DOCTOR OF PHILOSOPHY

The Rad9.M50 Variant of the DNA Damage Checkpoint Protein Rad9 Regulates the MAP Kinase pathway in the Response to Heat Stress

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The Rad9.M50 Variant of the DNA Damage Checkpoint Protein Rad9 Regulates the MAP Kinase pathway in the Response to Heat Stress

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A thesis submitted for the degree of

Doctor of Philosophy

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Dedication

To the memory of my Father.…

To the love of my life, my Mother...…

To my lovely brothers: Khaled, Abdulbaset, Salah, Adnan and Hamza…

I dedicate my dissertation work with a special gratitude feeling to you all,

Rabiaa Oun

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Most importantly, I would not have been able to do my PhD without my family love and patience. My family whom my thesis is dedicated to, has been a constant source of support, concern, strength and love during all these years. I express my heartfelt thanks to my family.

Finally, I would like to thank the Libyan government represented by Libyan ministry of higher education and scientific research, Libyan embassy-cultural Attaché (United Kingdom-London).

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List of abbreviation

APS	Ammonium sulphate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BRCA1	Breast cancer 1
Cds1	CDP- diacylglycerol
Cdc2	Cell division cycle 2
Cdc25	Cell division cycle 25
CDK	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
CPT	Camptothecin
Crb2	Crumbs homolog 2
DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
DSBs	Double strand breaks
EDTA	Ethaylenediamine tera-acetic acid
EMM	Edinburg Minimal Media
H2AX	H2A histone family, member X
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
HU	Hydroxyurea
IR	Ionise Radiation
KDa	Kilo Dalton
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
ME	Malt Extract
MEA	Malt Extract Agar
$MgCL_2$	Magnesium Chloride
MMS	Methyl methane sulfonate
mRNA	Messenger ribonucleic acid
NaCl	Sodium Chloride
P53	Tumour suppressor p53
PARP	poly-ADP-ribose polymerase

PBS	Phosphate-buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PI3	phosphatidylinositol 3-like enzymes
RAD proteins	Radioresistance protein
RFC	Replication Factor C
RPA	Replication Protein A
S345	Serine 345
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfat- Polyacrylamide gel
S.pombe	*Schizosaccharomyces pombe*
S.cerevisiea	*Sachaaromyces cerevisiae*
TCA	Trichloroacetic acid
UV	Ultraviolet
WT	Wild Type
YEA	Yeast Extract Agar
YEL	Yeast Extract Liquid

Abstract

Environmental stress activates the MAP kinase pathway to regulate transcription and cell division. In this pathway, a MAP kinase kinase kinases (MAPKKK) activates the MAP kinase kinase (MAPKK) which in turn phosphorylates the MAP kinase (MAPK) simultaneously at a serine or threonine residue and a tyrosine residue in close vicinity. In the fission yeast *Schizosaccharomyces pombe*, the dual specific MAPKK Wis1 phosphorylates the MAPK Sty1/Spc1 at threonine 171 and tyrosine 173. Active Sty1 phosphorylates and activates the transcription factor Atf1. Sty1 is closely related to Hog1 in the budding yeast *S.cerevisiae* and p38 in human cells.

This thesis reveals a novel regulation of Sty1 by the alternative translation product of the DNA damage checkpoint protein Rad9. Alternative translation from the internal AUG codon at position 50 produces an N-terminally truncated protein variant (Rad9-M50), expression of which is restricted to dividing cells. The basal level of this variant is low and increases in the response to heat stress and DNA alkylation by the DNA damaging drug methyl-methanesulfonate (MMS).

The key finding reported here is the ability of Rad9 variant to shield Sty1 from dual specific kinases other than Wis1. Loss of Wis1 eliminates the phosphorylation of Sty1 at T171 and Y173. However, removal of the Rad9 variant (*rad9-M50A*) restores Sty1 phosphorylation in the absence of Wis1 at high temperature and in the presence of oxidative stress. This aberrant stimulation enables Sty1 to phosphorylate Atf1. A model is presented in which Rad9 variant shields Sty1 from an alternative dual-specific kinase, possibly Wee1, Hhp1 (CK1) or Mps1/TTK.

The thesis also reports the requirement of the DEAD-box RNA helicase Ded1 for the expression of Rad9 variant and presents genetic evidence linking Rad9 variant with the phosphatase Ptc1 in the response to heat stress and MMS induced DNA damage.

Thesis organisation

The thesis is organized as following:

- **Chapter 1:** Introduction
- **Chapter 2**: Materials & Methods used to perform the biomedical and the genetic analysis in yeast.
- **Chapter 3**: Ded1 RNA Helicase is required for the heat and MMS-induced induction of Rad9 variant in *S. pombe*. The aim of this chapter was to test whether the well-known RNA helicase Ded1 is required for the inducible variant of the checkpoint protein Rad9 in response to heat stress and MMS. I show that Ded1 RNA helicase is needed in order to induce Rad9 variant under heat and MMS conditions. I also show that Ded1 is required for the heat induction of a novel protein variant of the DNA replication checkpoint kinase Cds1/Chk2.
- **Chapter 4**: Dephosphorylation of the *S.pombe* DNA Damage Checkpoint Kinase Chk1 by Ppa2 and Ptc1 phosphatases at elevated temperatures. The aim of this chapter was to test whether the heat inducible variant of the DNA damage checkpoint Rad9 or any of the known phosphatases dephosphorylate the checkpoint kinase Chk1 in response to elevated temperature. I show that Ppa2 and Ptc1 phosphatases but not Rad9 variant dephosphorylate Chk1 at S345. This analysis also revealed a genetic interaction between Ptc1 phosphatase and Rad9 variant in the response to DNA damage.
- **Chapter 5**: The Rad9 variant regulates the MAP kinase pathway in the response to heat stress in *S.pombe*. Informed by the interesting genetic interaction between Rad9 variant and Ptc1 phosphatase, which has a reported role in the dephosphorylation of the MAP kinase Sty1, I extended my analysis to test whether Rad9 variant regulates Sty1 upon heat and oxidative stress. I show that Rad9 modulates Sty1 without the requirement of the up-stream MAPKK Wis1 which results in the phosphorylation of Atf1 in the absence of Wis1.
- **Chapter 6**: General Discussion. This chapter explains the key outcome of this project and presents a working model explaining the findings in the context of the literature.

Chapter 1: Introduction

1.1 Cell cycle, checkpoints and DNA damage responses

1.1.1 Cell cycle

The cell cycle is the important machinery for cells to grow and divide into two daughter cells. In order for cells to divide they have to replicate their genomic DNA before the separation of the duplicated chromosomes (Nurse, 1990). Growing cells undergo DNA synthesis (S phase) and mitosis in an orderly sequence to achieve this task. The cell cycle is ordered into G1 (GAP -1), S (synthesis), G2 (GAP-2) and M (mitosis) respectively (Harper & Brooks, 2005). Cells grow and prepare for DNA synthesis in the G1 phase, in S-phase cells replicate their genome, in G2 phase cells grow and prepare for mitosis and finally in M phase cells segregate their duplicated chromosomes (Harper & Brooks, 2005).

1.1.2 Checkpoints

The cell cycle is a series of events where cells cannot complete their latest events unless the earlier stage has been completed (Hartwell & Weinert, 1989; Russell, 1998). To achieve this level of accuracy, cells are evolved checkpoints that act between the cell cycle stages to ensure their successful completion. When DNA is damaged, cells activate a kinase cascade known as the DNA damage checkpoint which arrests the cell cycle or causes cell death depending on the extension of the problem. According to the stages at which they act, DNA damage checkpoints are defined as: G1/S (G1) checkpoint, intra-S phase checkpoint, G2/M checkpoint and mitotic (anaphase) checkpoint. Especially the DNA replication checkpoint (intra-S) received much attention over the recent years since many genomic lesions which trigger uncontrolled, cancerous growth are initiated by DNA replication stress (Macheret & Halazonetis, 20015). One of the key proteins, Rad9, is the subject of this PhD thesis. As explained in the subsequent chapters, Rad9 forms a ring complex with Hus1 and Rad1 (Figure 1-1 a) (Dore, et.al. 2009) which is only expressed in dividing cells (Janes et al., 2012). The 9-1-1 ring resembles the DNA replication factor PCNA (Figure 1-1 b) (Caspari, et.al. 2000) and enables DNA checkpoint proteins and DNA repair factors to associate with damaged DNA (Pichierri, et.al. 2012).

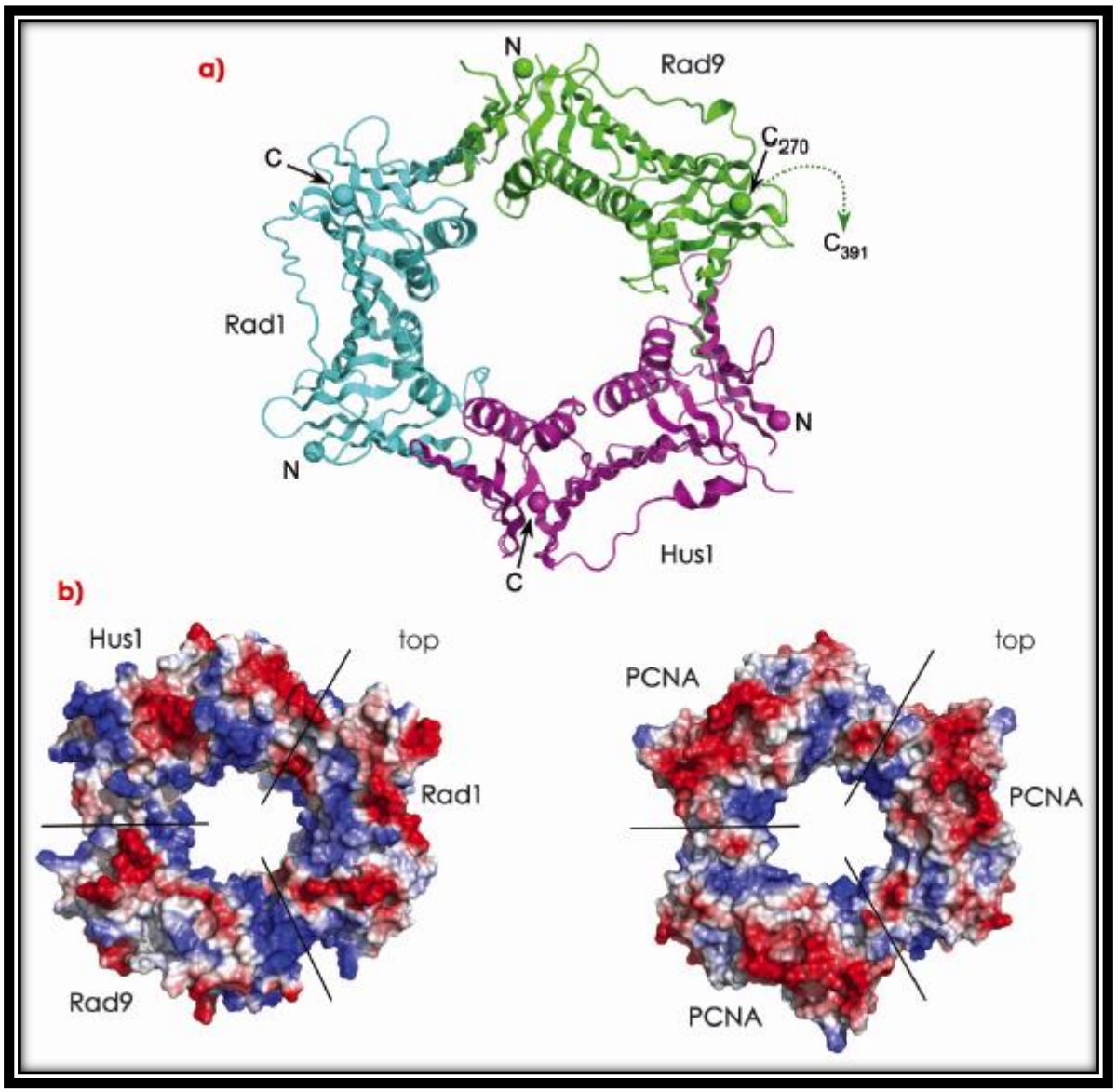


Figure 1- 1: Rad9-Rad1-Hus1 complex structure. (a) Cartoon of the Rad9-Rad1-Hus1 complex as a secondary structure where the green colour shows Rad9, the cyan colour shows Rad1 and the magenta colour shows Hus1. The marked label (C227-C391) shows the unstructured Rad9 C-terminal tail (approximately 120 residue). (b) On the left is the 9-1-1 electrostatic surface and on the right is the PCNA (http://www.esrf.eu/UsersAndScience/Publications/Highlights/2009/biology/sb05) (Sohn & Cho, 2009).

