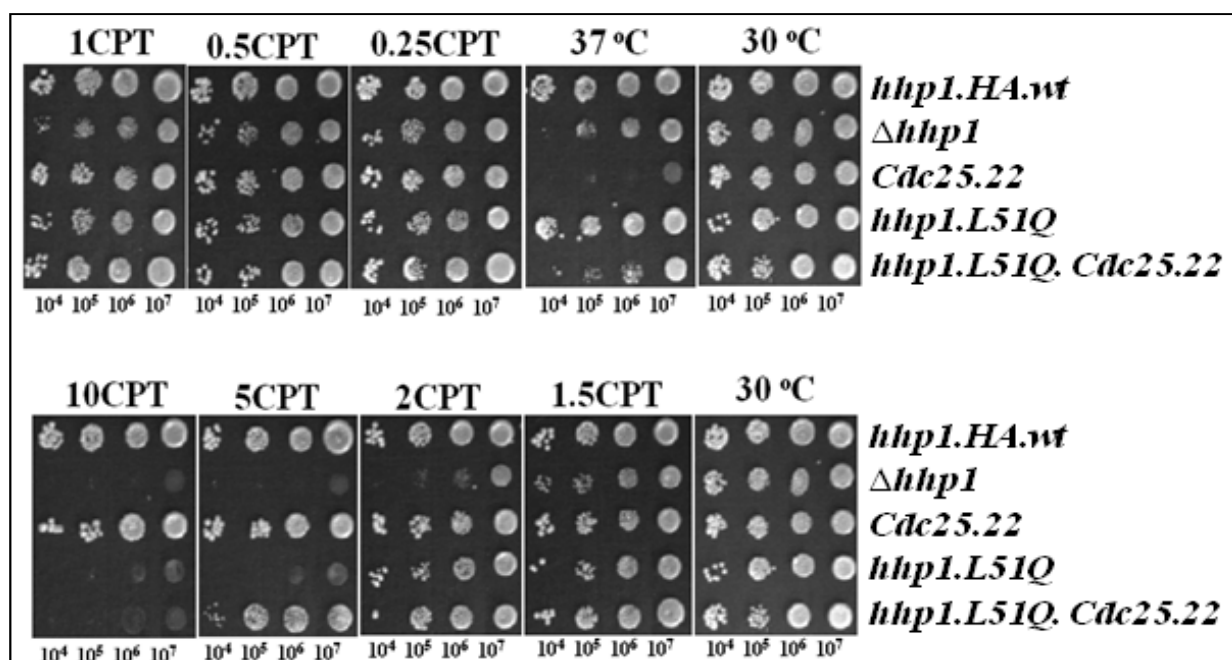


Figure: 7.3.2.2: CPT-Drop (Camptothecin-Drop) test for Hhp1.L51Q.cdc25.22. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C.

In the presence of the topoisomerase 1 inhibitor CPT, the genetic linkage between *cdc25.22* and *hhp1.L51Q* was however more complex. In addition to restoring growth at the restrictive temperature of 37°C, the *hhp1.L51Q cdc25.22* strain was slightly less CPT sensitive (Figure: 7.3.2.2).

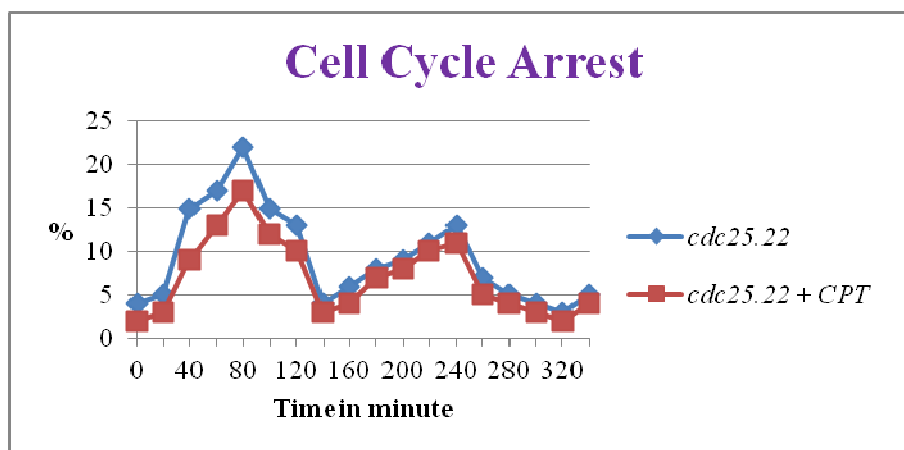


Figure: 7.3.2.3: CPT-induced (camptothecin-induced) cell cycle G2 arrest for the *cdc25.22* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C with or without 40µM CPT. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

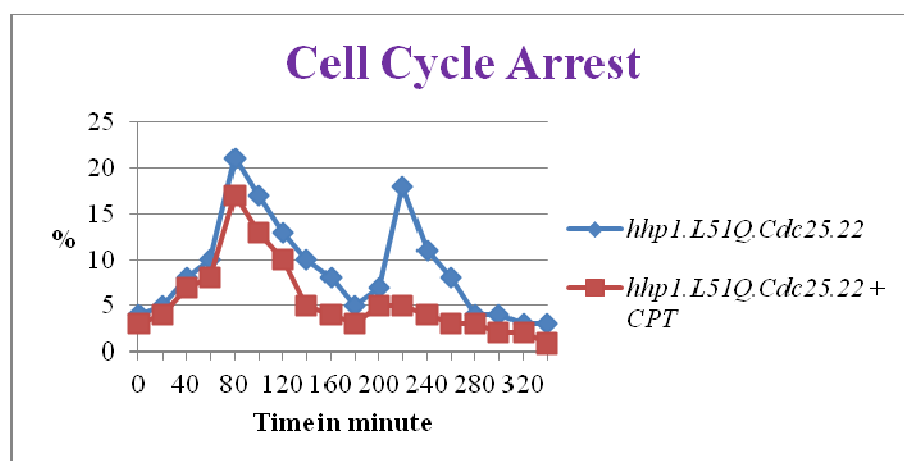


Figure: 7.3.2.4: CPT-induced (camptothecin-induced) cell cycle G2 arrest for the *cdc25.22 hhp1.L51Q* strain. Cells were synchronised in G2 by lactose gradient centrifugation and released into rich medium at 30 °C with or without 40µM CPT. Samples were withdrawn at the indicated time points (20 min). Cells were fixed in methanol and stained with DAPI (DNA) and calcofluor (septum). The percentage of septated cells, which are a readout for G1/S cells, was scored (%).

Since Cdc25 regulates the G2-M transition (Figure: 7.3.2.7), *cdc25.22* and *hhp1.L51Q cdc25.22* cells were synchronised in G2 and released in medium with and without 40 μ M CPT. As shown in Figure: 7.3.2.3, the reduction in Cdc25 activity on its own had little impact on the G2 arrest. The short 20-40 min delay observed in wild type cells was not evident in this experiment which may be the consequence of the slower progression through the cell cycle of this mutant strain. While the *cdc25.22* single mutant delays in G2 for 20-40 min (Figure: 7.3.2.3), the *hhp1.L51Q cdc25.22* double mutant displayed an extended arrest which resembles the long arrest of *hhp1* deletion cells (Figure: 7.3.2.4).

To test whether the Hhp1 protein is required for this genetic link, the DNA sensitivity tests were repeated with a Δ *hhp1 cdc25.22* strain in which no Hhp1 protein is expressed. Loss of the kinase suppressed the growth defect of the *cdc25.22* mutant at 37°C (Figure: 7.3.2.6), but had only a very small impact on the CPT sensitivity of the double mutant (Figure: 7.3.2.5). This indicates that a low Hhp1 activity is necessary to restore some of the CPT resistance of the *hhp1.L51Q cdc25.22* double mutant and to allow *cdc25.22* cells to over-come the G2 block at the restrictive temperature of 37°C. A loss of Hhp1 kinase activity could either reactivate Cdc25.22 or it could reduce the inhibitory function of Wee1. In both cases the inhibitory Y15 modification of Cdc2 would decline allowing cells to grow again (Figure 7.1.4.7).

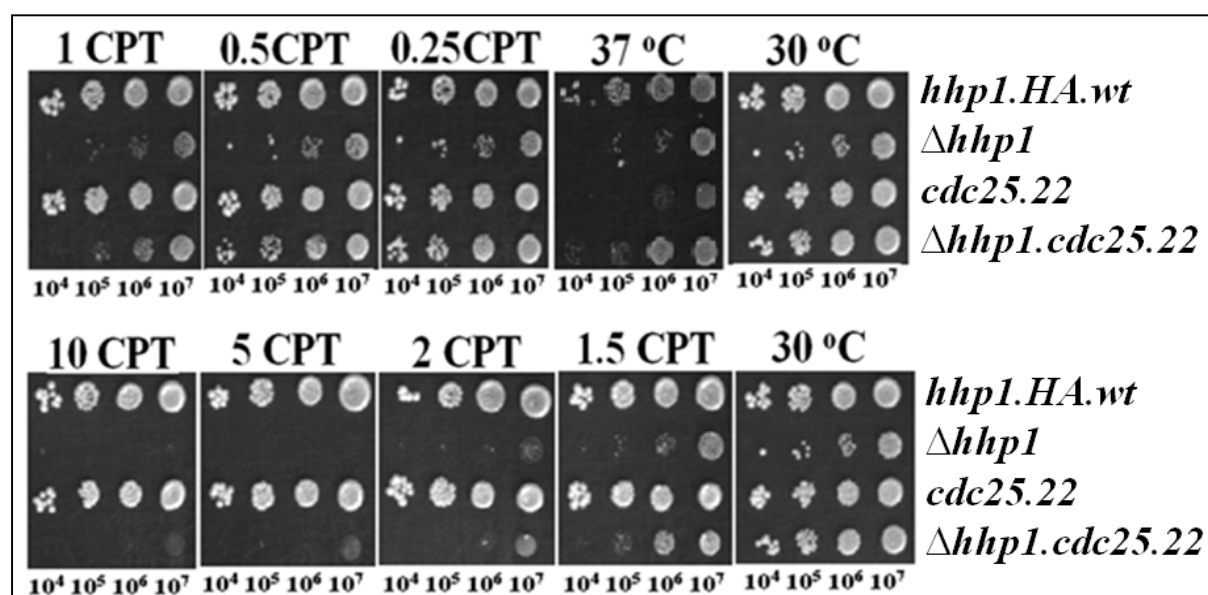


Figure: 7.3.2.5: Chronic CPT (camptothecin) exposure for *Cdc25.22 Δ hhp1*. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated CPT concentrations. Plates were incubated for 4 days at 30°C.

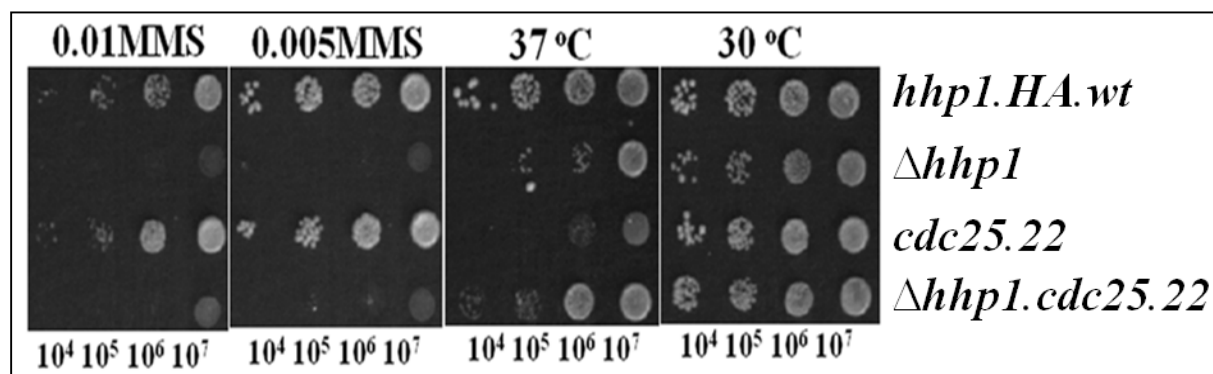


Figure: 7.3.2.6: Chronic MMS (methyl-methanesulfonate) exposure for *Cdc25.22Δhhp1*. Serial dilutions of the indicated strains were spotted onto rich medium plates containing the indicated MMS concentrations. Plates were incubated for 4 days at 30°C.

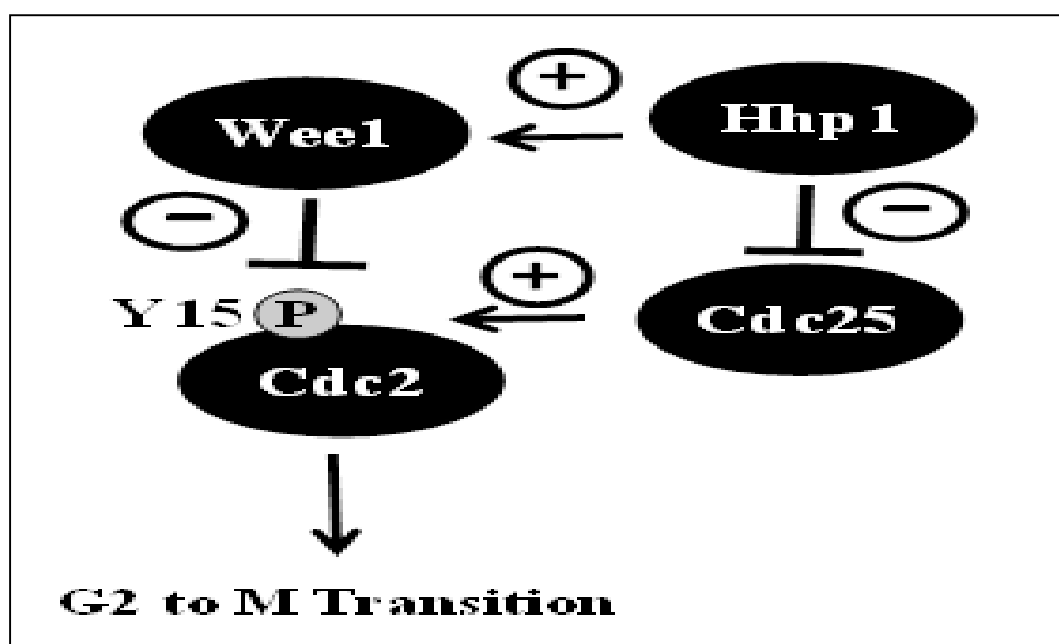


Figure: 7.3.2.7: Model of Hhp1 kinase activities with Wee1 and Cdc25. The impact of a reduction in Hhp1 activity could allow cells with a low Cdc25 phosphatase level to grow again at the restrictive temperature if Hhp1 either activates Wee1 or inactivates Cdc25. The drop in Hhp1 activity by the breast cancer mutation L51Q could reduce the accumulation of the inhibitory tyrosine 15 (Y15) phosphorylation of Cdc2 if Hhp1 activates Wee1. Alternatively, if Hhp1 blocks Cdc25, a reduction in its activity could allow the *Cdc25.22* phosphatase to be more active.