1.1.3 Initial responses to DNA damage

DNA replication problems, especially at fragile chromosomal sites, are a dominant source of DNA breaks and one of the earliest signs of cancer development (Barlow, et.al. 2013). The processes of DNA damage detection and repair are known as DNA damage response (DDR) (Zhou & Elledge, 2000). If cells cannot repair the DNA lesions, the prolonged arrest can lead to cell death (apoptotis) or senescence (Norbury & Zhivotovsky, 2004). The signaling process within the DDR happens through a distinct series of pathways identified as a network (Alyson

& Alvaro, 2010). This system contains proteins identified as sensors, signal transducing proteins, effector kinases, mediators and effector proteins (Figure 1-2).

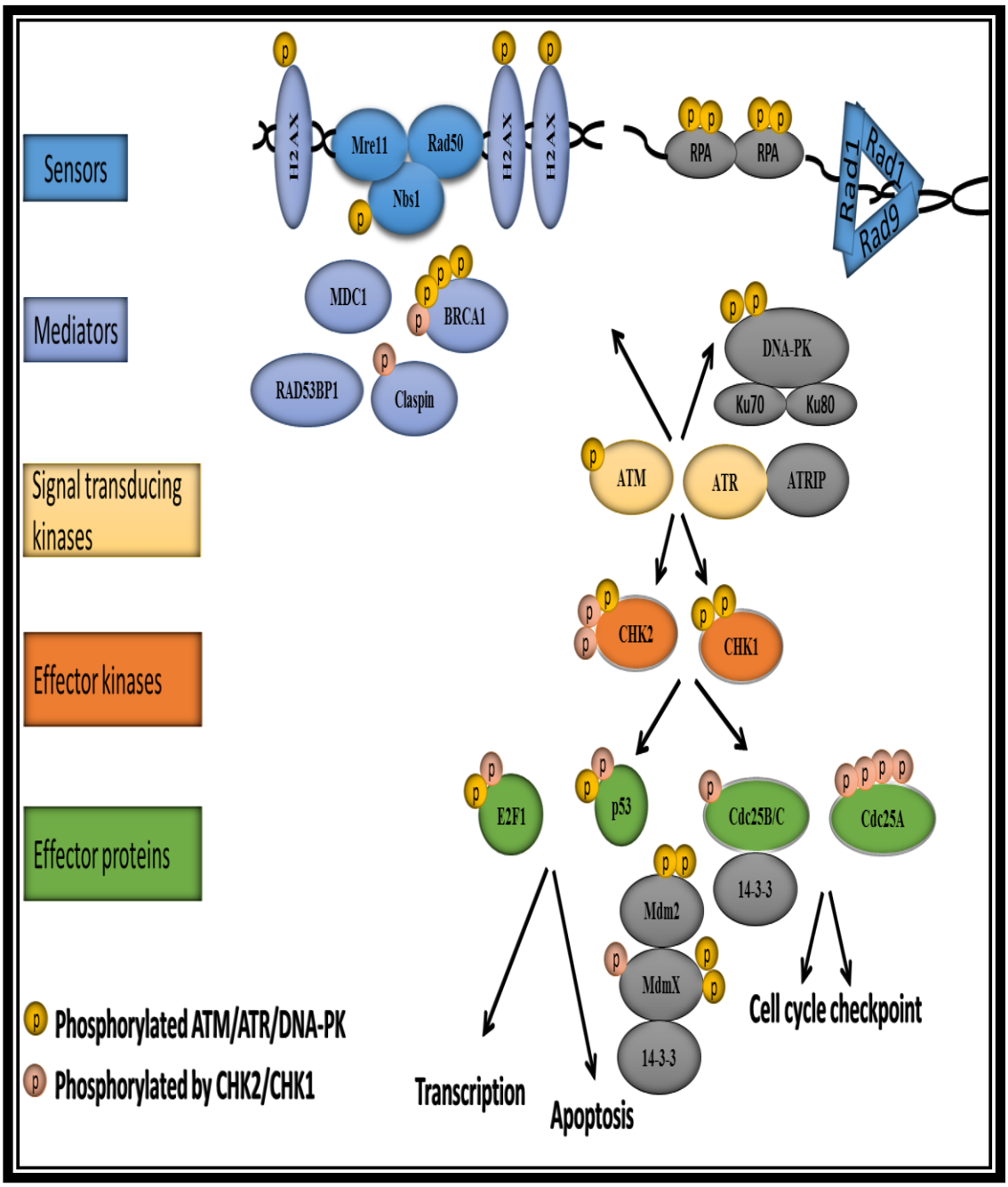


Figure 1- 2: Diagram of how cells response to DNA damage in human cells (Adapted from Alyson & Alvaro, 2010).

The response of cells to DNA double-strand breaks (DSBs) is imitated by ATM/Tel1 kinase, which belongs together with ATR/Rad3 and DNA-PK to the family of phosphatidylinositol 3-like kinases (PI3) (Lee & Chowdhury, 2011). In mammals, these kinases phosphorylate approximately 700 proteins in the response to DNA damage (Matsuoka, et.al. 2007). In simple eukaryotic cells like the fission yeast, *Schizosaccharomyces pombe*, the model organism used

in this study, Rad3/Mec1/ATR kinase is the main DNA damage detector as Tel1/ATM is less important due to the rapid conversion of broken DNA ends to single-stranded DNA in a process known as end resection (Limbo, et.al. 2011). While human ATM binds to broken DNA ends through the MRN complex (Mre11-Rad50-Nbs1) and signals to Chk2 kinase (Figure 1-2), ATR associates with single-stranded DNA through its subunit ATRIP/Rad26 and signals to Chk1 kinase (Reinhardt & Yaffe, 2009). The outputs of these signaling pathways include the stabilization of the DNA replication fork through ATR-Chk1 (Rad3-Cds1 in *S.pombe*) , a transient cell cycle arrest, the activation of DNA repair, and the promotion of apoptosis if the cell is beyond repair (Reinhardt & Yaffe, 2009).

While activation of these pathways by phosphorylation received much attention over the last two decades, its regulation by targeted dephosphorylation is much less well understood. Phosphatases have a key role in the regulation of the DDR (Bennetzen, et.al. 2010). A study of the phosphorylation dynamics in human cells after the occurrence of DNA damage showed that there is a substantial change in the phosphorylation pattern and its kinetics (Bensimon, et.al. 2010). In response to a radiometric agent that causes DNA breaks, 753 phosphorylation sites mapped to 394 proteins, and 342 sites showed events of dephosphorylation. More interestingly, the phosphopeptides are not only contained PI3-like kinases substrates, but they also contained substrates of cyclin-dependent kinases and casein kinase family members. This suggests that phosphatases have a key role in the process of DNA repair by protein dephosphorylation (Claudia, et.al. 2009; Bensimon, et.al. 2010).

1.1.3.1 Sensors of DNA damage

The proteins that start the signalling response and sense the abnormal structure of the DNA are still largely unknown. Recent work revealed an interesting insight into how single-stranded DNA breaks activate poly-ADP-ribose polymerase (PARP). Recognition of the unusual structure of the ssDNA break by the domains F1 and F2 triggers the re-organisation of the disorganised PARP domains and its catalytic activation (Eustermann, et.al. 2015).

DNA double-stranded breaks are recognised in human cells by the MRN complex which recruits ATM kinase (You, et.al. 2005) and single-stranded/double-stranded DNA junctions are the substrate for the ATR-ATRIP complex and the Rad9-Rad1-Hus1 ring which is independently loaded by Rad17 (Kondo, et.al. 2001). Rad17 can also recruit the MRN complex (Wang, et.al. 2014) which in turn can lead to ATR activation upon binding of the scaffold protein TopBP1 to the DNA lesion (Duursma, et.al. 2013). Intriguingly, ATM can also be

activated in the absence of the MRN complex through an as yet well-defined pathway (Hartlerode, et.al. 2015).

Rad9 is a subunit of the 9-1-1 complex. Its N-terminal section binds to Hus1 and Rad1 while its C-terminal extension is unstructured and highly phosphorylated (Figure 1-3 A). This unstructured region of Rad9 can bind to the 9-1-1 ring core complex and negatively regulate its DNA binding activity (Takeishi, et.al. 2015). The C-terminal domain binds also to TopBP1 in a manner regulated by its phosphorylation at two serine residues (S341, S387) in human cells (Ueda, et.al. 2012). The important activities of the tail domain are very interesting as this section is present in the *S.pombe* Rad9 variant (Figure 1-3 B). Interestingly, only growing *S.pombe* cells express this alternative translation product which initiates at AUG-50 (Janes, et.al. 2012). Its basal levels are very low but increase sharply in the response to heat stress. Loss of the variant reduces the duration of a heat-induced G2 arrest by 20-30min without rendering cells heat sensitive. The variant is phosphorylated by an unknown kinase independently of the DNA damage checkpoint and independently of the MAP kinase pathway which detects heat stress (Janes, et.al. 2012).

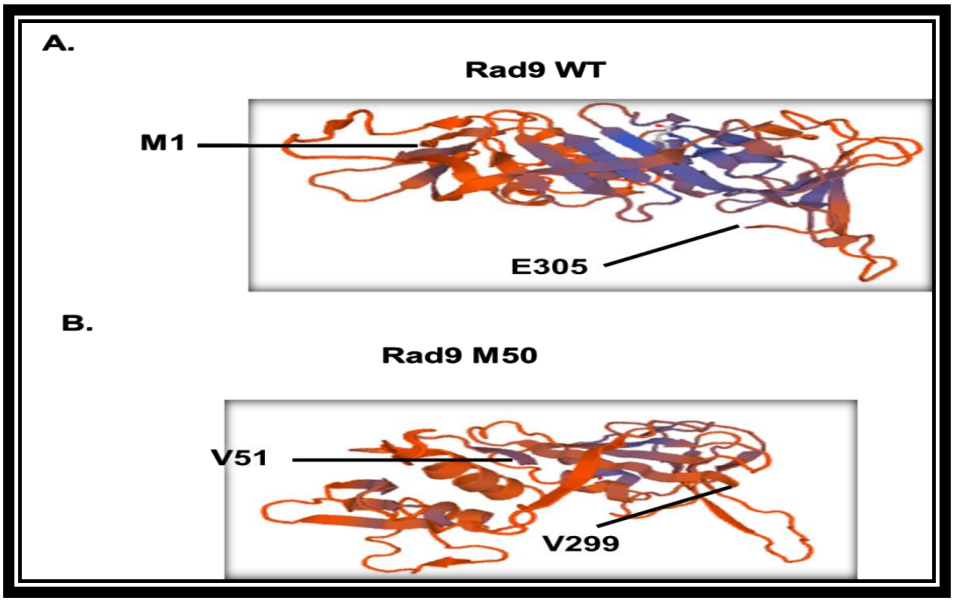


Figure 1- 3: Cartoon view of *S.pombe* Rad9 and its translational variant Rad9-M50 structure.
A: Rad9. The first and last amino acid in the structure are indicated. Please note that the unstructured C-terminal domain of Rad9 and Rad9-M50 is absent from the structure. B: Rad9 variant. Loss of the first 49 amino acids significantly affects the structure of Rad9-M50 indicating that the protein does not assemble in the 9-1-1 complex like full-length Rad9.The models were produced using the Swiss Model tool. They are based on the crystal structure of human Rad9 (Dore et al., 2009)

The 9-1-1 ring is loaded at sites of DNA damage by Rad17, a protein homologous to the Replication Factor C which associates, like RFC, with the 4 smaller RCF subunits 2-5 (RFC2-5) (O'Connell, et.al. 2000). Table 1-1 summarises the conserved proteins of the DNA damage checkpoints in fission yeast (*S.pombe*), budding yeast (*S.cerevisiae*) and mammals.

Table 1- 1: Conserved genes respond to DNA damage (Adapted from Bin-Bing & Stephen 2000).

Functional class	**Fission Yeast** ***S. pombe***	**Budding Yeast** ***S.cerevisiae***	**Mammals**
PCNA-like proteins	Rad1, Rad9, Hus1	Rad17, Ddc1,Mec3	Rad1, Rad9, Hus1
RFC-like proteins	Rad17	Rad24	Rad17
BRCT proteins	Crb2/Rhp9, Cut5	Rad9, DPB11	BRCA1? , 53BP1?
P13K-like proteins	Rad3, Tel1	Mec1, Tel1	ATM, ATR
Effector kinases	Chk1, Cds1	Chk1, Rad53	Chk1, Chk2
Coiled-coil proteins	Rad26	Ddc2/Lod1	ATRIP

In response to DNA damage, the Rad17-RFC complex interacts directly with the damaged DNA before loading the Rad9-1-1 complex onto DNA which results in the activation of Rad3/ATR kinase. The latter kinase is independently recruited by its subunit Rad26/ATRIP to single-stranded DNA (Figure 1-4) (Bermudez et.al. 2003; Ellison and Stillman, 2003). In fission yeast it is well known that Rad3-dependent phosphorylation of Chk1 kinase at serine 345 and Cds1 kinase at T11 within its N-terminal SQ/TQ cluster domain activates the G2-M and inter-S phase checkpoint, respectively (Nam & Cortez, 2011).

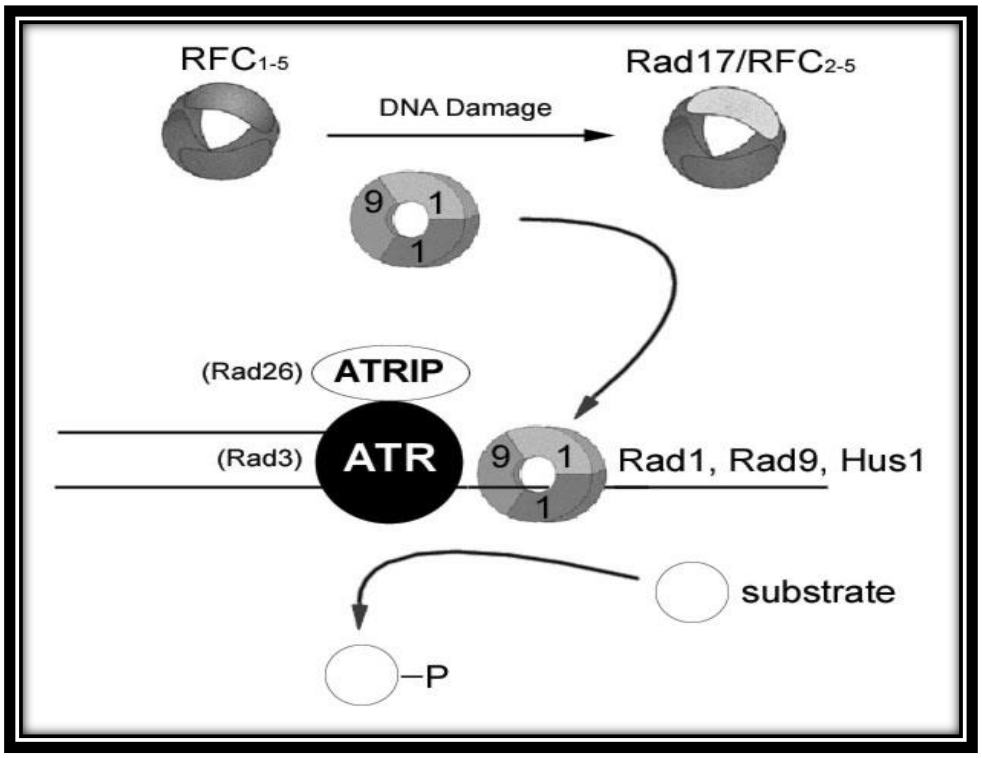


Figure 1- 4: Sensing the DNA damage by the Rad9-Rad1-Hus1 (9-1-1) complex. (Renthal, 2002).

1.2 Activation of Checkpoint Kinase 1 (Chk1)

In human and yeast cells, ATR/Rad3 kinase activates the effector kinase Chk1 by pho-phosphorylating its regulatory C-terminal domain at serine 345 (Han, et.al. 2016).

While yeast Chk1 is only phosphorylated at serine 345, human Chk1 is also phosphorylated at serine 317 (Liu, et,al, 2000; Capasso, et.al, 2002). Phosphorylation has been suggested to function as a catalyst to relieve an intermolecular interaction that blocks the access of substrates to the catalytic site of Chk1 (Liu, et.al. 200; Zhao & Piwnica-Worms, 2001; Lopez-Girona, et.al. 2001). A crystal structure of human Chk1 shows that its catalytic site assumes an open conformation. However, Chk1 does not achieve its full activity in the absence of DNA damage, thus indicating the existence of an inhibitory mechanism that blocks its maximum activity. Because the C-terminal domain of Chk1 interacts with the N-terminal kinase domain and since the N-terminal kinase domain alone has a high *in vitro* activity (Katsuragi, et.al. 2004; Walker, et.al. 2009), it is likely that the intra-molecular interaction delivers a physical interference of the catalytic site which is released in the presence of DNA damage (Figure 1-5, 1-6). In the absence of DNA damage, the N-terminal section (31-87aa) associates with the C-terminal part of Chk1 (particularly Leu-449), an interaction which is interrupted by the phosphorylation of S345 by Rad3/ATR in the presence of DNA damage (Figure 1-6) (Walworth, et.al. 1993).

It should also be noted that human Chk1 responds to DNA damage in S phase, whereas yeast Chk1 acts mainly in G2 (Bartek, et.al. 2003).

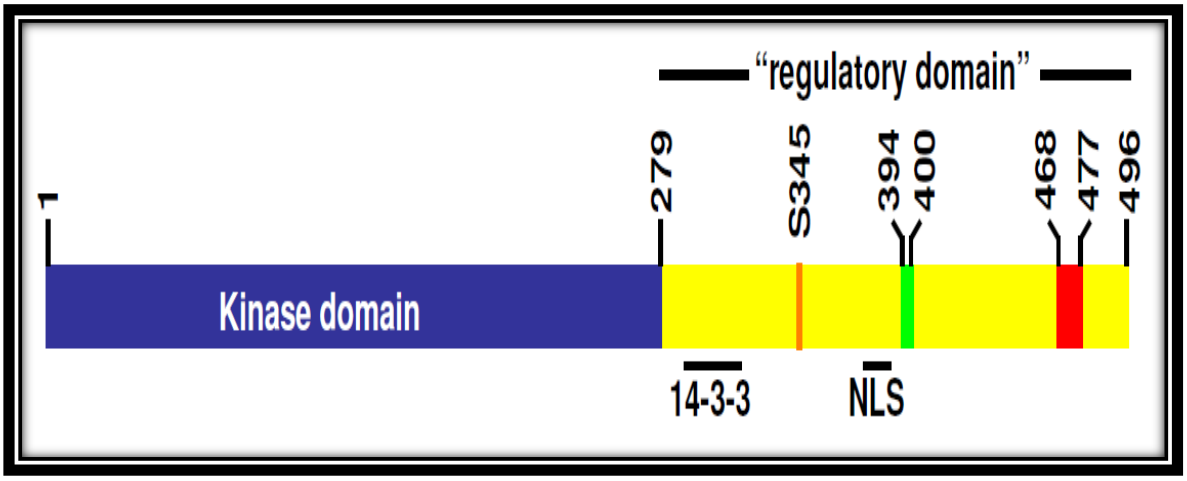


Figure 1- 5: The domain structure of Chk1 in the fission yeast *S.pombe.*

The N-terminal kinase domain is shown in blue and the C-terminal regulatory domain is shown in yellow. The domains indicated under the diagram are the important interaction domains with 14-3-3 proteins and the nuclear localization signal (NLS). The activating phosphorylation site serine-345 (S345) is shown in orange in the C-terminal regulatory domain (Adapted from Claudia, et.al. 2009).

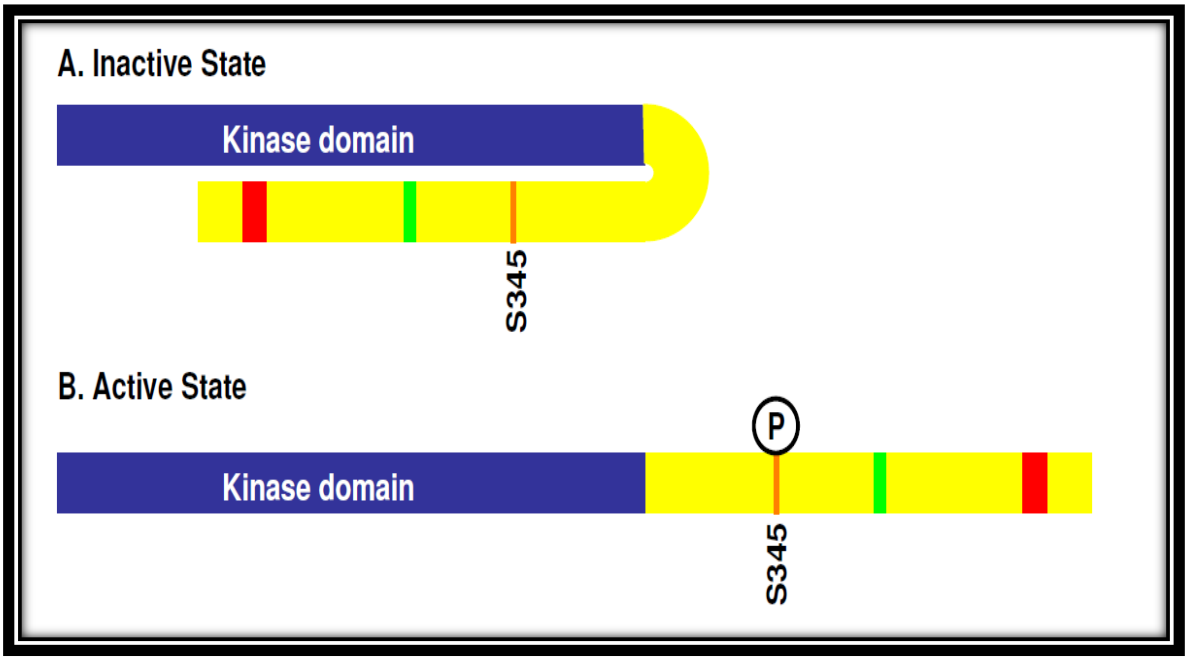


Figure 1- 6*:* The Chk1 regulation auto-inhibition model.