Chapter 8: Final Discussion and Conclusion

8.1. Discussion of Key Findings

The aim of this study was to dissect how CK1 (Hhp1) regulates DNA repair and cell cycle progression. Although CK1 enzymes play important roles in the circadian clock, Wnt signalling, cell cycle regulation and disease development, little is known about their DNA repair roles. Early work by Dhillon N, and Hoekstra M (15) reported that *S.pombe* cells without Hhp1 kinase are sensitive to ionising radiation and DNA alkylation by methyl-methanesulfonate (MMS). They concluded that the kinase is important for the repair of broken replication forks. This work confirms initial findings and extends the sensitivity profile of *hhp1* deletion cells to the topoisomerase 1 inhibitor camptothecin (CPT). This anti-cancer drug stabilises the topoisomerase 1- DNA cleavage complex in front of advancing replication forks. The collision of forks with this obstacle leads to a S phase specific DNA (559). Eukaryotic cells employ two DNA damage checkpoint pathways which respond differentially dependent on the DNA replication problem. When replication forks stall in the absence of nucleotides, *S.pombe* cells activate Cds1 kinase which is recruited to the fork by the scaffold protein Mrc1 (194, 237). The breakage of forks triggers the activation of Chk1 kinase which is recruited by the scaffold protein Crb2 to the damaged chromosome (246). How cells change from one system to the other system is largely unknown. Work in *S.cerevisiae* indicates that the response to broken forks is delayed until cells exit S phase (558). One key player in the repair of damaged forks is the structure-specific endonuclease Mus81-Eme1 (515, 539). Stalled forks are protected by Cds1 which phosphorylates Mus81 to remove the endonuclease from the nucleus. How the enzyme returns later from the cytoplasm to repair broken forks is still unclear, but it requires the dual phosphorylation of the regulatory subunit Eme1 first by the cell cycle regulator Cdc2 and later by Chk1 kinase (515) (Figure: 8.1.1). Cdc2 phosphorylates the repair complex during each G2 phase which primes the enzyme for its activities in the nucleus. Chk1 activates the enzyme when broken forks are detected. To be fully active, the Mus81-Eme1 complex requires also the DNA helicase Srs2 although this requirement does not depend on its helicase activity (517).

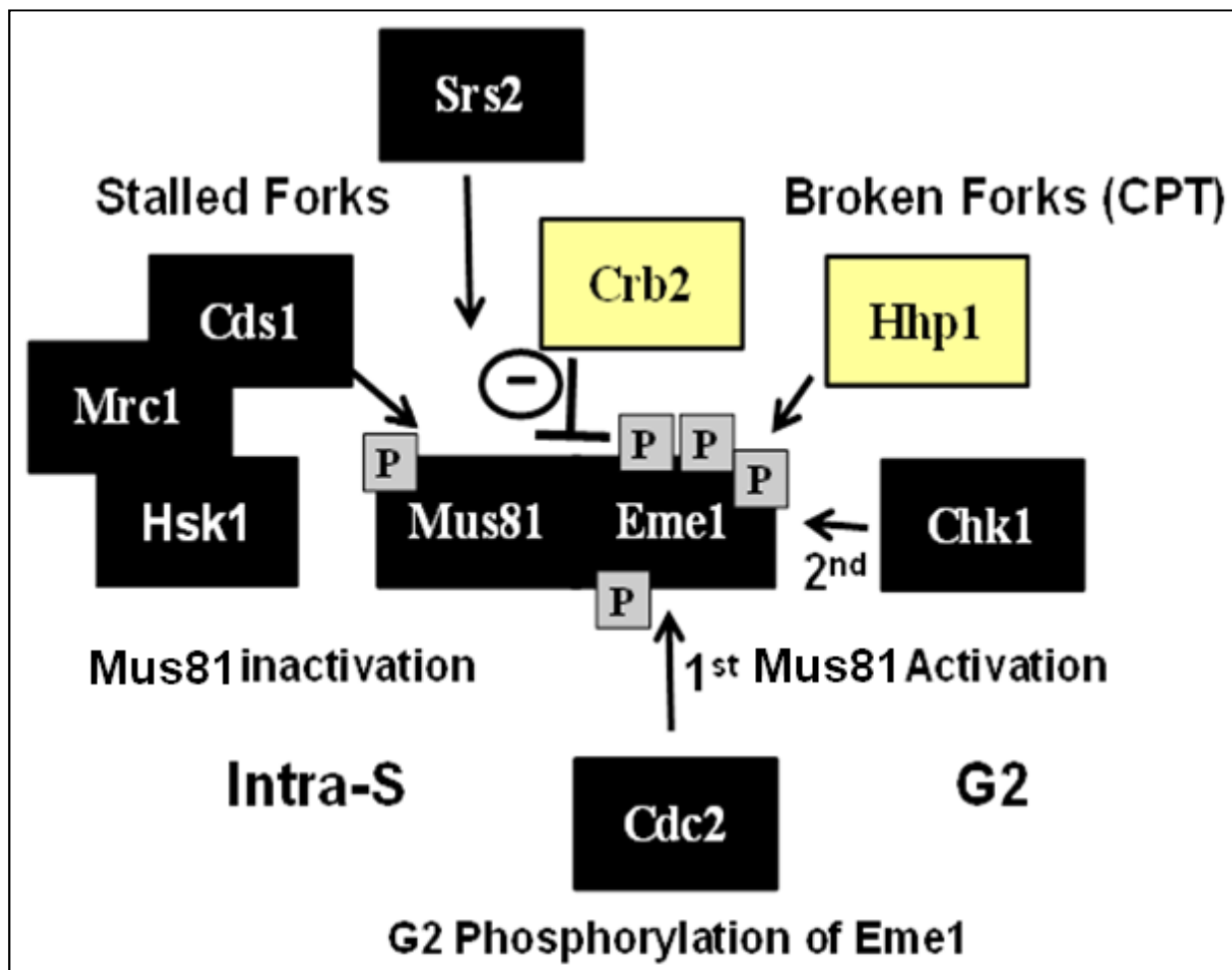


Figure: 8.1.1: How CK1 (Hhp1) may affect the repair of broken replication forks. Hhp1 is predicted to activate the endonuclease Mus81-Eme1 once it has been phosphorylated by Cdc2 and Chk1 kinase in G2. When DNA replication forks stall in the absence of nucleotides, Cds1 phosphorylates Mus81 to remove it from the nucleus. How the endonuclease returns to the nucleus to repair broken forks in G2 is not yet clear but may require the Cdc2-dependent phosphorylation of Eme1 which occurs during the normal cell cycle. Activation by Chk1 occurs in the presence of broken replication forks and is expected to initiate repair. The full activity of the endonuclease depends also on Srs2 DNA helicase in a manner which is independent on its helicase activity. The activating phosphorylation of the Mus81-Eme1 complex by CK1 (Hhp1) may promote, for example, the association of Srs2 with the primed Mus81-Eme1 complex.

The results presented in **Chapter 3** place Hhp1 genetically in the same pathway as Cds1, Mrc1, Srs2, Hsk1, Mus81 and Mus7. Interestingly the loss of Cds1, Mrc1 and Chk1 share the tendency to slightly reduce the sensitivity of *hhp1* deletion cells (**Figure: 3.3.1**, **Figure: 3.3.2.1**, **Figure: 3.3.3.1**). This indicates that the execution point of Hhp1 is after the activities of these three proteins. The involvement of Hsk1 (Cdc7) kinase and the fork protection protein Swi1 (Timeless) is in line with their requirements to activate and recruit Cds1 to stalled forks (118).

Consistent with a close link between Hhp1 and Mus81, Hhp1 kinase acts in parallel to the DNA recombination factor Rad51 and the DNA end processing protein Ctp1 (Figure: 3.4.8, Figure: 3.4.9). The same characteristics also apply to Mus81 (Figure: 3.3.1.1). This raises the interesting question of how Hhp1 kinase could activate the Mus81-Eme1 complex? As reported in Chapter 4, Srs2 DNA helicase and Hhp1 kinase act in the same camptothecin response pathway (Figure: 3.3.3.1). Since Srs2 is important to fully activate Mus81-Eme1, Hhp1 phosphorylation of either the Mus81-Eme1 endonuclease or the Srs2 DNA helicase could promote the association between these proteins thereby promoting the repair of broken forks. An alternative explanation is provided by the interesting observation that loss of Crb2 abolishes the extended G2 arrest of *hhp1* deletion cells (Figure: 4.7.3). While wild type cells arrest only briefly (20-40 min) in G2 after cells were treated with CPT (Figure: 3.1.5A), Δ *hhp1* cells arrest for 120-180 min (Figure: 3.1.5B). This prolonged arrest could be linked with the failure to activate DNA repair by Mus81-Eme1 or it could be a failure to switch off the checkpoint signal. Since the G2 delay of Δ *srs2* cells, which display a slightly longer G2 arrest of approximately 60 min (Figure: 4.6.3), is also suppressed upon deletion of *crb2* (272), it is possible that Crb2 needs to be removed from DNA to either allow the activation of Mus81-Eme1 or to enable an alternative repair pathway to take over. Crb2 binds directly to methylated DNA via its Tudor domain, and to DNA which is phosphorylated at break sites at histone H2A via its two C-terminal BRCT domains (216). Chapter 4 also provides evidence for a second important role of Cdc2 kinase in the regulation of this repair process. During mitosis, Cdc2 phosphorylates Crb2 at threonine 215 (272, 557), and the removal of this phosphorylation site (*crb2.T215A*) is as efficient as the deletion of *crb2* to abolish the extended G2 arrest in Δ *hhp1* and Δ *srs2* cells (Figure: 4.6.5, Figure: 4.7.3). This implies that the T215 phosphorylated Crb2 protein blocks the repair of broken replication forks by Mus81-Eme1 possible because it still carries the mitotic imprint. Hhp1 kinase (CK1) may be required for the removal of this imprint thereby switching the DNA repair system from its M/G1 mode to its G2 mode (272) (Figure: 8.1.2).

In Chapters 5, 6 and 7, different Hhp1 point mutants have been studied. This work identified two important separation-of-function mutants. The enlargement of the ATP binding site upon replacement of methionine 84 by a smaller glycine residue (M84G) has very little impact on the DNA damage sensitivity, but results in a prolonged G2 arrest when DNA replication forks break (Figure: 5.1.8). This difference implies that different levels of Hhp1 kinase activity are required

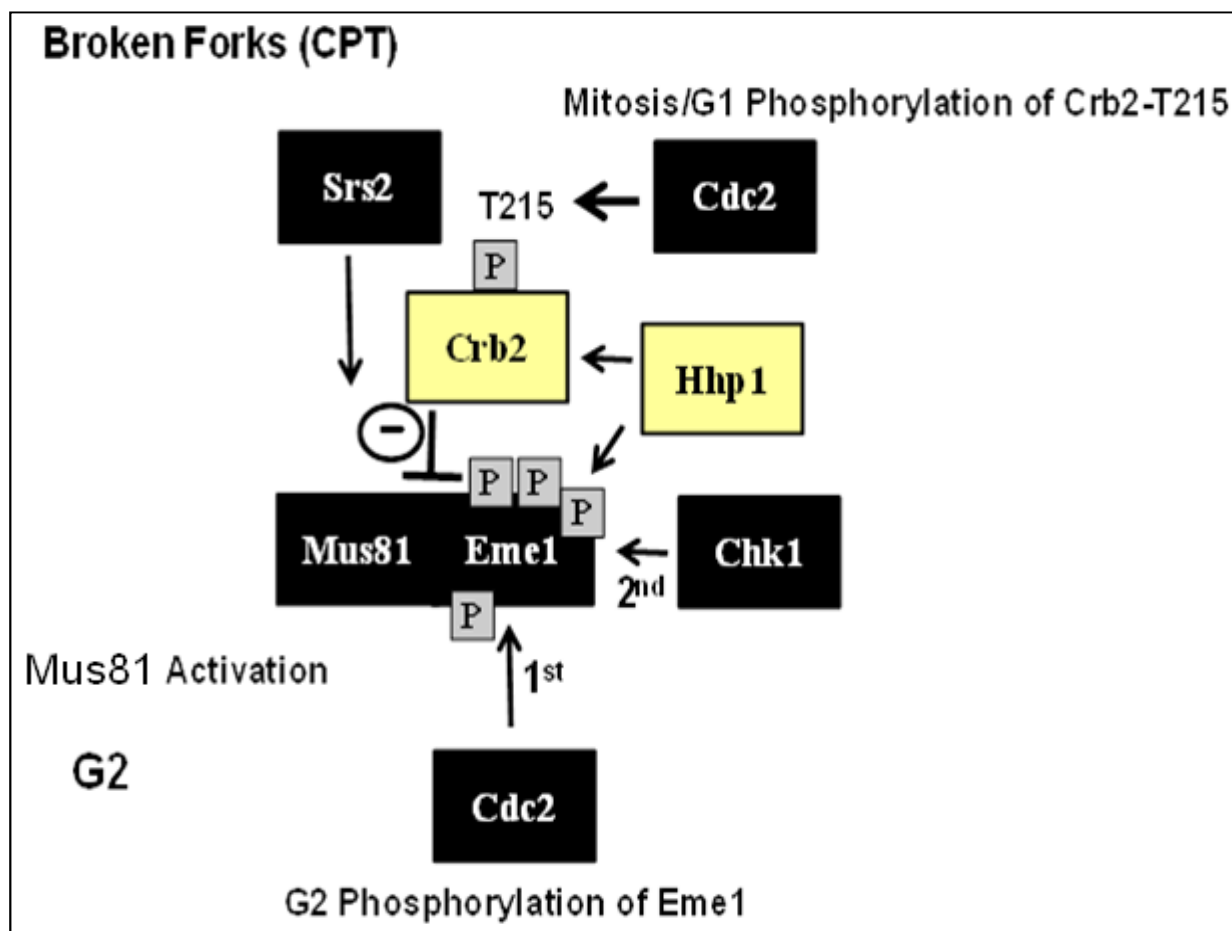


Figure: 8.1.2: The alternative activity of Hhp1 (CK1) kinase could be related to the removal of the mitotic phosphorylation of Crb2 at threonine 215 by Cdc2 as this modification may allow Crb2 to block the repair of broken replication forks by Mus81-Eme1.

for Hhp1 kinase activity are required for the regulation of DNA repair and the cell cycle. While a small drop in activity affect the cell cycle delay, it does not affect DNA repair. One way to explain this may be provided by the different modes of target recognition by CK1 (Hhp1). While most CK1 sites require a priming kinase, which phosphorylates the N-terminal serine or threonine of the S/T (P)-X1-X2-S/T motive first, this priming event can be substituted by acidic, negatively charged amino acids (1). If the phosphorylation of the primed sites were to be more efficient than the modification of the acidic motifs, a limited decline in Hhp1 activity may affect the acidic motives first. If this were to be the case, two different key targets are important for the DNA repair and cell cycle activity of Hhp1. The DNA repair target is most likely Mus81-Eme1, whereas the cell cycle target could be linked with Cdc25.