(A) When the C-terminal domain and the kinase domain physically associate, the kinase is inactive. (B) The phosphorylation of S345 opens and activates the kinase (Adapted from Claudia, et.al. 2009).

An early study on yeast Chk1 by Walworth found that overexpression of Chk1 leads to the arrest of the cell cycle in G2 without any need of the other *rad* checkpoint genes, which

suggests that Chk1 acts close to the end of the signaling cascade to halt cell cycle progression (Walworth, et.al. 1993). The cell cycle regulator Cdc2 kinase is phosphorylated at tyrosine 15 by Wee1 kinase, which keeps Cdc2 in a low activity state (Russell & Nurse, 1987). Wee1 is the target of Chk1 in yeast and human cells, and Wee1 phosphorylation by Chk1 increases the inhibitory tyrosine-15 modification of Cdc2 thereby temporarily halting the cell cycle in G2 (O'Connell, et.al. 1997). Full activation of Chk1 by ATR/Rad3 requires two scaffold proteins with BRCT domains (BRCA1 C-terminal domain), Crb2/53BP1 and Rad4/Cut5/TopBP1 since both interact with Chk1 and Rad3/ATR to form the fully active DNA damage checkpoint complex (Parrilla-Castellar, et.al. 2004). Fission yeast Crb2 recruits Chk1 closer to Rad3, which leads to Chk1 phosphorylation at S345 by Rad3 (Lopez-Girona, et.al. 2001; Capasso, et.al. 2002). This requires also the 9-1-1 ring that binds to both ATR/Rad3 and Rad4/Cut5. Chk1 activation requires Rad9 S/TQ-site phosphorylation to promote association with C-terminal BRCT domains of Rad4/TOPBP1 (Furuya, et.al. 2004). In humans, Chk1 is also phosphorylated at a second site, S317, but the analogous site (T323) in *S.pombe* Chk1 is not modified (Capasso, et.al. 2002). S345 phosphorylation stimulates Chk1 activity approximately 5 to 10 fold over a basal level that has been measured *in vitro* (Walworth, et.al. 1993). Whether this basal activity plays a key role in regulating, the progression of the cell cycle has not been thoroughly tested. It has been reported that one of the important Ser/Thr phosphatases in fission yeast, Dis2 removes the phosphate from S345 to inactivate the Rad3-Chk1 signal allowing cells to re-enter the cell cycle (Den & O'Connell, 2004). In human cells PP2A phosphatase is involved in the inactivation of Chk1 (Den & O'Connell, 2004). However, the precise mechanism of how these phosphatases inactivate Chk1 is still unknown.

1.3 Heat stress and DNA breaks

Heat stress not only hinders DNA replication, but also acts as a DNA damaging agent (Beck, et.al. 1995). It is known that heat stress can lead to the buildup of 8-oxoguanine, delaminated cytosine, and apurinic DNA sites (AP-sites) in a cell. Depending on the cell cycle stage, heat causes either single-stranded DNA breaks (SSBs) in S phase or double-stranded breaks in G1 and G2 (Velichko, et.al. 2012). A recent study found that heat stress prompts SSBs by the inhibition of DNA topoisomerase I Top1) in S phase (Kantidze, et.al. 2016). This is an enzyme that relaxes DNA supercoils in front of moving DNA replication forks by introducing transient SSBs into DNA. The catalytic cycle of Top1 comprises the cleavage of one DNA strand, and this enzyme-DNA cleavage complex which is stabilized at high temperatures (Beck, et.al.

1995). Collision of DNA replication forks with these immobilized complexes would normally lead to the formation of DNA double-strand breaks, but they are absent in heat-stressed human cells since heat delays at the same time fork progression (Wang, et.al. 2001REF). While the ATR-Chk1 pathway is activated by heat stress and Chk1 is phosphorylated at S345, some other ATR-dependent DNA damage responses do not take place. For example ATR does not modify the single-stranded DNA binding protein RPA32 (Tuul, et.al. 2013). Activation of ATM-Chk2 at elevated temperatures is delayed because the DNA damage sensors, the Rad50-Mre11-Nbs1 complex and 53BP1, are inactivated at high temperatures (Seno & Dynlacht, 2004).

1.4 Serine/ threonine phosphatases

Recent work in the fission yeast (*S.pombe*) revealed the up-regulation of a N-terminally truncated Rad9 variant which acts outside of the 9-1-1 complex and may dephosphorylate Chk1 at S345 in the presence of DNA breaks at elevated temperatures (Janes et al., 2012). Given the possible link between Rad9 variant and the dephosphorylation of Chk1, the different types of phosphatases are introduced here.

Serine/Threonine phosphatases are grouped according to their structure, sequence, and biochemical properties, such as metal dependency. Amongst these enzymes are seven metal-independent protein phosphatases (PP1/Dis2, PP2A/PPA2, PPA2B, PP4, PP5, PP6 and PP7) and one group of $Mg^{-2+/}Mn^{2+}$-dependent phosphatases (PP2C/Ptc1-4) (Lee & Chowdhury, 2011).

1.4.1 Protein phosphatase Dis2 (PP1 type)

Protein phosphatases of type 1 (PP1) have been identified as Ser/Thr phosphatases which are highly expressed and involved in many cellular functions such as mitotic progression, RNA processing, checkpoint activation, and DNA repair (Lee & Chowdhury, 2011). These protein phosphatases exist in three catalytic isoforms (PP1α, PP1β, and PP1γ) which assemble in about 650 different phosphatase complexes with different PP1-interacting proteins (Lee & Chowdhury, 2011). These interacting proteins are substrate-targeting proteins or substrate-inhibitors (Bollen, et.al. 2010). Some PP1 phosphatases play important roles in DNA damage signaling. For example, human ATM kinase is maintained in its inactive state by dephosphorylation of Ser1981 by the Repo-Man-PP1γ complex (Peng, et.al. 2010). In the presence of a double-stranded DNA break, Repo-Man disconnects from ATM thereby allowing for the auto-phosphorylation of ATM at Ser1981 (Peng, et.al. 2010). The PP1 phosphatase Dis2 is directly involved in the inactivation of Chk1in the fission yeast *S.pombe* to permit cell cycle

re-entry after DNA damage has been repaired (Den & O'Connell, 2004). The required PP1-interacting protein is not yet known.

Den and coworkers have found that Dis2 dephosphorylates Chk1 at S345 after DNA damage induced by UV light (Den & O'Connell, 2004). Intriguingly, Chk1 is still phosphorylated in the absence of Dis2, although at a much lower level after DNA damage. This implies that Dis2 is not the only phosphatase involved in Chk1 regulation.

The inactivation of Chk1 is likely to act as the primary mechanism to release the checkpoint arrest in *S.pombe*. Hyper-phosphorylation at Ser-345 and activation of Chk1 decreased both coincidentally with the entry into mitosis (Harvey, et.al. 2004).

Interestingly, Dis2 does not influence the function of the Rad proteins (i.e. Rad3, & the 9-1-1 ring) which act upstream of Chk1 in checkpoint signaling (Den & O'Connell, 2004). The Rad proteins and Rad3/ATR kinase work normally regardless of the levels of Dis2 (Den & O'Connell, 2004). Consistent with this idea, phosphorylation of the Rad3 substrate, Rad9 was unaffected by the over-expression of Dis2. Dis2 appears to be specific to the down-stream elements of the pathway as only Chk1 and its scaffold protein Crb2 were affected in cells with high Dis2 protein levels. (Den & O'Connell, 2004).

In human cells, the hyper-phosphorylation of the BRCA1 scaffold protein is decreased by the over-expression of PP1α, and a BRCA1 derived peptide is dephosphorylated by PP1α *in vitro* (Murakami & Okayama, 1995). BRCA1 acts in the context of DNA break repair and is important to mediate between ATM kinase and the MRN complex (Alli & Ford, 2015).

How human cells coordinate phosphorylation and dephosphorylation of Chk1 is as yet unclear. Mammalian PP1α and PP1δ show high activity in the response to DNA damage in a manner dependent on ATM kinase. The latter is probably related to a reduction in their inhibitory phosphorylation by the cell cycle regulator Cdk2 which is the target of ATM (Den & O'Connell, 2004). It is thought that the activity of Dis2 can also be inhibited by Cdc2/Cdk1 in *S.pombe* (Den & O'Connell, 2004).

Another study suggested that S345 is protected from Dis2 either by the association of Chk1 with other proteins like the 14-3-3 proteins or by a change in localization (Figure 1-7) (Heiko, 2003). The phosphoserine-binding protein family, known as 14-3-3 proteins, have been shown to protect phosphoserine motifs, as well as to alter the subcellular localization of proteins. The Rad24 and Rad25 proteins, the two14-3-3 proteins in *S.pombe* are bound to the phosphorylated form of Chk1 (Dunaway, et.al. 2005). Another protein that may shield S345 from dephosphorylation is Crb2 which associates with the phosphorylated form of Chk1 kinase and is itself a target of Rad3 kinase (Satoru, et.al. 2004).

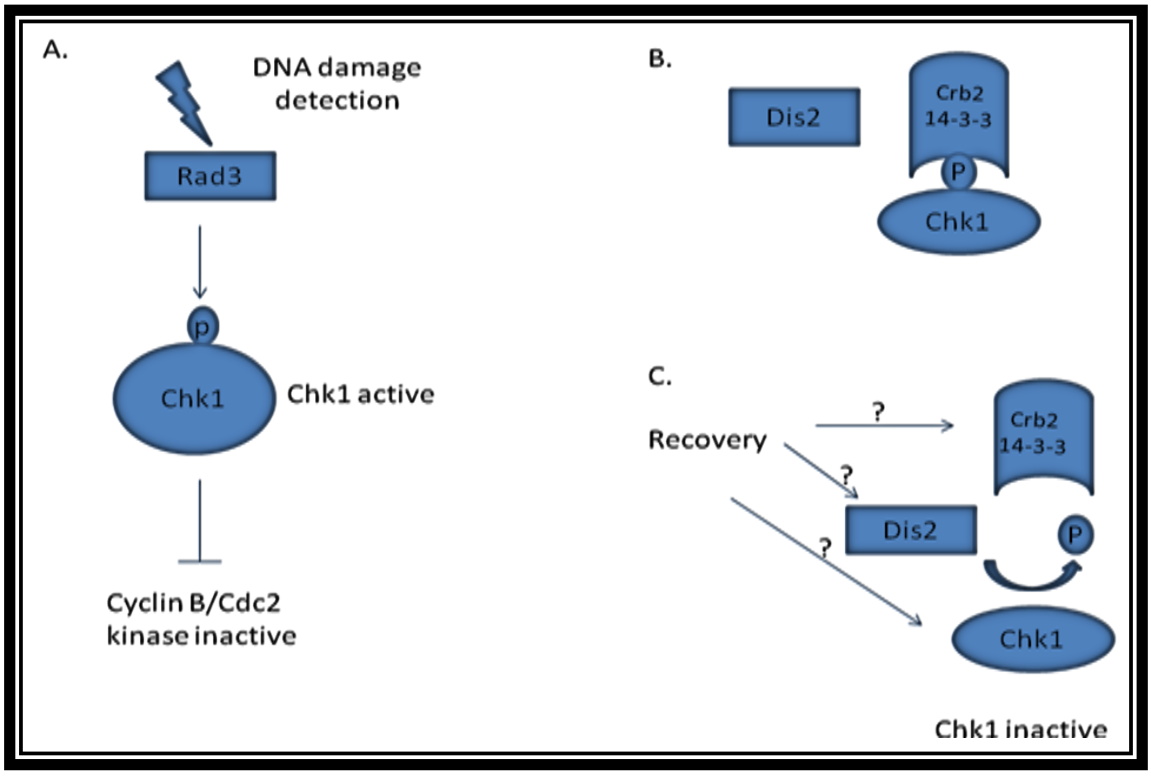


Figure 1- 7: Model for the de-phosphorylation of Chk1 in the fission yeast *S.pombe*.

(A) DNA damage leads to the phosphorylation of Chk1 at S345 by Rad3 (ATR) kinase. (B) The binding of the phosphorylated Chk1 kinase to either the 14-3-3 proteins Rad24 and Rad25, or to the scaffold protein Crb2 (53BP1) may shield the modified S345 from Dis2 phosphatase. (C) The release of the 14-3-3 proteins and Crb2 allow Dis2 to remove the phosphate from S345 thereby terminating the DNA damage signal.

1.4.2 Phosphatase type2-A (PP2A) or PPA2

In contrast to PP1 phosphatases, phosphatases of type2-A form hetero-trimeric complexes that consist of one catalytic subunit (α or β), one scaffolding A subunit (a or b) and one regulatory B subunit (PR55, PR61, PR72) (Lee & Chowdhury, 2004). *S.pombe* PPA2 is a serine/threonine protein phosphatase which shares 80% sequence identity with mammalian type 2A phosphatases. This phosphatase controls entry into mitosis by targeting Wee1 and Cdc2 kinase (Kinoshita, et.al 1993). Human PP2-A plays a key role in the response to DNA damage primarily through the regulation of ATR and ATM (Freeman, et.al. 2010). At broken chromosomes, PP2-A dephosphorylates downstream targets of both kinases including the histone variant γ-H2AX which is phosphorylated by ATM at serine 139 (Chowdhury, et.al. 2005).

In yeast cells, PP2A is a complex of three different subunits known as the C (catalytic), B (regulatory), and A (scaffold) subunit (Yu, 2006). PP2Ac (the C subunit) is the catalytic subunit, and its interaction with the scaffold subunit A forms the dimeric core enzyme. PP2Ab

(the B subunit) is the regulator subunit that defines the substrate specificity and intracellular localization of the core enzyme (Yu, 2006).

In higher eukaryotes, as well as in the fission yeast *S. pombe*, PP2A regulates mitotic entry (Kinoshita, et.al. 1993) by inactivating CDK1/Cdc2 activity which promotes mitosis (Tina, et.al. 19991). A similar role is executed by PP2A in the budding yeast *S.cerevisiae* **(**Yu, 2006). Human Ppa2 has been shown to dephosphorylate Chk1 at S317 and S345 *in vitro*, and its activity is regulated by Chk1 in a feedback loop (Leung-Pineda, et.al. 2006).

1.4.3 Phosphatase type 2-C (PP2C or Ptc1-4)

PP2C is the second major group of the phosphatases that dephosphorylate serine and threonine residues. This type of phosphatase is Mg^{+2} or Mn^{+2} dependent and has no sensitivity to okadaic acid, unlike the other two groups. Mammalian PP2C is involved in $Ca2^{+}$ signalling as well as in cell differentiation induced by vitamin D3 (Fukunaga, et.al. 1993; Leung-pineda, et.al. 1994). *Ptc1*$^{+}$, *Ptc2*$^{+}$ and *Ptc3*$^{+}$ are the three genes encoding PP2C-type phosphatases in the fission yeast *S.pombe* (Shiozaki & Russell, 1995 a-c). It has been reported that yeast and mammalian PP2C enzymes negatively regulate stress-induced signals through the MAPK pathways utilising p38 kinase in mammals, Hog1 in budding yeast *S.cerevisiae* and Spc1/Sty1 in fission yeast *S.pombe* (Maeda, et.al. 1994; Shiozaki, et.al. 1994, 1995a-c; Gaits, et. al. 1997).

The deletion of any of the three *ptc* genes in the fission yeast *S.pombe* is viable and approximately 10% of PP2C activity can be retained which is dependent on a fourth *PP2C* gene which is called *ptc4* (Shiozaki & Russell, 1995). Ptc4 is implicated in the signaling of nutrient deprivation (Gaits & Russell, 1999) and regulates the MAPK Sty1 in the response to oxidative stress (Di, et al., 2012**).** Sty1 MAPK is also regulated by Ptc1 under heat shock conditions (Nguyen & Shiozaki, 1999). Sty1 is phosphorylated at a tyrosine and a threonine in close proximity in its kinase subdomain VIII (T171 + Y173) by the MAPK kinase Wis1 (Zaskiewicz & Cooper, 1995; Brewster, et.al. 1993; Millar, et.al. 1995; Shiozaki and Russell, 1995; Kato, et.al. 1996).

Upon its activation by Wis1, Sty1 phosphorylates the transcription factor Atf1 to promote the expression of stress-induced genes (Shiozaki & Russell, 1996; Chen, et.al. 2003; Paredes et.al. 2003) and targets the Cdc2 phosphatase Cdc25 to regulate onset of mitosis (Lopez-Aviles, et.al. 2005). Interestingly, the impact on mitosis depends on the type of stress. While osmotic and oxidative stress advances *S.pombe* cells into mitosis, heat stress blocks mitosis (Shiozaki & Russell, 1995; Kishimoto & Yamashita, 2000). How Sty1 achieves these opposing outcomes is not fully understood. While the phosphatases Ptc1 and Ptc3 regulate Sty1 in the response to

osmotic and oxidative stress by dephosphorylating T171 (Shiozaki & Russell, 1995), Pyp1 phosphatase has a major role at elevated temperatures by dephosphorylating Tyr-173 (Degols, et.al. 1996; Samejima, et.al. 1997). Interestingly, the simultaneous deletion of *pyp1+* and *pyp2+* leads to the lethal hyper-activation of Sty1 which supports the idea that Tyr173 dephosphorylation by Pyp1 and Pyp2 is the key regulation of Sty1 (Millar, et.al. 1995; Shiozaki and Russell, 1995). Interestingly, Tyr-173 stays phosphorylated in the response to heat stress since Pyp1 and Pyp2 are both blocked while Thr-171 is dephosphorylated by Ptc1 and Ptc3 (Figure 1-8) (Nguyen & Shiozaki, 1999). This implies that under heat conditions, Sty1 regulation is governed only by Ptc1 and Ptc3. How Pyp1 and Pyp2 are inactivated at the same time is still unknown.

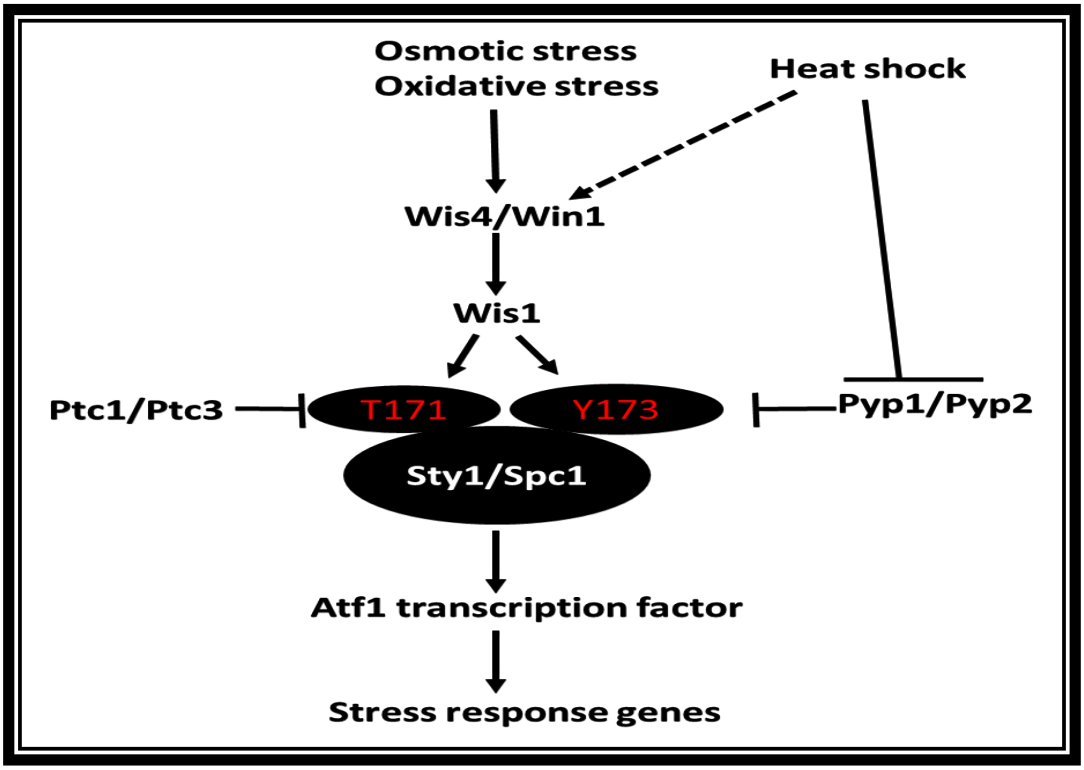


Figure 1- 8: Sty1/Spc1 activation in the response to osmotic, oxidative and heat stress and its regulation by the serine phosphatases Ptc1 and Ptc3, and the tyrosine phosphatases Pyp1 and Pyp2 in *S.pombe* (Adapted from Nguyen & Shiozaki, 1999).

Active Sty1 modifies the CREB transcription factor Atf1 at eleven serine and threonine residues which are directly followed by a proline (S/TP) (Figure 1-9) (Wilkinson, et.al. 1996; Gaits, et.al. 1998). Phosphorylation of Atf1 induces its association with the bZIP transcription factor Pcr1 and the translocation of the heterodimeric complex into the nucleus (Takeda, et.al. 1995; Wahls & Smith, 1994; Kanoh, et.al. 1996).

Atf1 is the mammalian Atf2 paralogoue which is phosphorylated by p38 MAP kinase (Takeda, et.al. 1995). The heteromeric Atf1-Pcr1 transcription factor binds to DNA sequences known as CRE (the cAMP response element) elements in promoter regions (Ziff, 1990).