Chapter 7 reports that the breast cancer mutation L51Q in Hhp1 suppresses the temperature sensitivity of the *cdc25.22* mutation (Figure: 7.3.2.2). Since the inhibitory tyrosine 15 modification of Cdc2 is removed by Cdc25 phosphatase (363, 395, 412), normally mutations which increase Cdc2 activity like the deletion of *wee1* kinase, which phosphorylates Y15, or the gain-of-function allele of *cdc2*, *cdc2.1w*, which renders Cdc2 insensitive to Wee1 inhibition (220, 221, 222, 224, 226, 272), suppress *cdc25* mutations, it is possible that loss of Hhp1 kinase activity increases Cdc2 kinase levels. If this were to be the case, loss of Hhp1 could lead to a prolonged G2 arrest indirectly by increasing Cdc2 activities (Figure: 8.1.3). This conclusion is supported by the extended G2 arrest of *cdc2.1w* cells in the presence of CPT (Figure: 4.3.3) which is abolished upon mutation of the T215 phosphorylation site in Crb2 (Figure: 4.5.6). Hence, deletion of Hhp1 may extend the mitotic imprint of Crb2 indirectly by elevating Cdc2 activity.

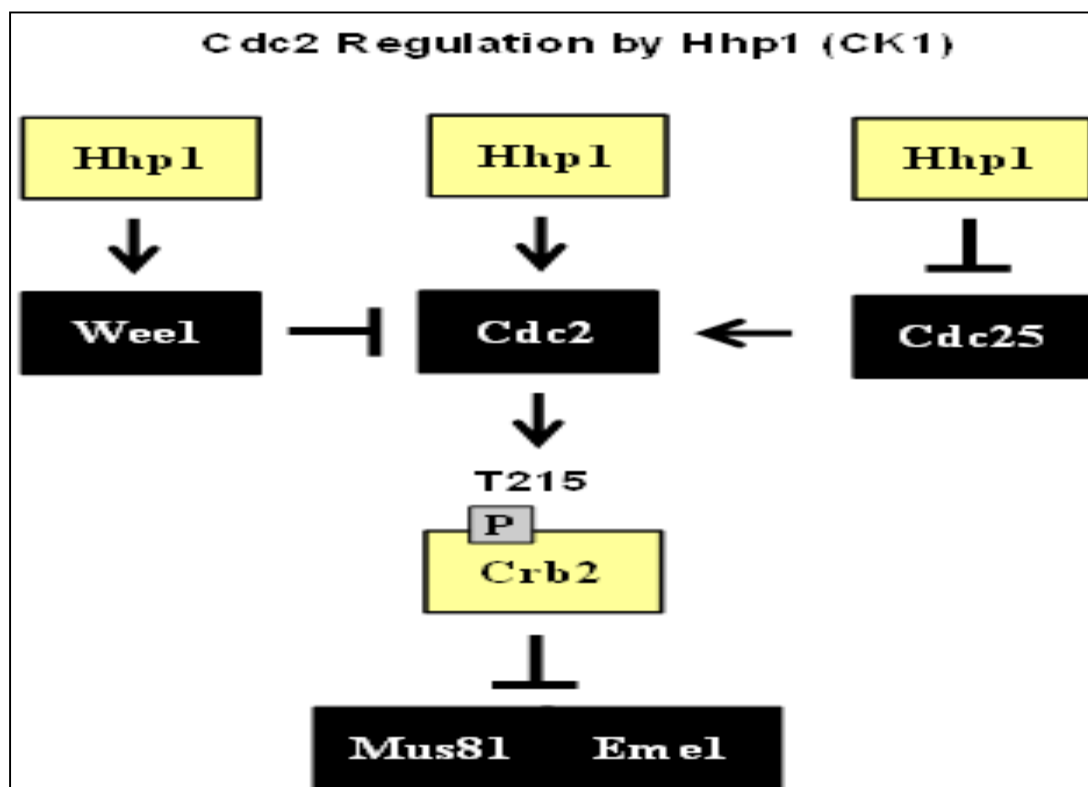


Figure: 8.1.3: Cell Cycle regulation by Hhp1 (CK1). Loss of Hhp1 (CK1) activity may either reduce the inhibitory function of Wee1 kinase or enhance the activating function of Cdc25 phosphatase. This would result in an increase in Cdc2 activity, as in the *cdc2.1w* mutant, and the accumulation of the mitotic imprint on Crb2 which blocks the repair activity of Mus81-Eme1. Hhp1 may also directly target Cdc2 as indicated by its association with the cell cycle regulator (272).

There is however one problem with this explanation since *Ahhp1* cells are elongated (i.e. delayed in G2) and not short (i.e. advance into mitosis like *cdc2.1w* cells), which shows that the cell cycle promoting activity of Cdc2 is not elevated. This conundrum could be resolved by the finding that *S.pombe* cells express five phosphorylated and two unphosphorylated forms of Cdc2 (554). Only two phosphorylated forms regulate cell cycle progression, whereas the two unphosphorylated forms regulate DNA repair. There is also one phosphorylated form with unknown function which is not the target of Wee1. Hhp1 may phosphorylate this form of Cdc2 directly to coordinate Crb2 and Mus81. A direct link between Cdc2 and Hhp1 is supported by the physical association between both kinases (272).

The second interesting separation-of-function mutant is the breast cancer mutation L51Q. As summarised in **Chapter 7**, this mutation affects the cell cycle delay only when DNA replication forks break in CPT medium but not when cells are exposed to heat stress. As reported previously (229), heat stress triggers an extended G2 arrest in wild type cells of approximately 180 min (**Figure: 7.3.10**) which is extended to 340 min in the absence of Hhp1 (**Figure: 7.3.11**) (**Table: 8.1.1**). Why the L51Q mutation in the vicinity of the ATP binding site only affects the DNA damage induced G2 arrest but not the heat induced G2 arrest is as yet unclear.

As shown in **Chapter 5**, the DNA repair and cell cycle functions of Hhp1 could also be separated by a mutation in the nuclear localisation sequence. Replacing tyrosine 227 (*hhp1.Y227F-HA*) at the bottom of the cleft which forms the nuclear localisation domain, had only a small impact on the cell cycle delay in CPT medium (**Figure: 5.3.4**) while turning the cells highly CPT sensitive (**Figure: 5.3.2**). Deletion of the entire NLS in frame caused a similar phenotype (**Figure: 5.3.6**, **Figure: 5.3.9**). This implies that nuclear localisation is important for the DNA repair activity but not for the cell cycle function of Hhp1. This is in line with the proposed regulation of the endonuclease Mus81-Eme1 in the nucleus (**Figure: 8.1.4**). Given that the cell cycle regulator Cdc2 resides at the spindle pole body in *S.pombe*, which is accessible from the cytoplasm (210, 556), there may be no need for Hhp1 to enter the nucleus to target Cdc2.

	Resistance to DNA damage		Lethal rate	G2 delay (CPT)	G2 delay (40 °C)	Kinase activity (high/ low)
	DSBs-CPT	MMS				
<i>hhp1.HA.wt</i>	yes	yes	low	20-40 min control	180 min control	normal
Δ <i>hhp1</i>	no	no	high	>120 min	>340 min	---
<i>hhp1.M84G</i>	yes	yes	low	180 min	60 min	low
<i>hhp1.M84G.Y169F</i>	yes	yes	low	40 min	200 min	low
<i>hhp1.M84G.Y227F</i>	no	no	high	160 min	220 min	high
<i>hhp1.M84G.NLS.del</i>	no	no	high	80 min	200 min	high
<i>hhp1.L51Q</i>	no	no	high	80 min	220 min	high
<i>hhp1.S183A</i>	yes	yes	high	>120 min	240 min	low
<i>hhp1.R180C</i>	yes	yes	low	60 min	>240 min	high
<i>hhp1.R180C.K40R</i>	no	no	high	>120 min long	240 min	low
<i>hhp1.R180C.M84G</i>	no	no	high	>120 min short	---	low
<i>hhp1.R180C.C-terminal.del</i>	yes	yes	low	60 min	180 min	Very low
<i>hhp1.K40R</i>	no	no	high	>120 min long	>280 min	low
<i>hhp1.M82I</i>	no	yes	high	>120 min	220 min	low
<i>hhp1.P49S</i>	no	no	high	80 min	160 min	high

Table: 8.1.1: Characterizing summary of Hhp1 kinase mutants. Some Hhp1 mutants are sensitive to DSBs and that enhanced lethal ratio, as consequence they will have a long G2-arrest. This is theory maybe right for almost the Hhp1 mutants, but existing of mutant at any site of the kinase could make a phenotype and change the kinase activities i.e., Hhp1.S183A, and Hhp1.M84G.

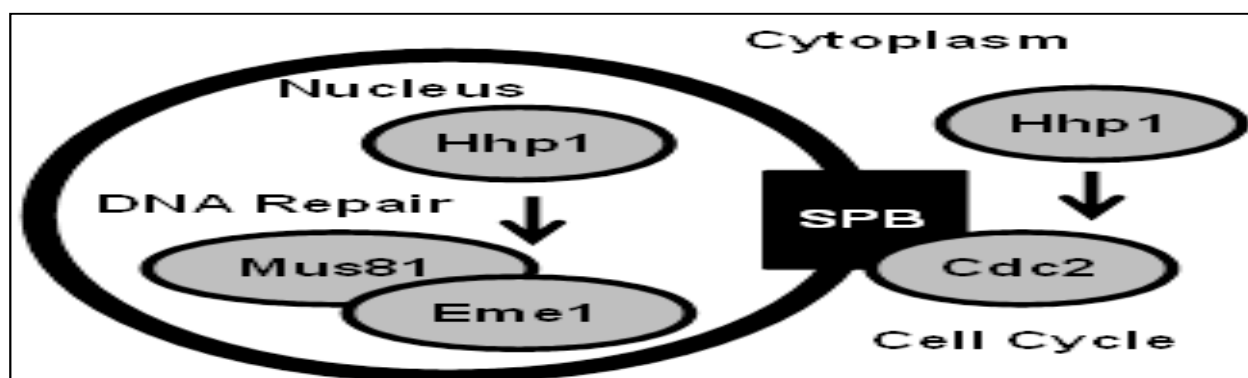


Figure: 8.1.4: Mutations in the Nuclear Localisation Sequence separate the DNA repair and cell cycle activities of Hhp1 kinase. An intact NLS is important to regulate the repair enzyme Mus81-Eme1 in the nucleus, while the cell cycle regulator resides at the spindle pole body (SPB) and may be accessible from the cytoplasm. Loss of Chk1 partly suppressed the CPT sensitivity of the NLS mutants (Figure: 5.4.3) probably because Mus81-Eme1 is not primed which may allow an alternative repair pathway to take over.

Chapter 6 reports the finding that the three circadian clock mutations (R180C, M82I and P49S) all affect the cell cycle activity but only to a very small extent the DNA repair function. This finding is in line with the earlier discussion point that enlargement of the ATP binding site (M84G) results in a similar separation of function. All four mutations probably only reduce the kinase activity of Hhp1, which is consistent with similar observations in *Drosophila* (62, 64), and could be explained by a different affinity of Hhp1 for primed versus acidic target sequences. What was however novel are the biochemical changes caused by the circadian clock mutations. Isoelectric focusing revealed four to five forms of wild type Hhp1 which did not change in the response to DNA damage (Figure: 6.2.1.9, Figure: 6.2.1.10). In the tau mutation R180C, which shortens the clock in hamster (69), caused the change of one form of isoelectric points (Figure: 6.2.1.9) which became more positive. This could identify this form of Hhp1 as the form which regulates Cdc2 as the R180C mutant had a cell cycle defect but a normal DNA repair response. Two of these forms, the two more negative forms, disappeared upon deletion of the C-terminal tail domain which places both post-translational modifications in this part of Hhp1. It was also interesting to find that the kinase dead mutant (K40R) contained all forms of the kinase which strongly implies that auto-phosphorylation is not the cause of these modifications.

8.2. Conclusion

The main conclusions from this work are **(i)** that CK1 (Hhp1) has two important cellular execution points: the repair of broken DNA replication forks and G2 cell cycle arrests in the presence of DNA damage or when cells are exposed to heat stress; **(ii)** that CK1 is an important regulator of the DNA repair enzyme Mus81-Eme1 which acts on broken replication forks; **(iii)** that mutations which are linked with cancer and disorders of the circadian clock cause a partial loss of kinase activity which could be explained by different affinities of CK1 to primed (first phosphorylated by a priming kinase) and acidic phosphorylation sites; and **(iv)** that CK1 exists in at least six forms in fission yeast with different isoelectric points which may explain how one kinase can cover such a broad range of functions.

Further work is however required to translate these important findings from the model organism *Schizosaccharomyces pombe* to human cells.