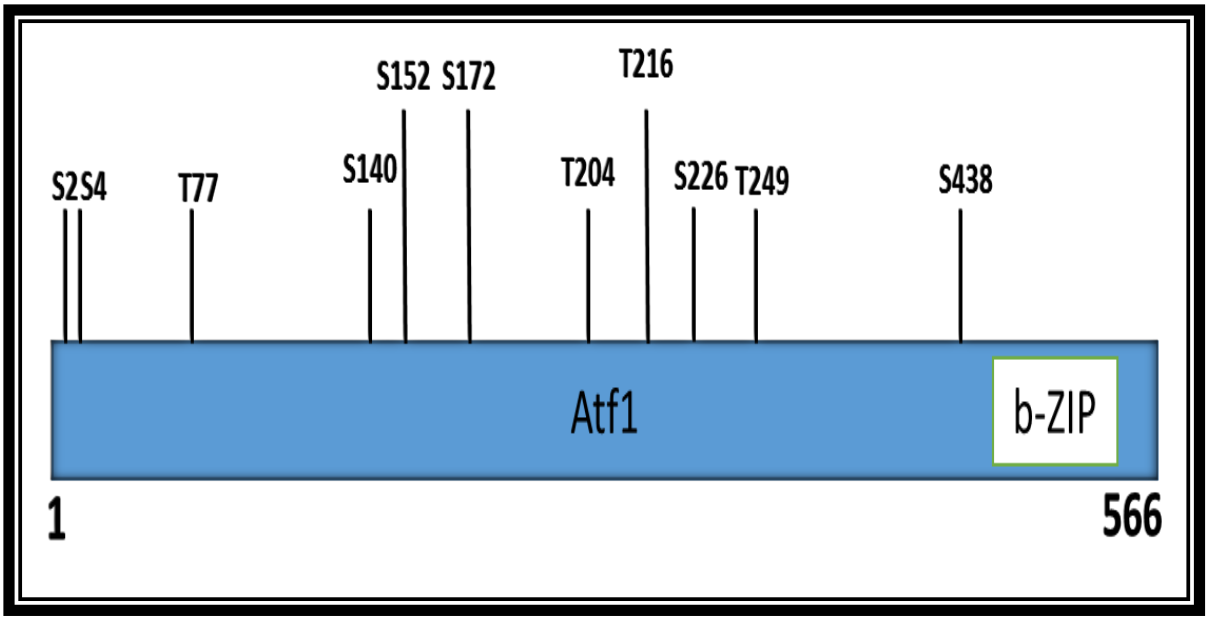


Figure 1- 9: The potential 11 MAPK sites on Atf1 protein.
This protein consist of 566 amino including the domain of b-ZIP and the essential 11 sites of MAPK which are either Thr-Pro or Ser-Pro (Adapted from Lawrence, et.al. 2007).

Both yeast Sty1 and human p38 are activated by γ-irradiation and UV light that break DNA in addition to the other environmental stress factors (Bulavin, et.al. 2002; Mikhailov, et.al. 2004). The activation of p38 in the response to DSBs is closely related to the ATM and ATR checkpoint kinases (Reinhardt, et.al. 2007). Activation of p38 requires ATM but not by a direct phosphorylation event as the p38 kinase lacks the SQ/TQ motif that is recognised by ATM kinase. It is believed that ATM directly targets the Tao kinases (Thousand and one) which activates p38 via the upstream kinase MKK3/6 (Raman, et.al. 2007). An alternative mechanism, distinct from ATM and ATR, may also exist as p38 is stimulated in the presence of the ATR/ATM inhibitor caffeine in the response to UV and topoisomerase II inhibitors (Mikhailov, et al., 2004; Reinhardt, et al., 2007). Sty1 and p38 both regulate G2-M transition through the Cdc2 phosphatase Cdc25B (Bulavin, et.al. 2001). Although human Cdc25B is directly phosphorylated by p38 *in vitro*, other studies indicated that p38 indorsed Cdc25B phosphorylation indirectly via activation of MK2 kinase (Manke, et.al. 2005; Lemaire, et.al. 2006). MK2 activation is believed to be important for the G2/M checkpoint in the response to

UV light (Manke, et.al. 2005; Lemaire, et.al. 2006). It has been shown that in the response to γ-irradiation, all isoforms of p38 MAPK are activated in an ATM dependent manner except the p38γ isoform (Wang, et.al. 2000).

The functional relationship between Sty1 and Rad3 in the fission yeast *S.pombe* is much less clear. Although activation of Rad3 by osmotic stress has not been reported, it is evident that Sty1 is activated by DNA damage triggered by UV light and ionizing radiation (IR) (Degols & Russell, 1997; Watson, et.al. 2004). Microarray studies revealed that IR increases the transcription of some stress response genes (*gpx1+, meu8+, obr1+, plr1+ and tms1+*) in a manner partly dependent on Sty1 and Rad3. While these genes were up-regulated in *rad3Δ* or *sty1Δ* single mutants, their expression was blocked in the *rad3Δsty1Δ* double mutant (Watson et al., 2004). Another cross-over point is Cdc25 phosphatase which associates with Sty1 and is indirectly targeted by Rad3 through the down-stream kinases Cds1 and Chk1 (Sundaram, et.al. 2008).

1.5 Aim of the project

Heat stress remodels the DNA damage response in human cells so that DNA breaks are no longer recognised. Heat also arrests cells in G2 and blocks DNA replication (Turner & Caspari, 2014). These temperature induced changes are now used in the clinic to treat cancer patients. Especially the delayed response of ATM kinase to broken chromosomes sensitizes cancer cells to radiotherapy. New nanotechnology approaches aim to locally generate high temperatures inside cancer tissues when the devises are activated by microwaves or magnetic waves (Datta, et.al. 2016). Heat is now an established treatment regime for localised cancers like prostate tumours (Zhang, et.al. 2016). It is therefore important to explore how heat affects the efficacy of the DNA damage response as this is directly correlated with tumour recurrence and drug resistance (Curtin, 2013). The DNA damage detection system acts also closely with the stress-responsive MAP kinase pathways. Indeed, both pathways are activated by the same treatments like UV light and ionising radiation (Alao & Sunnerhagen 2008). How both systems interact and how they prevent cancerous cell growth is however not well understood.

The fission yeast *Schizosaccharomyces pombe*, serves as a very good model to understand how heat affects the response to DNA breaks and how the two pathways interact. Especially the heat-induced induction of an N-terminally truncated protein variant from a cryptic translation initiation site (AUG-50) in the *rad9* mRNA may open a window to analyse these processes (Janes, et.al. 2012). Full-length Rad9 forms a DNA detection complex with Rad1 and Hus1, known as the 9-1-1 DNA damage checkpoint clamp. The Rad9 variant acts independently of

this ring complex in the regulation of the heat-induced G2 arrest and its expression correlates with the heat-induced removal of the activating serine 345 phosphorylation of Chk1 kinase which is one of the main readouts of ATM/Tel1 and ATR/Rad3 activation by DNA damage (Janes et al., 2012).

This PhD thesis did set out to obtain further information about the cellular roles of Rad9 variant in the regulation of Chk1 and in the modulation of the heat-activated Wis1-Sty1 MAP kinase pathway.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Yeast Media

Rich Medium (YEA and YEL): 3% (30g/L) glucose, 0.5% (5g/L) yeast extract and 200 mg/L adenine (for plates 2% (20g/L) agar was added.

Minimal Medium (EMM): 3% (30g/L) glucose, 0.67% (6.7g/L) yeast nitrogen base without amino acids and the following supplements were added as required (adenine 200mg/L, leucine=uracil=histidine=100mg/L) and the pH was adjusted to 5.5-6.0 with NaOH. For plates 2% (20g/L) Melford Agar was added.

Malt Extract (ME and MEA): 30g/L Malt extract, 225mg/L (adenine, uracil, histidine and leucine) and for plates 20g/L agar was added. The pH was adjusted to 5.5.

2.1.2 Stock solutions and Buffers

50x Tris-acetate-EDTA (50x TAE): 242g Tris base, 37.2g $Na_2EDTA.2H_2O$, 57.1ml glacial acetic acid and filled up to 1L with distilled water.

10x Phosphate Buffered saline (10x PBS): 43.6g $Na_2HPO_4.7H_2O$, 12.8g NaH_2PO_4, 360g NaCl and filled up to 4L with distilled water.

Ethylenediamine tetra acetic acid (EDTA) 0.5M (pH=8): 93.05g EDTA in 500ml distilled water. To adjust the pH to 8, 5M NaOH was added.

1M Tris: 60.57g Tris in 500ml distilled water. pH adjusted to 8

1x Tris-EDTA (1xTE): 10mM Tris-HCl (pH 7.5) and 1mM EDTA (pH 8).

100% Trichloroacetic Acid (100% TCA): 500g TCA in 350ml distilled water.

10x SDS buffer : 40g SDS , 576g glycine, 121g Tris-HCl and filled up to 4L with distilled water.

10x transfer buffer: 56g glycine, 124g Tris-base and filled up to 1L distilled water.

10x Tris-EDTA (10x TE): 0.1M Tris-HCL and 0.01M EDTA. pH adjusted to 7.5.

4x SDS loading protein: 16ml of 100% glycerol, 9.6ml of 1M Tris-base (pH 8), 16ml of 20% SDS and 2ml of beta mercaptoethanol and bromphenol blue.

5M NaCl: 146g NaCl in 500m distilled water.

3% Milk buffer: 15g milk powder, 50ml of 10x PBS, 250µl tween 20 and up to 500ml distilled water.

2.1.3 Strains used in this study

Table 2- 1 : Genotypes of strains used in this study.

Strains	Genotype	Source
WT 804	*h- ade6-M216 leu1-32 ura4-D18*	Janes et al., 2012
rad9-HA	h- ade6-M216 leu1-32 ura4-D18 rad9-3HA-kanMX4	Janes et al., 2012
rad9.M50A-HA	*h- ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat*	Janes et al., 2012
Δrad9	h- ade6-M216 rad9::ura4+ leu1-32 ura4-D18	Janes et al. 2012
chk1.Myc.HA.His	*h- leu1-32 ura4-D18 chk1 9myc2HA6His::ura4+*	Qu et al., 2012
chk1-Myc. rad9-HA WT	*leu1-32 ura4-D18 chk1 9myc2HA6His::ura4+* rad9-3HA-kanMX4	This study
chk1-HAHisMyc. rad9.M50A-HA	*leu1-32 ura4-D18 chk1 9myc2HA6His::ura4+ rad9.M50A-3HA-nat*	This study
Δ dis2	*dis2::ura4+ ade6-M216 leu1-32 ura4-D18*	(Japan Fission Yeast Strain Collection)
Δ ppa2	*ppa2::ura4+ ade6-M216 leu1-32 ura4-D18*	(Japan Fission Yeast Strain Collection)
Δ ptc1	*ptc1:: ura4+ ade6-M216 leu1-32 ura4-D18*	FY17634 (Japan Fission Yeast Strain Collection)
Δ pyp1	*pyp1::* kanMX4 *ade6-M216 leu1-32 ura4-D18*	Bioneer *S.pombe* deletion library
chk1-HA. Δ dis2	*chk1-HA dis2::ura4+ ade6-M216 leu1-32 ura4-D18*	This study
chk1-HA. Δ ppa2	*chk1-HA ppa2::ura4+ ade6-M216 leu1-32 ura4-D18*	This study

chk1-HAc. Δ ptc1	*chk1-HA ptc1:: ura4+ ade6-M216 leu1-32 ura4-D18*	This Study
chk1-HA. Δ pyp1	*chk1-HA pyp1::* kanMX4*ade6-M216 leu1-32 ura4-D18*	This Study
Δ dis2. rad9.M50A-HA	*ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat dis2::ura4+*	This Study
Δ ppa2. rad9.M50A-HA	*ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat ppa2::ura4+*	This Study
Δ ptc1. rad9.M50A-HA	*ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat ptc1:: ura4+*	This Study
Δ pyp1. rad9.M50A-HA	*ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat pyp1::* kanMX4	This Study
ded1.1D5	*ded1.1D5 ura4.D18 ade6 leu1.32*	Liu et al., 2002
rad9-HA. ded1.1D5	*ded1.1D5 ura4.D18 ade6 leu1.32 rad9-3HA-kanMX4*	This Study
rad9-HA. ded1.1D5 pRep41-Myc-Ded1-wt	*ded1.1D5 ura4.D18 ade6 leu1.32 rad9-3HA-kanMX4 pREP41-Myc-Ded1*	This Study
cds1-HAHis	*cds1-2HAHis6-ura4+ ura4-D18, leu1-32 ade6-M216*	FY11080 (Japan Fission Yeast Strain Collection)
cds1-HAHis ded1.1D5	*ded1.1D5 cds1-2HAHis6-ura4+ ura4.D18 ade6 leu1.32*	This Study
cds1-M159A	*cds1::loxP-cds1-M159A-HA-loxM leu1-32 ura4-D18 ade6-M216*	(Fletcher and Caspari, unpublished)
Δ sty1	*sty1::ura4+ ade6-M216 leu1-32 ura4-D18*	Millar et al., 1995
Δ wis1	*wis1:ura4+ ade6-M216 leu1-32 ura4-D18*	Millar et al., 1995
Δ hus1	*hus1::Leu2+ ade6-M216 leu1-32 ura4-D18*	Caspari et al., 2000
Δ sty1. rad9.M50A-HA	*ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat sty1::ura4+*	This Study

Δ wis1. rad9.M50A-HA	*ade6-M216 leu1-32 ura4-D18 rad9.M50A-3HA-nat wis1::ura4+*	This Study
Δ hus1 Δ wis1	*hus1:Leu2+ wis1::ura4+ ade6-M216 leu1-32 ura4-D18*	This Study

2.1.4 Plasmids used in this study

Plasmid name	Source
pRep41-Myc	**Grallert, et.al. 2000**

2.1.5 Antibodies used in this study

Table 2- 2: List of antibodies.