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10. Appendix 1

Epistatic Relationships Among <i>S. pombe</i> Proteins		
Protein Name	Protein Name	References
<i>hhp1</i>	<i>srs2</i> =ATP-dependent DNA helicase	Thesis data
	<i>chk1</i> =protein kinase	Thesis data
	<i>rad3</i> =ATR checkpoint kinase	Thesis data
	<i>cds1</i> =replication checkpoint kinase	Thesis data
	<i>rad22</i> =DNA recombination protein	Thesis data
	<i>mus7</i> =DNA repair protein	Thesis data
	<i>mus81</i> =Holliday junction resolvase subunit	Thesis data
	<i>ku70</i> =domain protein Pku70	Thesis data
	<i>sty1</i> =MAP kinase	Thesis data
	<i>hsk1</i> =Dbf4(Dfp1)-dependent protein kinase	Thesis data
	<i>mrc1</i> =mediator of replication checkpoint 1	Thesis data
	<i>fbh1</i> =DNA helicase I	Thesis data
	<i>cdc25</i> =M phase inducer tyrosine phosphatase	Thesis data
	<i>wee1</i> =M phase inhibitor protein kinase	Thesis data
	<i>mad2</i> =spindle checkpoint protein	Thesis data
	<i>mik1</i> =mitotic inhibitor kinase	Thesis data
	<i>crb2</i> =DNA repair protein	Thesis data
	<i>ku80</i> =domain protein Pku80	Thesis data
	<i>cdc2</i> = cyclin-dependent protein kinase	Thesis data
<i>srs2</i>	<i>hhp1</i> =serine/threonine protein kinase	Thesis data
	<i>chk1</i> =protein kinase	Thesis data
	<i>rqh1</i> =RecQ type DNA helicase	250, 254, 184
	<i>rad51</i> =RecA family recombinase	238, 255, 184
	<i>rad22</i> =DNA recombination protein	238, 255, 184
	<i>cdc25</i> =phase inducer tyrosine phosphatase	Thesis data
	<i>cdc2</i> = cyclin-dependent protein kinase	Thesis data, and 240
<i>chk1</i>	<i>hhp1</i> =serine/threonine protein kinase	Thesis data
	<i>rad3</i> =ATR checkpoint kinase	179, 293
	<i>cdc2</i> =cyclin-dependent protein kinase	Thesis data
	<i>srs2</i> =ATP-dependent DNA helicase	Thesis data
<i>tel1</i>	<i>cds1</i> =replication checkpoint kinase	Thesis data
<i>rad51</i>	<i>rqh1</i> =RecQ type DNA helicase	184
	<i>rad22</i> =DNA recombination protein	322, 277
	<i>fbh1</i> =DNA helicase I	318
<i>rad22</i>	<i>rqh1</i> =RecQ type DNA helicase	238, 255, 184
<i>crb2</i>	<i>cdc2</i> =cyclin-dependent protein kinase	Thesis data
<i>mus81</i>	<i>rad22</i> =DNA recombination protein	277
	<i>mus7</i> =DNA repair protein	257

Table: 10.1: Discovery of Epistatic Relationships Among *S. pombe* Proteins. Here in this is table above an epistatic genetic co-operations were identified among yeast proteins in association of cellular DNA repair events.

11. Appendix 2

7734-7747 *Nucleic Acids Research*, 2014, Vol. 42, No. 12
doi: 10.1093/nar/gku452

Published online 26 May 2014

Hyperactive Cdc2 kinase interferes with the response to broken replication forks by trapping *S.pombe* Crb2 in its mitotic T215 phosphorylated state

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Received October 20, 2013; Revised May 03, 2014; Accepted May 7, 2014

ABSTRACT

Although it is well established that Cdc2 kinase phosphorylates the DNA damage checkpoint protein Crb2^{53BP1} in mitosis, the full impact of this modification is still unclear. The Tudor-BRCT domain protein Crb2 binds to modified histones at DNA lesions to mediate the activation of Chk1 by Rad3^{ATR} kinase. We demonstrate here that fission yeast cells harbouring a hyperactive Cdc2^{CDK1} mutation (*cdc2.1w*) are specifically sensitive to the topoisomerase 1 inhibitor camptothecin (CPT) which breaks DNA replication forks. Unlike wild-type cells, which delay only briefly in CPT medium by activating Chk1 kinase, *cdc2.1w* cells bypass Chk1 to enter an extended cell-cycle arrest which depends on Cds1 kinase. Intriguingly, the ability to bypass Chk1 requires the mitotic Cdc2 phosphorylation site Crb2-T215. This implies that the presence of the mitotic phosphorylation at Crb2-T215 channels Rad3 activity towards Cds1 instead of Chk1 when forks break in S phase. We also provide evidence that hyperactive Cdc2.1w locks cells in a G1-like DNA repair mode which favours non-homologous end joining over interchromosomal recombination. Taken together, our data support a model such that elevated Cdc2 activity delays the transition of Crb2 from its G1 to its G2 mode by blocking Srs2 DNA helicase and Casein Kinase 1 (Hhp1).

INTRODUCTION

Despite the importance of cyclin-dependent kinases (CDKs) for the regulation of the DNA damage response (DDR) (1,2,3), it is still enigmatic how CDKs act as activators of DNA repair while being down-regulated by the DNA damage checkpoint. Two

possible answers to this puzzle may lie in the temporal or spatial organization of CDKs.

The activity of CDK1-cyclin B kinase peaks early in mitosis (4) and this rise could prime DDR proteins for their roles in the next cell cycle. Between the end of G2 and the start of the subsequent S phase, cells repair broken chromosomes by non-homologous end-joining (NHEJ) (5,6). To facilitate NHEJ, the Ku70-Ku80 DNA binding complex and the chromatin protein 53BP1^{Crb2, Rad9} prevent the resection of broken DNA ends (7,8,9). It is widely assumed that the transition from NHEJ to homologous recombination (HR) in S phase requires the activity of CDKs. Interestingly, neither progression through S phase nor the presence of the sister chromatid are necessary to initiate HR (10).

Alternative to this temporal regulation, the DNA repair choice could be controlled by CDKs directly at the chromatin. In fission yeast, Cdc2-cyclin B kinase associates with origins of replication on chromosomal pool of Cdc2 also activates HR is currently unknown. In human cells, the Mre11 subunit of the MRN (Mre11-Rad50-Nbs1) complex recruits CDK2-cyclin A to broken chromosomes to promote the formation of a complex between BRCA1 and the endonuclease CtIP^{Ctp1, Sae2} (12,13). This BRCA1-CtIP complex stimulates end resection and HR. Such a spatial separation would allow the DNA damage checkpoint to stop progression into mitosis by targeting CDK at the centrosome (14) while leaving the chromosomal CDK pool active to promote DNA repair.

A DDR protein which exhibits an oscillating change in its activity throughout the cell cycle is the BRCT and tudor domain protein 53BP1^{Crb2, Rad9}. In G1, human 53BP1 recruits Rif1 to broken DNA to block end resection, a step which is antagonized in S/G2 by BRCA1 in association with CtIP^{Ctp1, Sae2} (13,9,15). After the G1/S transition

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tion, 53BP1 turns into an activator to promote the association between the checkpoint kinases ATM^{Te11} and Chk2^{Cds1} (16). This activator role is later switched off by Polo-like kinase when cells enter mitosis (17). Despite this cyclic regulation, the relationship between human 53BP1 and CDKs is only poorly understood (17). At the mechanistic level, much more is known about the CDK1-dependent regulation of the 53BP1 paralog, Crb2, in fission yeast (*Schizosaccharomyces pombe*). Cdc2 phosphorylates the N-terminus of Crb2 at threonine-215 in mitosis which triggers the formation of a complex between Crb2 and the BRCT domain protein Rad4^{Cut5, Dpb11, TopBP1} on the chromatin (18,19). The cellular activities of the mitotic Crb2-Rad4 complex are not known and are not related to the recruitment of Crb2 to a broken chromosome. The latter process depends on the association of Crb2 with phosphorylated histone H2A through its C-terminal BRCT domains and with K-20 methylated histone H4 through its Tudor domains (20). The mitotic T215 phosphorylation primes, however, the Crb2-Rad4 complex for its rearrangement in G2. Once Cdc2 activity has increased again at the start of G2, Rad4 recruits the kinase to the complex where it modifies a non-canonical Cdc2 site at T187. This enables Crb2 to bind to Chk1, and Rad4 to associate with the Rad9-Rad1-Hus1 checkpoint clamp (21).

Since most studies concerning the DNA repair roles of CDKs were conducted with inhibitors or proteins lacking a CDK phosphorylation site (22), we took advantage of the hyperactive *cdc2.1w* allele in *S. pombe* to investigate how the untimely activation of CDK1^{Cdc2} might affect the DDR. Like most *wee* mutations ('wee' cells are short), the *cdc2.1w* (*wee2-1*) mutant enters mitosis prematurely (23,24). The Cdc2.1w kinase carries a glycine-to-aspartate (G146D) replacement in the vicinity of its ATP binding site which renders it insensitive to the inhibition by Wee1 kinase (25). Although *cdc2.1w* and *wee1* deletion cells share the same cell cycle phenotype, only *Δwee1* cells are known to be sensitive to the DNA replication inhibitor hydroxyurea, UV light and ionizing radiation (26,27).

We report here that elevated Cdc2 activity renders *S. pombe* cells specifically sensitive to the topoisomerase 1 poison camptothecin (CPT), an anti-cancer drug which breaks DNA replication forks. While wild-type cells delay mitosis only briefly in G2 when replication forks collapse by activating the Rad3^{ATR}-Crb2^{53BP1}-Chk1 checkpoint pathway, *cdc2.1w* cells enter a prolonged G2 arrest independently of Chk1. Our data suggest that hyperactive Cdc2.1w traps Crb2 in its G1 mode by blocking Srs2 DNA helicase and Casein Kinase 1 (Hhp1). As this correlates with an increase in NHEJ and a decrease in interchromosomal recombination, the repair of broken replication forks may be delayed leading to the aberrant activation of Cds1 and an extended G2 arrest.

MATERIAL AND METHODS

Strains

The following *S. pombe* strains were used in this study: wild-type 804 (*h- ade6-M210 leu1-32 ura4-D18*), *Δchk1* (*h- ade6-M210 chk1::ura4+ leu1-32 ura4-D18*), *cdc2.1w* (*h- ade6-M210 leu1-32 ura4-D18 cdc2.1w*), *cdc25.22* (*h-*

cdc25.22 ade6-M210 leu1-32 ura4-D18), *Δhhp1* (*h- ade6-M210 hhp1::hphMX6 leu1-32 ura4-D18*), *Δwee1* (*h- ade6-M210 wee1::ura4+ leu1-32 ura4-D18*), Hhp1-HA₃ (*h- ade6-M210 leu1-32 ura4-D18 hhp1::hhp1-HA3-kanMX4*), Ku70-GFP-HA₃ (*h90 ade6-M210 leu1-32 lys1-131 ku70::ku70-GFP-HA3-kanMX4*), Ku80-HA₃ (*h90 ade6-M210 leu1-32 ura4-D18 ku80::ku80-HA3-ura4+*), Rad16-GFP-HA₃ (*h90 ade6-216 leu1-32 lys1-131 ura4-D18 rad16::rad16-GFP-HA3-kanMX4*), Srs2-Myc₁₃ (*h- ade6-216 leu1-32 ura4-D18 srs2::srs2-HA3-kanMX4*), Cdc13-HA₃ (*h- ade6-216 leu1-32 ura4-D18 cdc13::cdc13-HA3-ura4+*), Chk1-HA₃ (28), Myc₁₃-Rqh1 (2), Mus81-Myc₁₃ (29), Hhp1-GFP (*hhp1-GFP-kanMX4 ade6-216 leu1-32 ura4-D18*) (30). To construct multiple deletion strains, the following gene deletions were employed: *Δcds1* (*ade6-M210 cds1::ura4+ ura4-D18*), *Δchk1* (*ade6-M210 chk1::kanMX4 leu1-32 ura4-D18*), *Δsrs2* (*ade6-M210 srs2::kanMX4 leu1-32 ura4-D18*), *Δrad3* (*ade6-M210 rad3::ade6+ leu1-32 ura4-D18*), *Δcrb2* (*ade6-M210 crb2::ura4+ leu1-32 ura4-D18*). The *crb2-T215* mutant is described in (18).

Biochemical techniques

Isoelectric focusing, native protein extracts and total protein extracts are described in (31) and size fractionation is documented in (32).

Cell synchronisation and DNA repair assays

The preparation of lactose gradients has been reported in (31) and the inter-sister recombination assay is described in (33). The strains used in the recombination tests were: wild type (*h- ade6-M210 leu1-32 ura4-D18 ade6-L469-ura4+ ade6-M375*), *Δsrs2* (*h- ade6-M210 srs2::kanMX4 leu1-32 ura4-D18 ade6-L469-ura4+ ade6-M375*), *cdc2.1w* (*cdc2.1w ade6-M210 leu1-32 ura4-D18 ade6-L469-ura4+ ade6-M375*) and *cdc2.1w Δsrs2* (*h- cdc2.1w srs2::kanMX4 ade6-M210 leu1-32 ura4-D18 ade6-L469-ura4+ ade6-M375*).

Break-induced recombination: The assay uses the *S. cerevisiae* HO (Homothallic switching) endonuclease to cleave the Ch¹⁶-MG minichromosome at one defined DNA sequence as described in (34). The assay was performed with the following changes: wild-type cells and *cdc2.1w* cells containing Ch¹⁶-MG were transformed with the plasmid pREP81X-HO-*Leu2+* and maintained on minimal medium plates (+thiamine, + uracil). Single colonies were grown into stationary phase either in the absence or presence of thiamine (15 M thiamine (5ug/ml)) at 30°C in 10 ml of minimal medium (+ uracil). Dilutions were plated on minimal medium plates (+thiamine, +uracil). Colonies were then replica-plated onto rich medium plates with 75 g/ml G418 to determine the ration of recombinogenic cells. Adenine was omitted at all stages to avoid false-positive colonies which can arise due to the loss of the mini-chromosome.

Plasmid repair assay: cells were transformed with equal amounts of either cut (SacI) or uncut pREP41-*Leu2+* plasmid. Cells were plated on minimal medium plates with uracil and adenine and *leu2+* colonies were counted after 4-5 days at 30°C.

Antibodies

The following antibodies were used: anti-HA (Santa Cruz: SC7392), anti-Myc (Santa Cruz SC40), anti-Cdc2 (Abcam AB70860), anti-GFP (Roche Applied Science 11814460001).

GFP-Trap

Soluble protein extracts were prepared by breaking 5×10^8 cells in lysis buffer (50 mM HEPES pH 8.0 200 mM KoAC, 20 mM NaCl, 1 mM EDTA, 0.1% Nonidet, 20 mM beta-glycerol phosphate, 0.1 mM NaF, 1:100 diluted Melford protease inhibitors (IV, P2402), 1 mM DTT). Proteins fused to the Green Fluorescent Protein (GFP) were purified by adding 10 l of GFP-nAb Agarose (Insight Biotechnology, ABP-NAB-GFPA025) to 1 ml of the lysis buffer containing 100–200 ul soluble protein extract.

RESULTS

Elevated Cdc2 activity renders *S. pombe* cells sensitive to CPT

We took advantage of the *cdc2.1w* (*wee2-1*) mutation in fission yeast to find out whether elevated CDK levels interfere with the DDR. The Cdc2.1w kinase is hyperactive due to a glycine-to-aspartate (G146D) replacement at the entrance to its ATP binding site (Figure 1A). This mutation renders Cdc2.1w insensitive to the inhibition by Wee1 kinase (23,24). Although loss of Wee1 (*Δwee1*) advances entry into mitosis like the *cdc2.1w* mutation, only *Δwee1* cells are known to be DNA damage sensitive (26,27).

Our own survival assays performed with wild type, *Δwee1* and *cdc2.1w* cells confirmed the previous findings for *Δwee1* cells, but also revealed a yet unknown sensitivity of *cdc2.1w* cells to the topoisomerase 1 (Top1) poison camptothecin (CPT) (Figure 1B). CPT immobilises Top1 at the DNA in front of advancing replication forks which break upon their collision with this obstacle (35). To test whether the CPT sensitivity is a consequence of elevated Cdc2 activity, we introduced a temperature-sensitive loss-of-function allele of Cdc25 phosphatase (*cdc25.22*) into the *cdc2.1w* strain. This mutant allele is known to lower Cdc2 activity due to the reduced removal of the inhibitory tyrosine-15 phosphorylation (Figure 1A) (36). As shown in Figure 1C, the *cdc2.1w cdc25.22* double mutant was CPT resistant strongly indicating that high Cdc2 activity interferes with the repair of broken replication forks. We also constructed a *cdc2.1w Δwee1* double mutant to test whether Cdc2.1w and Wee1 act jointly in the response to CPT. Although the *cdc2.1w Δwee1* strain grew more slowly in the presence of the drug, the sensitivity of the double mutant was not increased thus placing both kinases in the same pathway (Supplementary Figure S1A).

Hyperactive Cdc2 prolongs the G2/M arrest when replication forks break

Yeast cells differ in their response to collapsed replication forks from human cells which arrest in S phase, as they progress through S phase without a delay before briefly (20–40 min) arresting in G2 (37).

We used lactose gradients to synchronize wild type, *Δchk1*, *cdc2.1w* and *cdc2.1w cdc25.22* cells in G2 to analyse their cell-cycle response to 40 M CPT. Synchronized cells were released into rich medium with and without the drug, and samples were withdrawn every 20 min over 6 h. As shown in Figure 1D, wild-type cells progressed with the same rate through the first G1/S phase (first peak of septation), but delayed entry into the second cycle by 20 min in CPT medium. As expected, this checkpoint response was abolished upon deletion of *chk1* (Figure 1E). Cells with elevated Cdc2 activity showed, however, an unexpected behaviour. They postponed entry into mitosis for 2 h before slowly continuing to cycle (Figure 1F). This extended G2 arrest could be caused by unrepaired replication forks, which continue to send a Rad3^{ATR}-Chk1 signal, or by the inability of *cdc2.1w* cells to restart the cell cycle. In contrast to the CPT sensitivity, a reduction in Cdc2.1w activity by the introduction of the *cdc25.22* allele failed to suppress the extended arrest (Figure 1G). This intriguing observation implies that the CPT sensitivity and the prolonged G2/M arrest are two distinct manifestations of the *cdc2.1w* mutation. A possible explanation for the inability of Cdc25.22 to correct the cell-cycle defect lies within mitosis. Although the point mutation lowers the phosphatase activity during interphase, this effect may be neutralised by the 10-fold increase in Cdc25 activity in mitosis (4). If this were to be the case, Cdc2.1w may trigger the cell-cycle defect while cells progress through mitosis, whereas the hyperactive kinase may affect the repair of collapsed forks in G2.

The G2 arrest in *cdc2.1w* cells is independent of Chk1

Intrigued by this finding, we asked whether hyperactive Cdc2 would also impose a G2/M arrest in *Δchk1* cells given the importance of the Rad3^{ATR}-Crb2^{53BP1}-Chk1 signalling pathway in the presence of CPT (38). Rather unexpectedly, synchronized *cdc2.1w Δchk1* cells showed a similarly extended G2 arrest in CPT medium as the *cdc2.1w* single mutant (Figure 2C). This shows that hyperactive Cdc2 bypasses Chk1 to block entry into mitosis when replication forks break. Since Rad3 kinase signals either through Chk1 or Cds1^{Chk2} (39), we also tested a *cdc2.1w Δcds1* double mutant. Elevated Cdc2 activity did also impose a G2 arrest in the absence of Cds1 but this arrest was ~1 h shorter compared to the *cdc2.1w Δchk1* mutant (Figure 2D). The ability of the *cdc2.1w Δcds1* mutant to arrest and the observation that Chk1 is not required in *cdc2.1w* cells (Figure 2C) could be explained in two ways. Either a third unknown kinase is involved, or Chk1 is not important in *cdc2.1w* cells as long as Cds1 is intact. To distinguish between these possibilities, we measured the cell-cycle arrest of a *Δchk1 Δcds1 cdc2.1w* triple mutant in CPT medium. Interestingly, loss of both kinases completely abolished the G2/M arrest (Figure 2E) showing that Cds1 acts first in *cdc2.1w* cells when forks break rendering Chk1 redundant. This is an intriguing observation since Chk1 is normally the key kinase in the presence of CPT (38).

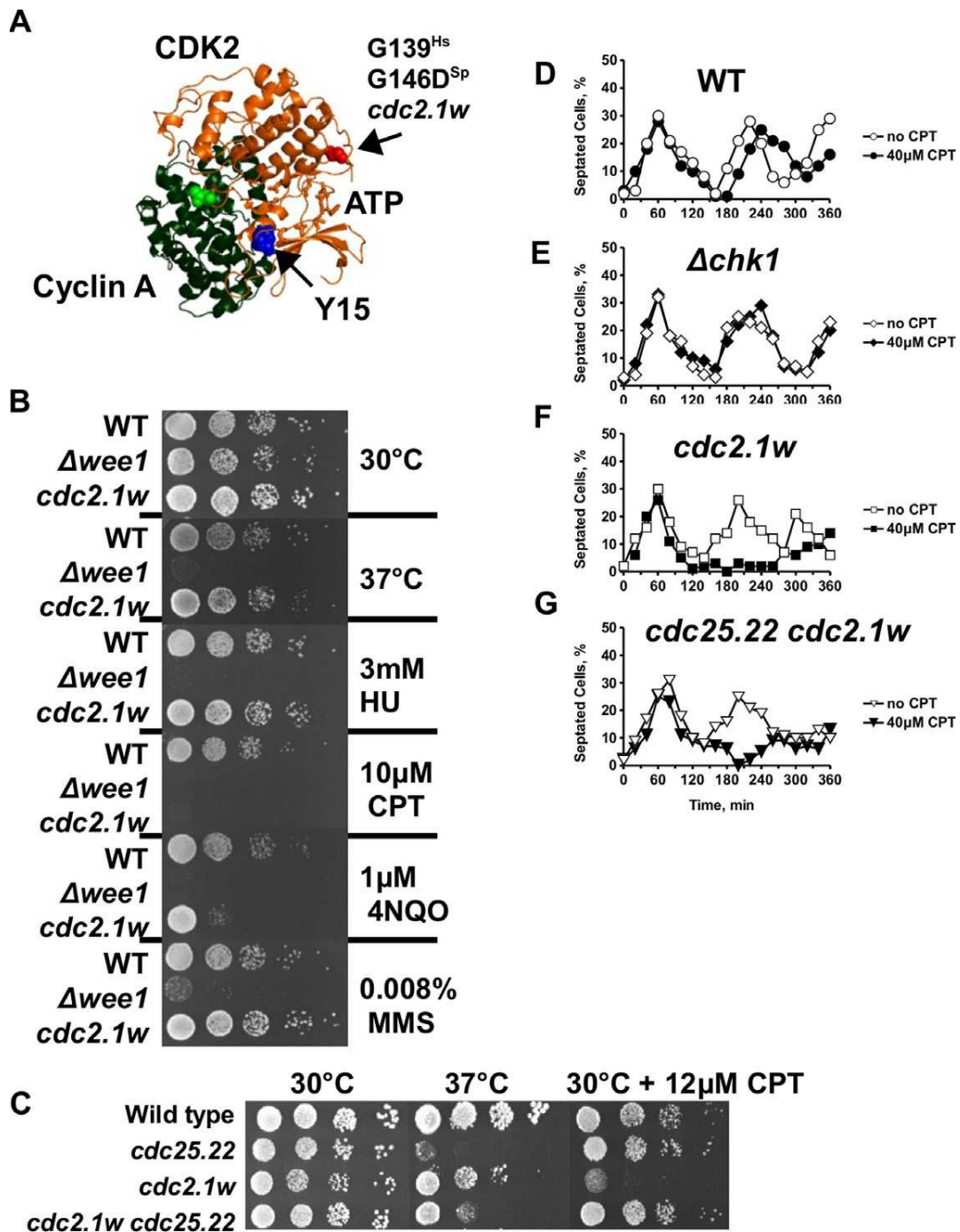


Figure 1. Cells with hyperactive Cdc2.1w kinase are CPT sensitive and enter an extended cell-cycle arrest. (A) The G146D mutation in the *S. pombe* Cdc2.1w kinase and the highly conserved Y15 phosphorylation site have been mapped onto the structure of the closely related human CDK2-cyclin A complex (PDB ID: 1FIN) using the program Polyview 3D. (B) Drug sensitivity of *cdc2.1w* cells. Serial dilutions (10-fold; starting with 10^7 cells/ml) of the listed strains were spotted onto YEA plates containing the indicated drugs. The plates were incubated at 30°C for 3 days. One YEA plate was incubated at 37°C. (C) Introduction of the *cdc25.22* allele into *cdc2.1w* cells suppresses the CPT sensitivity. (D–G) Hyperactive Cdc2.1w causes an extended cell-cycle arrest in the presence of CPT which is not suppressed by the *cdc25.22* allele. The indicated strains were synchronized by lactose gradient centrifugation in early G2 and released into YEA medium with or without 40 μM CPT at 30°C. 40 μl aliquots were withdrawn in 20 min intervals and added to 300 μl methanol. Cells were stained with hoechst (1:1000) and calcofluor (1:100) (calcofluor 1 mg/ml in 50 mM sodium citrate, 100 mM sodium phosphate pH 6.0; hoechst 10 mg/ml in water) prior to scoring under a fluorescence microscope. Open symbols: no CPT, closed symbols: 40 μM CPT.

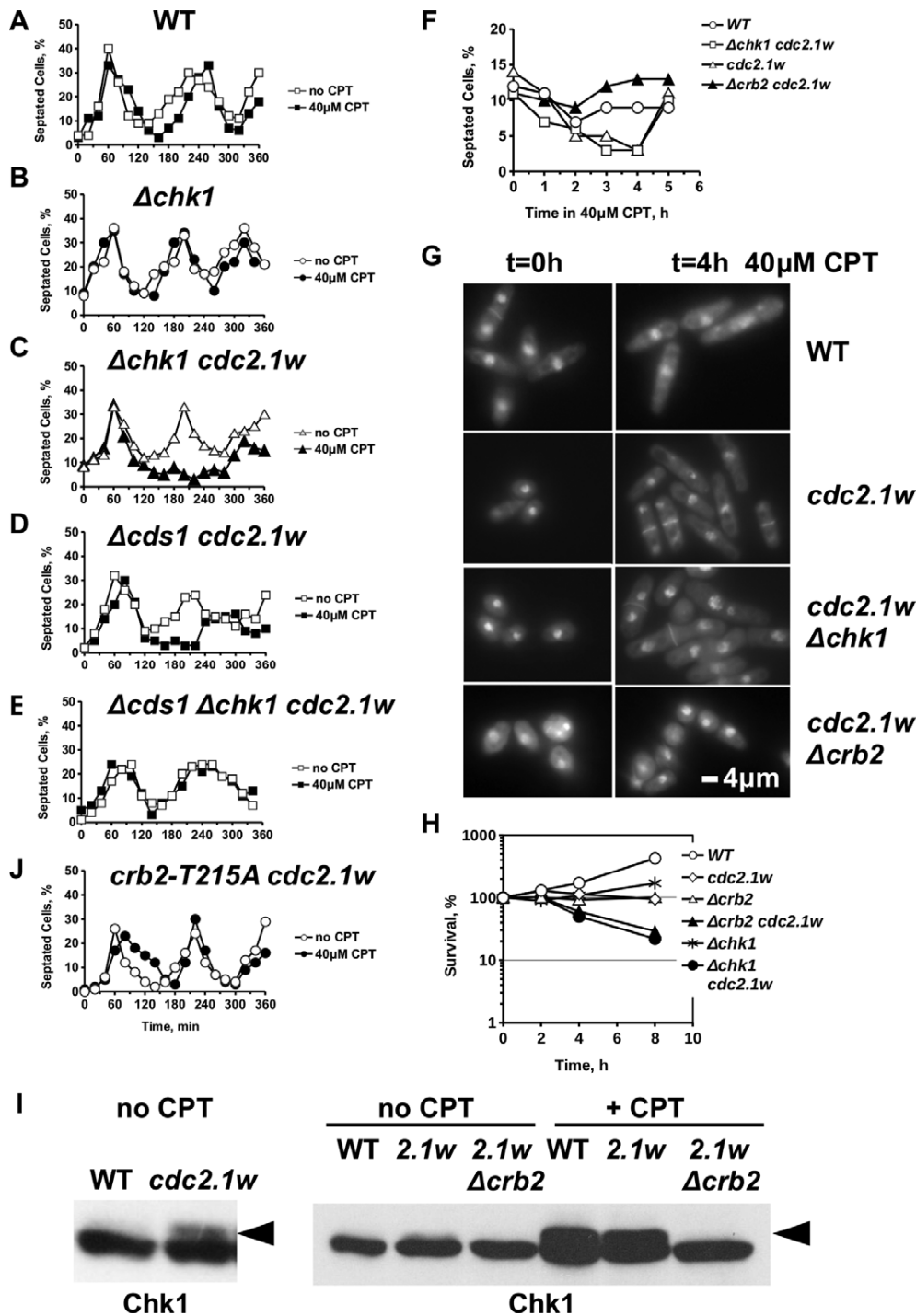


Figure 2. The extended cell-cycle-arrest of *cdc2.1w* cells is independent of Chk1 but requires Crb2 and its Cdc2 phosphorylation site T215. (A–E, J) Crb2, its phosphorylation site T215 and Cds1 are required for the extended arrest. The indicated strains were synchronized in G2 and released into YEA medium with or without 40 μM CPT at 30°C. Open symbols: no CPT, closed symbols: 40 μM CPT. (F) CPT (40 μM) was added to asynchronous cultures of the indicated strains in YEA medium at 30°C and the septation index was analysed over 5 h. (G) Loss of *crb2*, but not deletion of *chk1* prevents cell elongation in the presence of CPT. Cells harvested at the start of the experiment (*t* = 0 h) and after 4 h (*t* = 4 h) were inspected under a fluorescence microscope after staining with hoechst (DNA) and calcofluor (septum). (H) Cell survival was analysed by incubating 5×10^4 cells/ml of the indicated strains for 8 h in the presence of 40 μM CPT in YEA medium at 30°C. Samples were withdrawn at the indicated time points, plated on YEA plates and incubated for 3 days to analyse viability by colony formation. (I) Crb2 is required for the phosphorylation of Chk1 in *cdc2.1w* cells. Chk1-HA cells (WT), Chk1-HA *cdc2.1w* and Chk1-HA *cdc2.1w* Δ*crb2* cells were left untreated or were incubated for 4 h with 40 μM CPT in YEA medium at 30°C. Total protein extracts were separated on a 10% SDS PAGE and the Chk1 protein was visualized with an anti-HA antibody after western blot. The arrow highlights the slower migrating phospho-band of Chk1. The low levels of phosphorylated Chk1 in undamaged *cdc2.1w* cells as well as the DNA damage induced modification are dependent on Crb2.