Antibody	Company	Cat.no	Description	Concentration
Anti-HA	Santa Cruz	SC7392	Mouse-monoclonal	1:1000
Anti-Myc	Santa Cruz	SC40	Mouse-monoclonal	1:1000
Anti-P38-phosho	Abcam	Ab47363	Rabbit- polyclonal	1:2000
Anti-Atf1	Abcam	Ab18123	Mouse-monoclonal	1:2000
Anti-cdc2	Abcam	Ab5467	Mouse-monoclonal	1:2000
Anti-Rabbit secondary AB-HRP anti-light chain	Calbiochem	401315	Goat - polyclonal	1:10000
Anti-Mouse secondary AB-HRP anti-light chain	Jackson ImmuneResearch	115-035-174	Goat - polyclonal	1:10000

2.2 Methods

2.2.1 Construction of yeast strains

When *S.pombe* cells are starved for nitrogen they arrest in G1 and engage in mating between haploid cells of the opposite mating types h^+ and h^- in order to form spores as a result of meiosis. Cells of the opposite mating types were mixed on ME plates using a drop of sterile water and then incubated at 25°C for 4 days. Successful mating was detected under the microscope by asci formation. Random spores were selected by treating cells with 30% ethanol at room temperature for 30 min. Only spores survive this treatment, whereas vegetative cells are killed. The surviving spores were plated on YEA plates and incubated at 30°C for 4 days. The strains were tested for the desired genetic markers and by western blot. This protocol was adapted from Forsburg & Rhind (2006).

2.2.2 Storage of the strains

Long term storage was conducted in YEA liquid medium mixed with 50% glycerol at -80°C. To reactivate cells, a small amount of frozen cells was dispatch on a YEA plate before incubating the plates for 3 days at 30°C. The strains under examination were maintained on YEA plates and kept at 4°C for a maximum of one month.

2.2.3 Cell Counting

A haemocytometer (Thoma Double Cell) was used to count the cells by loading 10µl of liquid culture under a cover slip on the chamber and the cells were counted under a microscope. The cell number per large square was multiplied by 10^6 to calculate the number of cells per 1ml.

2.2.4 Protein extracts

2.2.4.1 TCA total protein extraction

Cells were grown in sterile YEA or MM liquid media as required in a 200 ml flask overnight at 30ºC in a shaking incubator up to 1-3x10^7 cells/ml. For one sample 5x10^8 cells were harvested in a 50ml tube which was spun for 3 minutes at 3000rpm. Cells were resuspended in a volume of 10ml of the required liquid medium in order to perform the treatments. After the treatment, cell pellets were washed in 20ml 1xPBS followed by centrifugation for 3 minutes at 3000rpm. The cell pellet was resuspended in 1ml 1xPBS, transferred to a 1.5 mL eppendorf tube and spun at 12000 rpm for 2 minutes at 4ºC. The supernatant was discarded and the cell pellet was resuspended in 200µL of 20% TCA, transferred to a 2ml flat-bottom microfuge tube.

After the addition of 8 small spoonful of glass or silica beads to break the cell wall, the tube was placed on the Genie Disruptor for 6 minutes in the cold room. The liquid cell extract was transferred to a 1.5 mL test tube and spun at 12,000 rpm at 4ºC for 5 minutes in a Sanyo Hawk 15/05 refrigerated bench top microfuge. The supernatant was discarded and the precipitated protein pellet was dissolved in 300µL of 4x SDS sample buffer and 300µl of 1M Tris (pH 8.8). Pellets were resuspended by pipetting prior to incubation at 95°C for 5 minutes and loading on SDS page. For further use, the samples were stored at -20°C.

2.2.5 SDS-PAGE

2.2.5.1 One dimensional SDS-PAGE

Different acrylamide percentages were used in this study to separate the proteins according to their sizes. A 20µl aliquot of the total protein extracts from the appropriate *S.pombe* strains was loaded and a 5µl aliquot of the protein molecular weight marker was included in one well. Either 8% or 10% SDS-PAGE gels were used (Tables 2-3 & 2-4). Electrophoresis was performed in 1x SDS running buffer at 120V using a Consort EV231 electrophoresis power pack.

Table 2- 3: Separation Gel

Component	**8%**	**10%**
Number of mini gels	4	4
H_2O	8.5 ml	7.5 ml
1M Tris HCl pH 8.8	7.5 ml	7.5 ml
40% acrylamide/ methylene-bis-Acrylamide (ratio 37.5:1 or 100:1)	4 ml	5 ml
20% SDS (Sodium Dodecyl Sulphate)	150 µl	150 µl
10% APS (Ammonium Persulfate)	100 µl	100 µl
TEMED (Tetramethylenediamine)	30 µl	30 µl

Table 2- 4: Stacking Gel

Component	Volumes
Number of mini gels	4
H_2O	15.5 ml
1M Tris HCl pH 8.8	1.5 ml
40% acrylamide/ methylene-bis-Acrylamide (ratio 37.5:1)	2 ml
20% SDS (Sodium Dodecyl Sulphate)	100 µl
10% APS (Ammonium Persulfate)	200 µl
TEMED (Tetramethylenediamine)	40 µl

Transfer of the proteins onto nitrocellulose membranes was performed in 1x Transfer buffer with 15% (v/v) methanol at 70V for 2 hours or at 10V over night for 15 hours. The membrane was carefully removed from the blotting apparatus, placed in a small plastic container and incubated with 3% milk buffer (blocking buffer) for at least 30min.

The blocked membrane was removed from the container and placed in a plastic wallet with 5mL of the diluted primary antibody in 3% milk buffer (Table2-2). The plastic wallet was sealed, and placed on the rocker platform in the 4ºC cold room for overnight incubation.

For development, the membrane was removed from the plastic wallet, washed in 1xPBS with 0.05% Tween 20 three times for 10min and re-incubated with 5 ml of the diluted secondary antibody in 3% milk buffer (Table 2-2). After an incubation for at least 45min, the washing steps were repeated and the washed membrane was rinsed in freshly prepared chemiluminescence HRP developing solution (Perkin Elmer Western Lightning Kit). Finally, the membrane was placed in a transparent plastic wallet in film cassette and exposed to film (Fuji Blue Medical X-Ray Film SuperRX) in the dark room.

2.2.5.2 Phos-tag SDS PAGE

Phos-tag™ electrophoresis is a powerful technology to detect the phosphorylation of proteins as their movement is retarded in the presence of manganese ions and the Phos-tag acrylamide reagent (Figure 2-1) (Kinoshita, et.al. 2006).

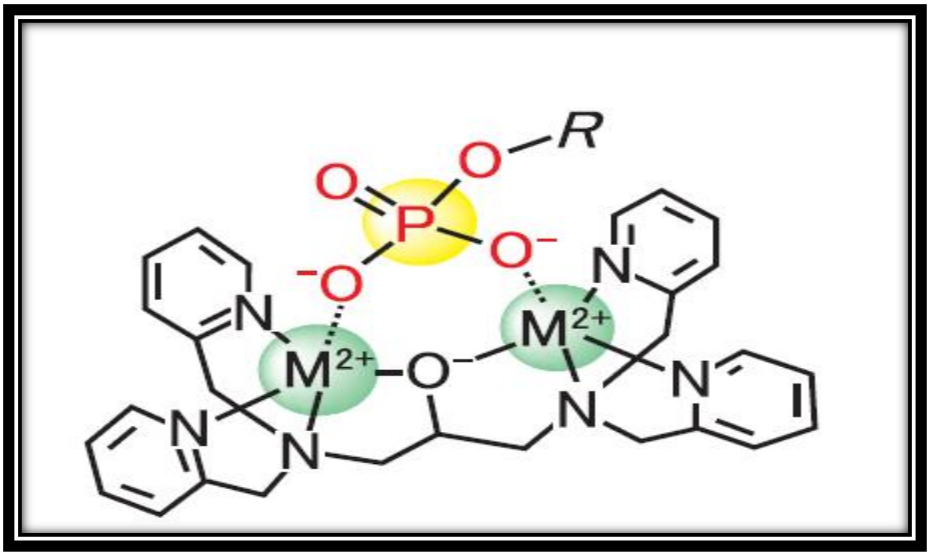


Figure 2- 1: The Phos-tag molecule (www.alphalabs.co.uk).

The Phos-tag gels were prepared according to the recipe listed in Table 2-5.

Table 2- 5: Composition of the Phos-tag Gels.

Component	8%
Number of mini gels	**4**
HO_2	4.5 ml
1M TrisHCl pH 8.8	5.0 ml
30% acrylamide/ methylene-bis-acrylamide (ratio 29:1)	3.5 ml
20% SDS (Sodium Dodecyl Sulphate)	80 µl
Phos-tag^{Tm}	280 µl
10mM $MnCl_2$	280 µl
10% APS (Ammonium Persulfate)	200 µl
TEMED (Tetramethylenediamine)	40 µl

For the top gel the normal protocol was used (2.2.5.1). After the electrophoresis step, gels were incubated three times for 10 minutes in transfer buffer with 20mM EDTA (pH8.0) to remove the Mn^{++} ions prior to the Western blot and the same conditions were used for transfer step (2.2.5.1).

2.2.6 Drop Test Survival

A small amount of cells material was transferred to 10 ml of YEA medium in a 50ml falcon tube and incubated at 30ºC in a Sanyo shaker overnight. The tubes were removed from the shaker on the following day and 10µl aliquots of each culture were counted. The appropriate volume of cell culture was diluted in 1 ml of YEA medium to a final concentration of $1x10^7$ cells/ml. A 10 times serial dilution was then prepared by mixing 100 µL of the appropriate culture with 900µL of YEA medium. A 5µL drop of each dilution ranging from $1x10^7$ to $1x10^4$ cells/ml were applied to YEA plates with or without the indicated drugs. The plates were left at room temperature to dry and then incubated at 30°C for 3 days.

2.2.7 Acute survival

Acute survival experiments were performed like the drop test but the strains were diluted to $5x10^4$ cells/ml in 500µl of YEA medium. 500µl of YEA medium with twice the concentration of the tested drug was added and cells were incubated at 30°C. Samples (75µl) were withdrawn at the indicated times and plated on YEA plates. After 3-4 days at 30°C surviving colonies were counted. For UV treatment, cells were directly plated on YEA plates and exposed to the different doses of UV light (254nm). Each experiment was repeated for three times and cells survival percentage average was estimated against each incubation time or concentration of tested agent.