The G2 arrest in *cdc2.1w* cells requires the CDK phosphorylation site T215 in Crb2

Although *cdc2.1w* cells bypass Chk1, we still wanted to test the requirement of the Chk1-adaptor protein Crb2^{53BP1, Rad9} given that Cdc2 phosphorylates Crb2 at T215 in mitosis (18). Deletion of *crb2* in *cdc2.1w* cells resulted in very short cells which were difficult to synchronise (Figure 2G). To circumvent this problem, we added 40 M CPT directly to asynchronous cultures of wild type, *cdc2.1w*, Δ *chk1 cdc2.1w* and Δ *crb2 cdc2.1w* cells and followed the septation index over 5 h. As shown in Figure 2F, the septation index of *cdc2.1w* and Δ *chk1 cdc2.1w* cells dropped after the first hour to 2% and started to rise again after 4 h. This decline in dividing cells is consistent with an extended G2/M arrest. We also took samples at the start of the experiment ($t = 0$) and after 4 h to examine cells under the microscope. Fission yeast cells which stop in G2 continue to grow and become elongated (27). While *cdc2.1w* and *cdc2.1w* Δ *chk1* cells clearly elongated in CPT medium, *cdc2.1w* Δ *crb2* cells maintained their short cell size (Figure 2G). Consistent with the absence of cell elongation, the septation index of this double mutant failed to drop (Figure 2F). These findings show that Crb2 is required for the arrest in *cdc2.1w* cells despite the independence on Chk1. Since *cdc2.1w* Δ *crb2* and *cdc2.1w* Δ *chk1* cells lost their viability to a similar extent in the presence of CPT (Figure 2H), the ability to postpone mitosis is not linked with enhanced cell survival.

To identify Crb2 but not Chk1 as a component of the extended G2 arrest in *cdc2.1w* cells was a surprise especially since our earlier observations implicated Cds1 kinase which is normally activated by Mrc1 and not by Crb2 (40). The requirement for Crb2 left us, however, with a conundrum since we and others have found that Chk1 is phosphorylated in undamaged cells with hyperactive Cdc2 as well as in the presence of CPT (Figure 2I) (41,31). Consistent with the role of Crb2 as a Chk1 adaptor, the band shift caused by the phosphorylation of Chk1 at S345 in response to CPT was abolished in *cdc2.1w* Δ *crb2 chk1-HA* cells (Figure 2I). An explanation of why Chk1 is modified in a Crb2-dependent manner in *cdc2.1w* cells in the presence of DNA damage despite its unimportance for the mitotic arrest could be provided by the two independent modes of Crb2 recruitment to DNA. Phosphorylation of Crb2 at T215 by Cdc2 in mitosis directs the protein to undamaged DNA (18), whereas its T215-independent interactions with methylated and phosphorylated histones direct Crb2 to damaged DNA in G2 (20). Hence, hyperactive Cdc2.1w may only affect the T215 phosphorylated pool of Crb2, but not the DNA damage activated pool. Consistent with this notion, replacement of T215 by an alanine residue (T215A) prevented *crb2-T215A cdc2.1w* cells from arresting the cell cycle in CPT medium (Figure 2J). This important finding suggests that the mitotic modification of Crb2-T215 by Cdc2 may interfere with the activation of Chk1 when replication forks break in *cdc2.1w* cells.

Elevated Cdc2 activity locks cells in a G1-like DNA repair state

Given the importance of Cdc2^{Cdc28} activity for the recombinogenic repair of broken chromosomes in *S.cerevisiae* (1),

we wanted to test whether elevated recombination levels interfere with the repair of collapsed replication forks in *cdc2.1w* cells. To test this idea, we measured break-induced HR by using a genetic system which allows for the genetic exchange between chromosome III and the homologous mini-chromosome Ch¹⁶-MG upon its cleavage by HO endonuclease (Figure 3A) (34). Prior to HO expression from the inducible *nmt81* promoter (pREP81X-HO), cells containing Ch¹⁶-MG grow in the absence of adenine (ade6⁺) and in the presence of the antibiotic G418 (G418^R). Following HO induction, the cleavage of Ch¹⁶-MG at its unique HO site within the engineered *rad21* gene will trigger DNA repair. While homologous recombination between the two *rad21* genes will result in the loss of the G418 resistance cassette due to DNA end resection (ade6⁺ G418^S), NHEJ of the HO break will retain the antibiotic resistance (ade6⁺ G418^R). Approximately 30%–40% of wild-type cells grown in minimal medium without thiamine (repressor of the *nmt81* promoter) underwent recombination, whereas only ~10% were recombinogenic in the presence of the repressor (Figure 3B). The latter is due to the leaky nature of the *nmt81* promoter. Unexpectedly, less than 2% of *cdc2.1w* cells underwent recombination in thiamine-free medium (Figure 3B). This intriguing finding suggests that elevated Cdc2.1w activity favours NHEJ over interchromosomal recombination as previously reported for *S. pombe* cells arrested in G1 (3). While this observation contradicts the importance of Cdc2^{Cdc28} activity for DNA end resection as observed in *S.cerevisiae* (1), it is consistent with a recent report showing that an aberrant increase in Cdc2 activity blocks interchromosomal recombination in human cells (42).

Since this finding suggests an increase in NHEJ in *cdc2.1w* cells, we measured NHEJ using a plasmid repair assay (3). The plasmid pREP41 was linearized at its unique SacI restriction site (Figure 3C) and equal amounts of cut and uncut plasmid were transformed into asynchronous wild type, *cdc2.1w* and NHEJ-deficient Δ *ku70* cells. While less than 20% of wild type cells were able to repair the plasmid, more than 90% of *cdc2.1w* cells were proficient in this assay (Figure 3D). As G1 cells utilize NHEJ over HR (3), we analysed the DNA content of asynchronous wild type and *cdc2.1w* cultures grown in rich and minimal medium using flow cytometry (43). Although G2 is ~20 min shorter in *cdc2.1w* cells than in wild type cells (Figure 1F, Supplementary Figure S1B–E), we did not detect an increase in G1 cells in *cdc2.1w* cultures independently of the growth medium (Figure 3E). Taken together, these experiments support the conclusion that elevated Cdc2 activity locks *S.pombe* cells in a G1-like DNA repair state which may compromise the recovery of collapsed replication forks.

Cdc2 associates with Srs2 DNA helicase, Hhp1 kinase, Chk1 kinase and the Ku70-Ku80 DNA binding complex

To find out how Cdc2.1w affects Crb2 and the DNA repair status of cells, we performed a small-scale immunoprecipitation screen to identify DDR proteins which bind to the kinase. Soluble extracts prepared from undamaged strains expressing affinity-tagged versions of Srs2 DNA helicase, Rqh1^{BLM} DNA helicase, Ku70, Ku80, Mus81 endonucle-

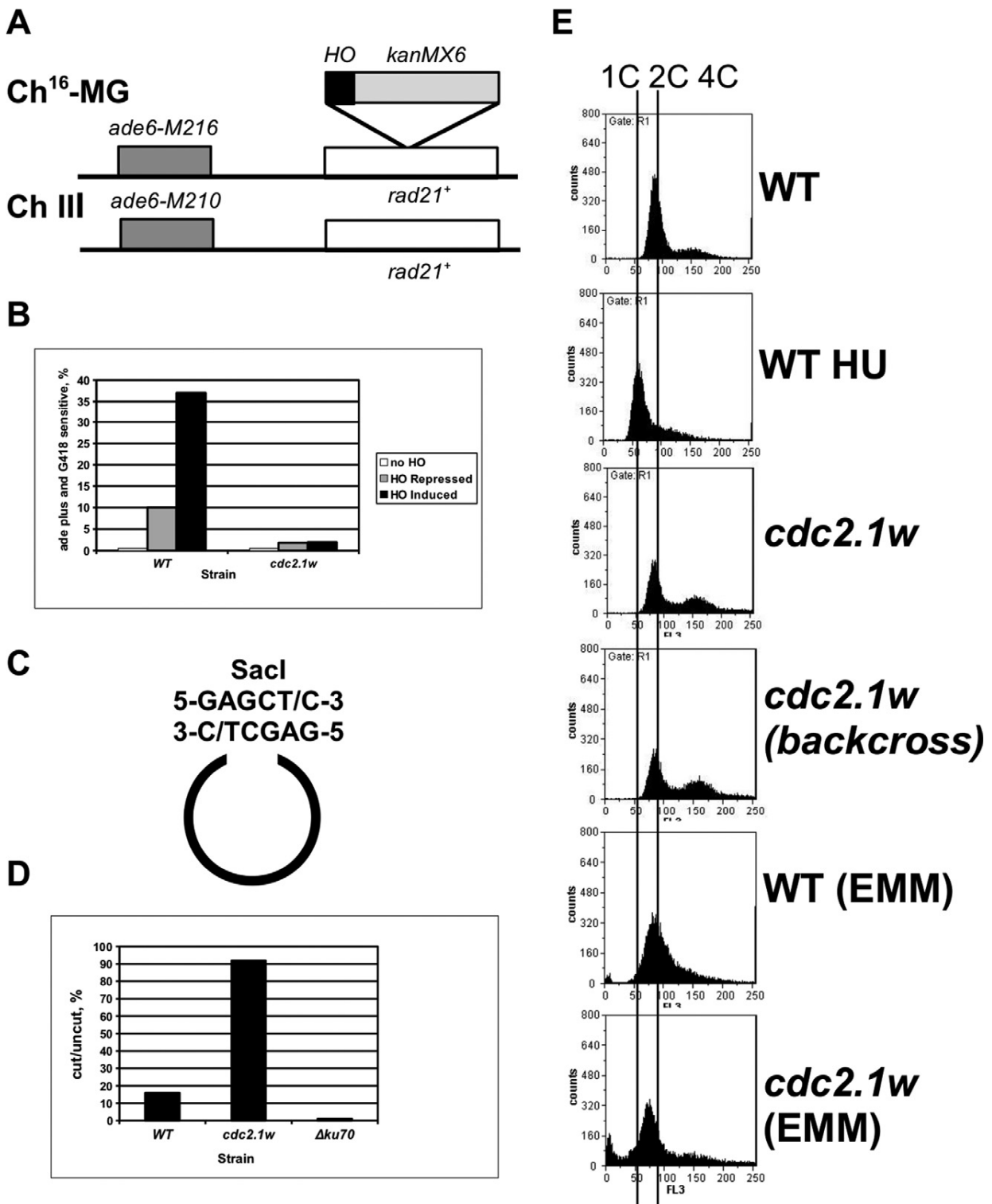


Figure 3. Elevated Cdc2 activity locks cells in a G1-like DNA repair mode. **(A)** Principle of the Ch¹⁶-MG Assay. **(B)** Wild-type cells and *cdc2.1w* cells containing the mini-chromosome Ch¹⁶-MG were grown in the absence of the HO endonuclease (No HO, pREP41 plasmid) or the presence of the HO enzyme either with (HO Repressed) or without thiamine (HO Induced). The averages of *ade6*⁺ *G418*^S colonies from three independent experiments are shown. **(C)** Principle of the plasmid repair assay. **(D)** The average ratios of transformants for the linearized vector normalized against the uncut plasmid from three independent experiments are shown. **(E)** The DNA content was measured using flow cytometry from asynchronous wild type and *cdc2.1w* cultures grown in rich medium or in minimal medium (EMM). Wild-type cells were arrested at the G1/S boundary with 12 mM HU for 4 h to have an internal standard for a 1C DNA content (WT HU). To exclude the presence of genetic alterations in the *cdc2.1w* strain, the strain was back-crossed against wild-type strains and the analysis was repeated (backcross).

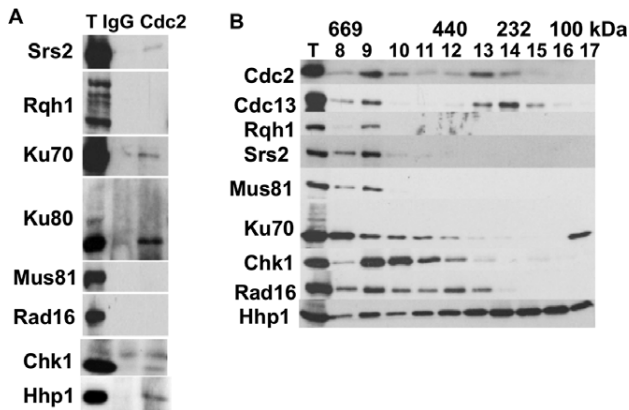


Figure 4. Cdc2 associates with Srs2, Ku70, Ku80, Hhp1 and Chk1. (A) Native protein extracts (T) (150 μ l) prepared from untreated cells were incubated with 5 μ l of an unrelated IgG antibody or 5 μ l of an anti-Cdc2 antibody over night. Protein-antibody complexes were harvested from the supernatant by the addition of 30 μ l protein A/G beads (Calbiochem) and analysed using an affinity tag-specific antibody (Srs2-Myc, Myc-Rqh1, Ku70-GFP-HA, Ku80-HA, Mus81-Myc, Rad16-GFP-HA, Chk1-HA, Hhp1-HA). (B) Size fractionation of the affinity tagged strains on a Superdex-200 column. (Total = native protein extract). The fractions obtained from the Cdc13-HA extract were also probed with an anti-Cdc2 antibody.

ase, Rad16^{XPF}, Chk1 kinase or Casein kinase 1 (Hhp1) were incubated with an anti-Cdc2 antibody and an unrelated immunoglobulin G (IgG) antibody and then precipitated with protein A/G beads. As shown in Figure 4A, small amounts of Ku70-Ku80, Srs2, Chk1 and Hhp1 were pulled down with the anti-Cdc2 antibody identifying these proteins as potential binding partners. Casein Kinase 1 was included in this experiment because we noted a strong genetic interaction between a loss-of-function *wee1* mutation (*wee1-50*) and the deletion of *hhp1* in an independent experiment (Figure 6A).

Size fractionation of soluble extracts obtained from these strains revealed two peaks of Cdc2 and cyclin B (Cdc13) at 600 kDa and 250 kDa, respectively (Figure 4B). All potential Cdc2 binding partners coeluted with Cdc2 in the higher molecular weight range of 600 kDa suggesting that they form larger protein complexes. Interestingly, monomeric protein was only detected for Ku70 and Hhp1, whereas all other proteins eluted with an apparent molecular weight well above their expected sizes.

For the rest of the project, we focused on Srs2 and Hhp1 since deletion of either gene was epistatic with the *cdc2.1w* mutation. The biology of the interaction between Ku70-Ku80 and Cdc2 will be reported somewhere else.

Loss of Srs2 DNA helicase prolongs the CPT-induced G2 arrest in a *crb2-T215A* dependent manner

Srs2 is a multifunctional DNA helicase which helps to join DNA ends with microhomologies during NHEJ (44), prevents unwanted HR by dismantling Rad51-ssDNA filaments (45), promotes HR by resolving D-loop structures during strand invasion (46) and binds to different replication structures *in vitro* (47). These opposing activities are

regulated by CDK1 in *S.cerevisiae* which phosphorylates Srs2 at multiple sites to stimulate HR in S/G2 (46).

Consistent with the association of Cdc2 with Srs2 (Figure 4A), we found an epistatic relationship between a *srs2* deletion and the *cdc2.1w* allele for the survival in the presence of CPT (Figure 5A). Since loss of *srs2* increases the spontaneous exchange between sister chromatids (48), we measured inter-sister recombination rates by employing an assay which monitors the restoration of adenine independence upon the recombination between two tandem *ade6-negative* heteroalleles which are separated by a functional *ura4+* marker (*ade6-L469-ura4+ -ade6-M375*) (33). The *ura4+* gene enabled us to distinguish *ade6+* recombinants that had lost (deletion type) or retained (conversion type) the intervening DNA between the *ade6* repeats. Deletion of the *ura4+* marker is indicative of recombination at collapsed forks (49), whereas spontaneous recombination between sister chromatids in G2 retains the intervening sequence (conversion type). As shown in Figure 5B, wild type and *cdc2.1w* cells both suffered from a 5-fold increase in gene deletions in the presence of 10 μ M CPT (WT: no drug: 0.8×10^{-4} ; CPT: 4.3×10^{-4} ; *cdc2.1w*: no drug: 0.8×10^{-4} ; CPT: 3.7×10^{-4}). This finding confirms the recombinogenic nature of collapsed forks, but also shows that uncontrolled genetic exchange at these structures is not the cause of death in *cdc2.1w* cells. Although recombination at broken forks is normal in *cdc2.1w* cells, the spontaneous exchange between sister chromatids in undamaged cells was 3-fold higher than in wild-type cells (WT: 1.3×10^{-4} ; *cdc2.1w*: 3.6×10^{-4}) (Figure 5B). Since such unwanted recombination is prevented by Srs2 (48), we measured the conversion rates in undamaged wild type, *cdc2.1w*, Δ *srs2* and Δ *srs2 cdc2.1w* cells. Cells without Srs2 had a 6-fold higher rate compared to wild type, which was not further increased in the Δ *srs2 cdc2.1w* double mutant (WT: 0.7×10^{-4} ; *cdc2.1w*: 2.7×10^{-4} ; Δ *srs2*: 4.5×10^{-4} ; *cdc2.1w* Δ *srs2*: 4.3×10^{-4}) (Figure 5C). In summary, these results imply that hyperactive Cdc2 has two effects on Srs2. Its anti-recombination activity is down-regulated in undamaged cells leading to elevated rates of spontaneous inter-sister exchange and also its DNA repair function is blocked when replication forks collapse resulting in CPT sensitivity. This close functional relationship between Cdc2 and Srs2 implies that the kinase modifies Srs2. Although *S.cerevisiae* Srs2 is phosphorylated by Cdc2^{Cdc28} (46), a similar modification has not yet been reported in *S. pombe*.

Given the close functional link between Cdc2 and Srs2, we measured the G2 arrest in synchronized Δ *srs2*, Δ *srs2 Achk1*, Δ *srs2 cdc2.1w* and Δ *srs2 crb2-T215A* cells. Intriguingly, loss of the helicase resembled the hyperactive *cdc2.1w* mutation because the extended G2 arrest of Δ *srs2* cells was independent of Chk1 (Figure 5E) but required the phosphorylation of Crb2 at T215 (Figure 5F). One important difference between *cdc2.1w* and Δ *srs2* cells was, however, the shorter G2 arrest when forks collapsed in the absence of the DNA helicase. While *cdc2.1w* cells delayed entry into mitosis for up to 2 h (Figure 1F), Δ *srs2* cells delayed only for 40–60 min (Figure 5D).

In summary, these findings imply that Cdc2 kinase targets Srs2 in *S.pombe* and that the hyperactive kinase may switch Srs2 activity from the prevention of spontaneous inter-sister

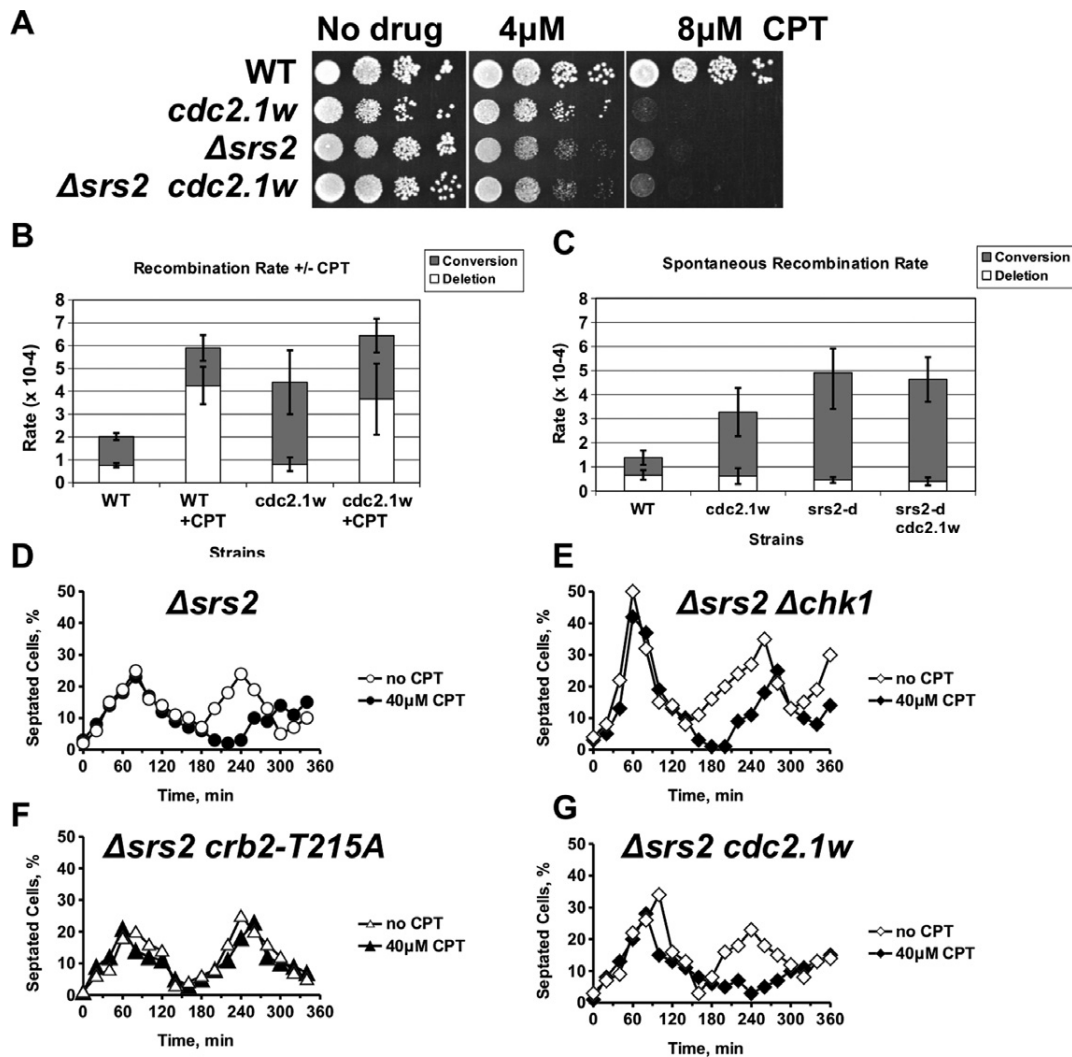


Figure 5. Hyperactive Cdc2.1w blocks Srs2 DNA helicase and loss of *srs2* arrests cell-cycle progression in a Crb2-T215 dependent manner. (A) Cdc2.1w and Srs2 act in the same CPT response pathway. (B) CPT induces deletion type recombination events. Wild type and *cdc2.1w* cells containing the recombination cassette (*ade6-L469-ura4⁺-ade6-M375*) were grown in 5 ml YEA medium with or without 10 μ M CPT from a single colony into stationary phase. Cell dilutions were plated onto minimal medium plates (100 mg/L uracil, 100 mg/L leucine, 200 mg/L guanine) to select for cells with a restored *ade6* gene (= recombination event). Loss of the *ura4* marker (= deletion event) was determined by replica-plating onto minimal medium plates (100 mg/L leucine, 200 mg/L guanine). Open boxes = deletion events, closed boxes = conversion events. (C) Cdc2.1w increases spontaneous gene conversion events in a Srs2-dependent manner. Strains of the indicated genotypes were grown in YEA medium without drug into stationary phase and analysed. (D–G) Loss of Srs2 delays cell-cycle progression in the presence of CPT independently of Chk1, but dependent on the Cdc2 phosphorylation site Crb2-T215. Cells of the indicated genotypes were synchronized in G2 and released in YEA medium with or without 40 μ M CPT at 30°C. Open symbols: no CPT, closed symbols: 40 μ M CPT.

recombination to the promotion of NHEJ. They also show that elevated Cdc2 activity has distinct effects on HR. While break-induced recombination between homologous chromosomes is blocked (Figure 3B), spontaneous recombination between sister chromatids, which are attached by the cohesion complexes, is increased (Figure 5B and C).

Casein kinase 1 (Hhp1) is aberrantly modified in *cdc2.1w* cells

We became interested in CK1 because the CPT sensitivity of the Δ *hhp1* deletion strain was partly suppressed by elevated Cdc2 activity (*cdc2.1w* or *wee1-50*) (Figure 6A). This

suppression was, however, limited to the acute exposure of cells to the topoisomerase 1 inhibitor as it was not evident when cells were grown for several days in the presence of the drug. This rescue places both kinases in the same CPT response, a conclusion supported by their physical association (Figure 4A). Since Hhp1 is known to undergo autophosphorylation (50), we employed isoelectric focusing to investigate the modification pattern of the kinase in wild type and *cdc2.1w* cells treated with 40 μ M CPT for 4 h or left untreated. Separation of soluble Hhp1-HA protein on a linear pH strip ranging from pH3 to pH10 revealed two species of CK1 (Figure 6B). The intensity of the more acidic form (number 1 in Figure 6B) increased in *cdc2.1w* *hhp1*-HA cells

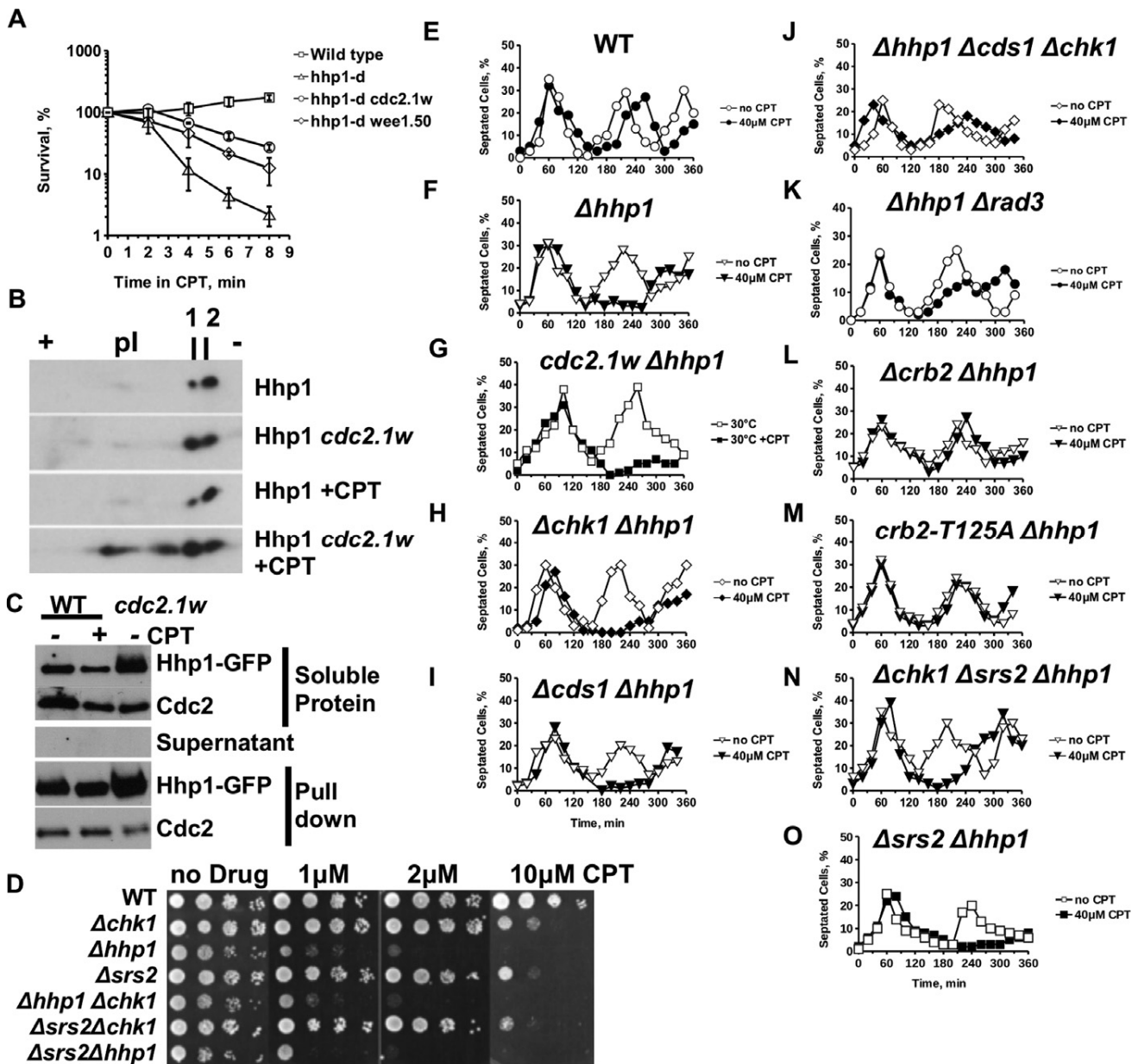


Figure 6. Cdc2 targets Casein kinase 1 (Hhp1) and loss of Hhp1 delays the cell cycle independently of Chk1 but requires Cds1 and the Cdc2 phosphorylation site Crb2-T215. (A) Hyperactive Cdc2.1w partly suppresses the CPT sensitivity of $\Delta hhp1$ cells (40 μ M CPT in YEA medium at 30°C). (B) Cdc2 targets Hhp1 kinase. Isoelectric focusing of total protein extracts prepared from Hhp1-HA and Hhp1-HA *cdc2.1w* cells either treated with 40 μ M CPT at 30°C for 4 h or left untreated. (C) Hhp1-GFP was purified from wild-type cells in the absence or presence of CPT (40 μ M, 4 h) and from untreated Hhp1-GFP *cdc2.1w* cells using the GFP-trap. Samples of the total soluble extracts and of the purified material were probed with an anti-GFP and an anti-Cdc2 antibody. Samples of the supernatant after the pull-down were probed with the anti-GFP antibody. (D) Hhp1 kinase acts in the same CPT response as Srs2 and Chk1. (E–O) Loss of Hhp1 delays cell-cycle progression in the presence of CPT independently of Chk1, but dependent on the Cdc2 phosphorylation site Crb2-T215, Rad3 and Cds1. Cells of the indicated genotypes were synchronized in G2 and released in YEA medium with or without 40 μ M CPT 30°C. Open symbols: no CPT, closed symbols: 40 μ M CPT.

independently of CPT. This observation, together with the direct association between the two kinases (Figure 4A), implies that Cdc2 phosphorylates Hhp1.

While Hhp1 was not further modified in CPT-treated wild-type cells, several hypermodified species appeared when *cdc2.1w hhp1-HA* were incubated with the drug (Figure 6B, panel 4). These additional modifications could arise

from aberrant Cdc2 activity in the presence of CPT, from a change in autophosphorylation of Hhp1 in *cdc2.1w* cells or from a yet unknown kinase targeting Hhp1 under these conditions.

To test whether the association of Cdc2 with Hhp1 is affected by CPT treatment or elevated Cdc2 activity, we purified Hhp1-GFP protein complexes from growing cells, cell

treated for 4 h with 40 μ M CPT or untreated *cdc2.1w* Hhp1-GFP cells using the novel GFP-trap. As shown in Figure 6C, the high affinity GFP-binding protein depleted the soluble Hhp1-GFP protein from the extract. The purified pool of Hhp1-GFP contained a significant amount of Cdc2 kinase independently of CPT treatment and high Cdc2 activity suggesting a stable interaction between the kinases.

Informed by the previous finding that hyperactive Cdc2 down-regulates Srs2 (Figure 5), we tested the CPT sensitivity of *Δsrs2 Δhhp1* cells and found an epistatic relationship between CK1 and the helicase. We also found an epistatic relationship between Chk1 and Srs2, and between Chk1 and Hhp1 (Figure 6D). Taken together, these observations imply that Hhp1 and Srs2 both have to be active for Chk1 to become stimulated by broken replication forks.

Deletion of Casein Kinase 1 prolongs the G2 arrest in a *crb2-T215A* dependent manner

To test whether Hhp1 acts in the G2 arrest of *cdc2.1w* cells, we synchronized wild type, *Δhhp1* and *cdc2.1w Δhhp1* cells and measured cell-cycle progression in the presence of 40 μ M CPT. Interestingly, deletion of *hhp1* on its own was sufficient to delay entry into mitosis by \sim 2 h (Figure 6F) in a Chk1-independent manner (Figure 6H). In light of the association of Hhp1 with Cdc2 (Figure 4A) and the Cdc2.1w-dependent change in its phosphorylation pattern (Figure 6B), it seems very likely that hyperactive Cdc2 alters Crb2 activities by blocking Hhp1 kinase. Consistent with this conclusion, loss of *crb2* (Figure 6L) or mutation of T215 to alanine (Figure 6M) abolished the G2 arrest of *Δhhp1* cells in the presence of CPT.

Since our earlier data on *cdc2.1w* strongly suggest that the mitotic Crb2-T215 modification allows Rad3 to activate Cds1 instead of Chk1 (Figure 2E), we deleted *cds1*, *rad3* or *cds1* and *chk1* in the *Δhhp1* mutant. While loss of Cds1 on its own had no effect on the arrest (Figure 6I), concomitant deletion of *cds1* and *chk1* or deletion of *rad3* abolished the G2 delay (Figure 6J and K). These observations are in agreement with our previous finding that *Δcds1 Δchk1 cdc2.1w* cells fail to stop in CPT medium (Figure 2E) and show that Chk1 becomes only important in *Δhhp1* cells when Cds1 is inactivated. In line with the epistatic relationship between Hhp1 and Srs2 (Figure 6D), loss of Srs2 in *Δhhp1* or in *Δhhp1 Δchk1 Δsrs2* triple mutant reentered the cell cycle earlier compared to the *Δhhp1 Δsrs2* double mutant (Figure 6N and O).

DISCUSSION

We report here that fission yeast cells with a hyperactive Cdc2 kinase (*cdc2.1w*) are specifically sensitive to the topoisomerase 1 inhibitor CPT (Figure 1B), enter a prolonged G2 arrest when replication forks break in the presence of CPT (Figure 1F) and maintain a G1-like DNA repair state with high levels of NHEJ and low levels of interchromosomal recombination (Figure 3). Our genetic data strongly suggest that the CPT sensitivity and the extended G2 arrest are two independent manifestations of elevated Cdc2 activity. While the introduction of a loss-of-function mutation in Cdc25 phosphatase (*cdc25.22*), which is known to

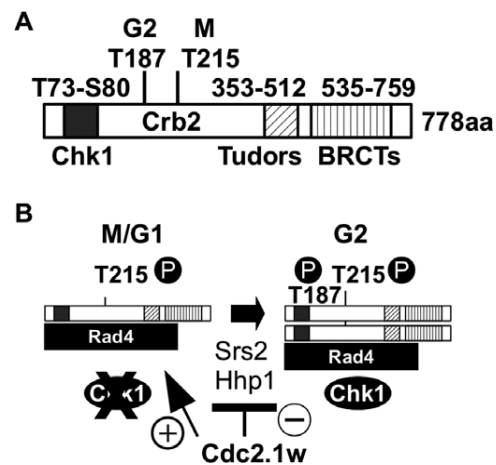


Figure 7. Model. (A) The domain structure of Crb2. In the presence of DNA damage, the C-terminal Tudor and BRCT domains allow Crb2 to bind to methylated and phosphorylated histones, respectively. Cdc2 phosphorylates Crb2 at threonine 215 (T215) in mitosis thereby promoting the association of Crb2 with Rad4 independently of DNA damage. Chk1 associates with Crb2 after the Rad3-dependent phosphorylation of T73 and S80 in the response to DNA lesions once Crb2 has been modified at T187 by Cdc2 in G2. (B) Model. In wild-type cells, the Crb2-Rad4 complex changes from its M/G1 configuration to its G2 configuration when cells exit S phase. This transition is promoted by Srs2 DNA helicase and Hhp1 (CK1) kinase, and by the G2 modification of T187 by Cdc2. In *cdc2.1w* cells, this transition is delayed due to the inhibition of Srs2 and Hhp1 by the hyperactive Cdc2.1w kinase. This locks the Crb2-Rad4 complex in its M/G1 mode. The repair of broken replication forks may be delayed as *cdc2.1w* cells favour NHEJ over interchromosomal recombination. This leads to the activation of Cds1 instead of Chk1, and an extended G2 arrest.

lower Cdc2.1w activity (24), suppresses the CPT sensitivity (Figure 1C), it fails to restore a normal G2/M delay (Figure 1G). This difference could be explained by the dynamics of Cdc2 throughout the cell cycle. *In vitro* kinase assays have shown that Cdc2 activity starts to increase half way through G2 in normal fission yeast cells, but starts very early in G2 and then rises at twice the rate in cells with hyperactive Cdc2 kinase (51). This change in Cdc2 levels throughout the cell cycle could have a profound effect on Crb2 as Cdc2 targets the protein at least twice in one cycle. At its activity peak in mitosis, Cdc2 phosphorylates Crb2 at T215 which allows Crb2 to bind to Rad4 (18,19). The mitotic Crb2-Rad4 complex may exist until the start of G2 when sufficient Cdc2 activity accumulated again to modify the complex at T187, a non-canonical Cdc2 site closer to the N-terminus of Crb2 (Figure 7) (21). The phosphorylation of T187 rearranges the Crb2-Rad4 complex so that Crb2 can bind to Chk1, and Rad4 to the Rad9-Rad1-Hus1 complex (21). This temporal order of modifications may be affected by the higher and faster rising levels of Cdc2 in *cdc2.1w* cells as they fail to activate Chk1 when replication forks collapse (Figure 2C). Intriguingly, *cdc2.1w* cells activate Cds1 kinase under these conditions (Figure 2D and E) which implies that the mitotic Crb2-Rad4 complex targets this kinase instead of Chk1. Elevated Cdc2 activity may either expand the mitotic pool of the T215 phosphorylated Crb2-Rad4 complex or it may initiate further modifications like the phosphorylation of T187 prematurely. As a result of this, the Crb2-Rad4 comple

may become trapped in its mitotic state (52) thereby promoting NHEJ over interchromosomal recombination (Figure 3). If replication forks were to break under these conditions, their repair may be delayed explaining the extended G2 arrest (Figure 7). Interestingly, the aberrant modification of the Crb2-Rad4 complex in *cdc2.1w* cells requires the inhibition of Hhp1 kinase and Srs2 DNA helicase as Δ *hhp1* and Δ *srs2* mutants both show an extended G2 arrest which is abolished upon mutation of T215 (Figures 5F and 6M). Since Hhp1 and Srs2 associate both with Cdc2 (Figure 4), the hyperactive kinase may either block or modulate their activities to promote NHEJ and/or to expand the pool of T215 modified Crb2-Rad4 complexes.

Budding yeast Srs2 possesses several functions which may help to explain how a multifunctional helicase could regulate Crb2. Srs2^{Sc} binds *in vitro* to junctions between single- and double-stranded DNA (47) and promotes NHEJ in G1 (44). If Crb2, like human 53BP1, prevents the resection of DNA ends in G1, Srs2 may terminate this activity by binding to these junctions at the G1/S transition. In normal cells, Srs2 may therefore be down-regulated by Cdc2 until cells initiate DNA replication. This inhibition may well be extended beyond the start of S phase in *cdc2.1w* cells causing problems with the activation of the DNA damage checkpoint kinases when forks break. Although hyperactive Cdc2.1w clearly blocks Srs2 as indicated by the increase in the spontaneous inter-sister recombination rate (Figure 5C), it is as yet unclear whether this is by a Cdc2-dependent modification of the helicase. The latter is, however, supported by the physical association between Cdc2 and Srs2 (Figure 4A), and by similar findings in *S.cerevisiae* (46). Alternatively, Cdc2 could regulate Srs2 indirectly via Hhp1 given the epistatic relationship between Δ *srs2* and Δ *hhp1* for the CPT sensitivity (Figure 6D) and the G2 arrest (Figure 6O).

Hhp1 is most closely related to human Casein kinase 1 (53), which performs diverse roles in the circadian clock, DNA repair and wnt- β -catenin signaling (54). CK1 enzymes are monomeric kinases which are regulated by autophosphorylation and require in many cases a priming kinase to recognize a substrate (54). There are several ways how Hhp1 could regulate Crb2. Hhp1 could act through Srs2, but this is less likely since the duration of the G2 arrest is significantly longer in Δ *hhp1* cells (Figure 6F) than in Δ *srs2* cells (Figure 5D). Alternatively, Srs2 may associate with and regulate Hhp1. This idea is based on the recent discovery that human CK1 associates with the RNA helicase DDX3 to control wnt- β -catenin signalling (55). A third possibility is that Cdc2 primes Crb2 for the phosphorylation by Hhp1 and that this modification is required for Srs2 to act on Crb2. In line with this notion, additional phosphorylation bands were observed once Crb2 was modified by Cdc2 at T215 (18). Whether this hyperphosphorylation of Crb2 is dependent on CK1 is not yet clear, but the *S.cerevisiae* paralog of Crb2, Rad9, is modified by Polo-like kinase and Casein kinase 2 when cells reenter the cell cycle from a G2 arrest (56).

In summary, our data entertain a model (Figure 7) in which Cdc2 retains the Crb2-Rad4 complex in its M/G1 mode by blocking Srs2 DNA helicase and Hhp1 kinase until cells enter G2 phase. This would silence Chk1 until suffi-

cient Cdc2 kinase has accumulated at the start of G2, which may be a prerequisite to promote NHEJ in G1 in wild-type cells. In *cdc2.1w* cells, this inhibition of Chk1 seems to continue beyond the start of G2 resulting in the aberrant activation of Cds1 and a long G2 arrest when forks break in a chromatin environment that favours NHEJ over interchromosomal recombination. Further work is, however, required to establish how Cds1 is activated in *cdc2.1w* cells and how Hhp1 and Srs2 are regulated by Cdc2 to modulate Crb2.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online

ACKNOWLEDGEMENTS

We would like to thank the Yeast Genetic Resource Center (YGRC) based at Osaka City University and Osaka University for strains. We are very grateful to Jacqueline Hayles for providing the *cdc2.1w*, *wee1.50*, *cdc25.22* and Δ *wee1* strains, to Kathleen Gould for providing the Hhp1-GFP strain and to Tim Humphrey for providing the Ch¹⁶-MG mini-chromosome assay.

FUNDING

Libyan Government [4839 to S.A.M.S.]; North West Cancer Research Fund [CR726 to K.E.-K.]. Source of open access funding: Research Grant.

Conflict of interest statement. None declared.

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