Chapter 3: Ded1 RNA Helicase is required for the heat and MMS-induced Induction of Rad9 Variant in *S.pombe*

3.1 Introduction

The *S.pombe* DNA damage checkpoint protein Rad9 is one subunit of the Rad 9-1-1 checkpoint clamp complex which plays an essential role for cell survival in the response to DNA damage and DNA replication stress (Parrilla-Casteller, et.al. 2004). In contrast to Rad9, Cds1 kinase is another DNA damage checkpoint protein that is phosphorylated by Rad3 and phosphorylates Cdc25 to mediate a cell cycle arrest (Furnari, et.al. 1999; Lindsay, et.al.1989; Ahn & Prives, 2002). Cds1 and Rad9 have both been shown to induce N-terminally truncated variants by alternative translation initiation from cryptic AUG codons within their mRNAs (Janes et al., 2012; Fletcher and Caspari, unpublished).

The variant of the DNA damage checkpoint protein Rad9 (Rad9-V) is induced when ribosomes utilise the cryptic translation initiation site AUG-50 at elevated temperatures (Janes et al., 2012) or in the presence of the DNA alkylation agent methyl-methanesulfonate (MMS) (this study) (Figure 3-1). Deletion of the first 49 amino acids allows Rad9-V to modulate Chk1 activity outside of the Rad9-Rad1-Hus1 ring (Janes, et.al. 2012). Work by Jessica Fletcher in the group identified AUG-159 as one internal translational start site within the *cds1* mRNA which is also activated when cells are exposed to elevated temperatures. It is however still unknown how the ribosomes can find AUG-50 in the *rad9* mRNA or AUG-159 in the *cds1* mRNA when the temperature rises.

Ded1 RNA helicase belongs to the family of DEAD-box RNA helicases that are ATP dependent enzymes with essential roles in mRNA translation (Linder, 2003) and pre-mRNA splicing (Gong, et.al. 2005). This protein family is found in prokaryotic and eukaryotic cells (Linder, 2003; Grallert, et.al. 2000). Furthermore, they have been shown to be involved in many physiological process of RNA metabolism (Table 3-1) (Cordin, et.al. 2006).

Ded1 unwinds duplexes of mRNA to allow ribosomes to scan the messenger RNA for initiation codons. In the fission yeast (*S. pombe*), Ded1 has been reported to be the regulator of general translation to mRNA molecules (Yang & Jankowsky, 2006).

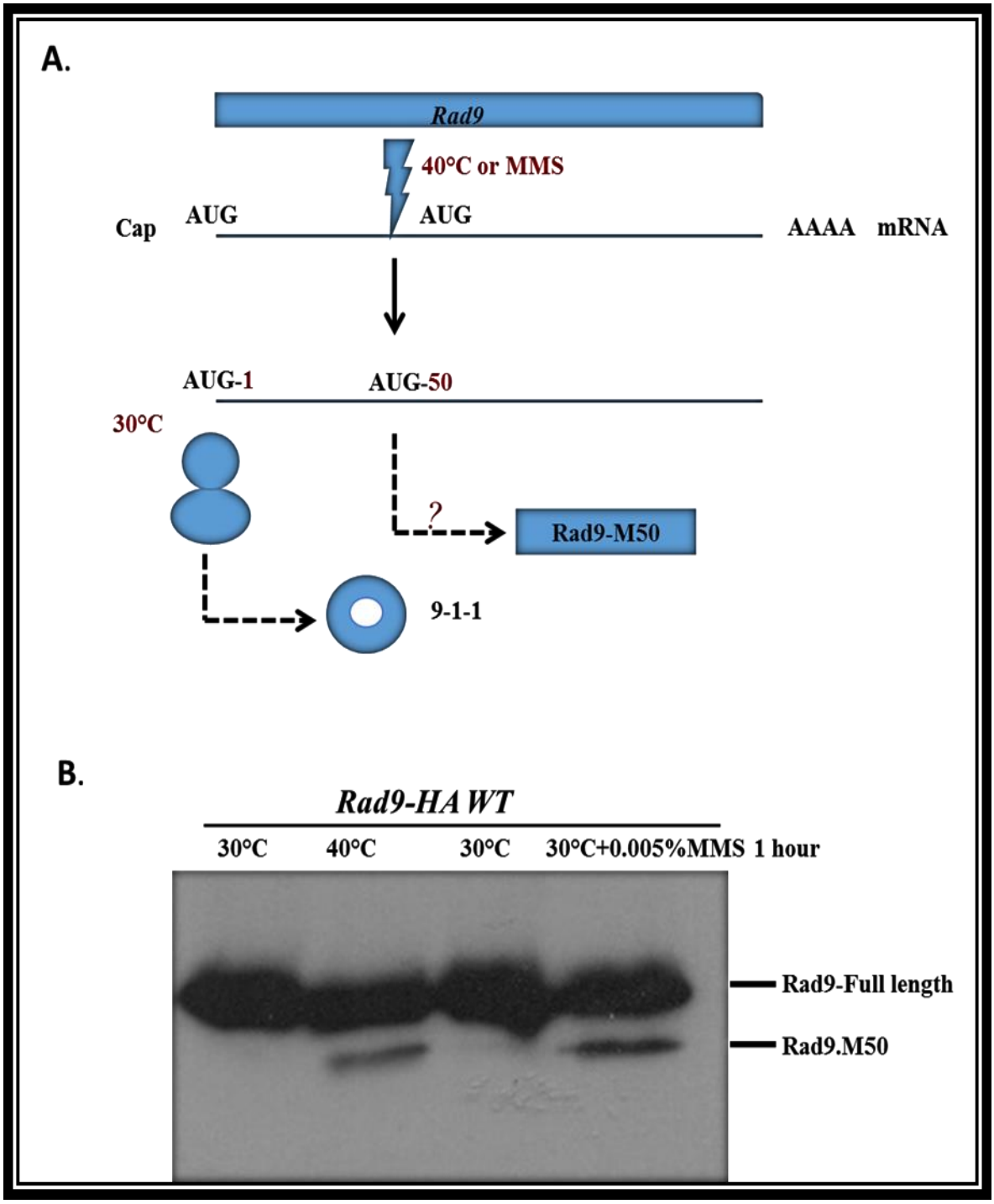


Figure 3-1: Rad9-M50 (Rad9-V) induction in the response to heat stress (40°C) and 0.005% methyl-methanesulfonate (MMS).

(A) Model. Induction of Rad9-V from the internal translation start site AUG-50 in the response to heat stress or MMS treatment. (B) Western blot showing induction of Rad9-V after the exposure of Rad9-HA cells to 40°C or 0.005%MMS at 30°C for 1 hour.

table 4- 1: Cellular functions performed by the DEAD box protein family and the cellular compartments where these activities are performed.

Cellular regions	DEAD box proteins functions within cellular regions
Nucleus	Transcription
	Pre-mRNA splicing
	RNA export
	Ribosome biogenesis
Cytoplasm	Translation
	miRNA processing
	RNA storage
	RNA decay
	snRNP biogenesis
	Nonsense mediated decay

The fission yeast *S.pombe* DEAD- box helicase Ded1 is highly related to Ded1 and DBP1 in the budding yeast *S.cerevisiae* and to mammalian DDX3Y and DDX4. The unwinding of an mRNA duplex starts at a region of single strand RNA so that Ded1 can scan towards the mRNA duplex. Opening of an mRNA duplex is not restricted to regions of single-stranded RNA as the helicase utilizes also blunt ends but at slower rate (Yang & Jankowsky, 2006). Unlike most RNA helicases, the translocation of Ded1 does not occur along the mRNA duplex, instead Ded1 plucks the mRNA at short open regions directly at the duplex (Yang, et.al. 2007). (Yang & Jankowsky, 2006). In the response to heat, Ded1 (*ded1.1D5*) inactivation leads to cell death due to impairments of translation (Grallert, et.al. 2000). The close similarity between the fission yeast *S.pombe* and budding yeast *S.cerevisiae* Ded1 lead to the possibility to rescue the phenotypes of the temperature sensitivity *ded1.1D5* point mutation in *S.pombe* by introducing

S.cerevisiae Ded1 on a plasmid (Grallert, et.al. 2000). When the activity of Ded1 is reduced, the protein levels of the cyclins Cig2 and Cdc13 drop significantly (Grallert, et.al. 2000) indicating that Ded1 is the general translation factor for cyclins (Chuang, et.al. 1997). Ded1 also enhances the efficiency of gene silencing by antisense RNA (Raponi & Arndt, 2002). It is also believed that Ded1 can anneal two RNA strands (Yang & Jankowsky, 2005).

Ded1 has been shown to interact with Moc1, Rp132-2 and Cpc2 to form a complex in order to regulate translation that later leads to sexual differentiation in fission yeast when the Ste11 transcription factor is induced under nitrogen starvation conditions (Paul, et.al. 2009). Another interesting interaction has been found between Ded1 and Chk1 in the fission yeast *S.pombe* although the cellular functions of this complex are still unknown (Liu, et.al. 2002). In response to heat stress, the fission yeast *S.pombe* Ded1 induces a larger protein variant with unknown function (Paul, et.al. 2009).

Given that Chk1 associates with the RNA helicase Ded1 and because a larger variant of Ded1 is induced by heat stress (Liu, et.al. 2002), I investigated the requirement of this translation factor for the activation of the cryptic initiation site AUG-50 in the *rad9* mRNA as well as for the induction of the Cds1 variant from AUG-159. Using a temperature sensitive allele of *ded1* (*ded1.1D5*) (Grallert, et.al. 2000), I can show that the RNA helicase is essential for the heat and MMS induced production of Rad9 variant and for the heat induced up-regulation of the Cds1 variant. This implies the presence of a secondary structure in the *rad9* and *cds1* mRNAs encompassing AUG-50 and AUG-159, respectively, which needs to be resolved by the RNA helicase to allow ribosomes to utilise this internal initiation codon under heat stress or MMS conditions.

3.2 Results

3.2.1 The Rad9 variant requires the Ded1 RNA helicase to be induced in response to heat stress and MMS treatment

In order to examine the requirement of Ded1 RNA helicase to induce Rad9 variant (M50) in the response to heat stress, the wild type *rad9-HA* strain C-terminally tagged with the hemagglutinin (HA) epitope expressed from its endogenous locus on chromosome I, the strain harbouring the temperature sensitive *ded1.1D5* mutant linked with the *rad9-HA* allele (*rad9-HA ded1.1D5*) and the *rad9-HA ded1.1D5* strain that expresses the wild type Ded1 RNA helicase from a *pREP41* plasmid (*rad9-HA ded1.1D5 pRep41-Myc-ded1*) were analysed in

response to heat stress. All strains were grown at 30°C in YEA liquid media overnight prior to the exposure of 10^7 cells aliquots to 30°C or 40°C for one hour (Figure 3-2). The transformed *rad9-HA ded1.1D5 pRep41-Myc-ded1* strain was inoculated from a selective minimal medium plate to ensure that all cells contained the plasmid.

The smaller band of the Rad9 variant of approximately 50 kDa was observed when the *rad9-HA WT* strain was exposed to 40°C, while it was absent in the *rad9-HA* strain with the temperature-sensitive *ded1* allele (*rad9-HA ded1.1D5*) indicating that Ded1 RNA helicase is required for the heat induction of the Rad9 variant (Rad9.M50). To support this conclusion, I used a strain that returned the expression of Ded1 to the *rad9-HA ded1.1D5* mutant from the pREP41-Myc plasmid (*rad9-HA ded1.1D5 pRep41-Myc-ded1*). Interestingly, I could re-establish the expression of Rad9 variant at 40°C in the complemented *rad9-HA ded1.1D5 pRep41-Myc-ded1* strain. This supports the conclusion that Ded1 RNA helicase is required for the heat induction of Rad9 variant (Rad9.M50).

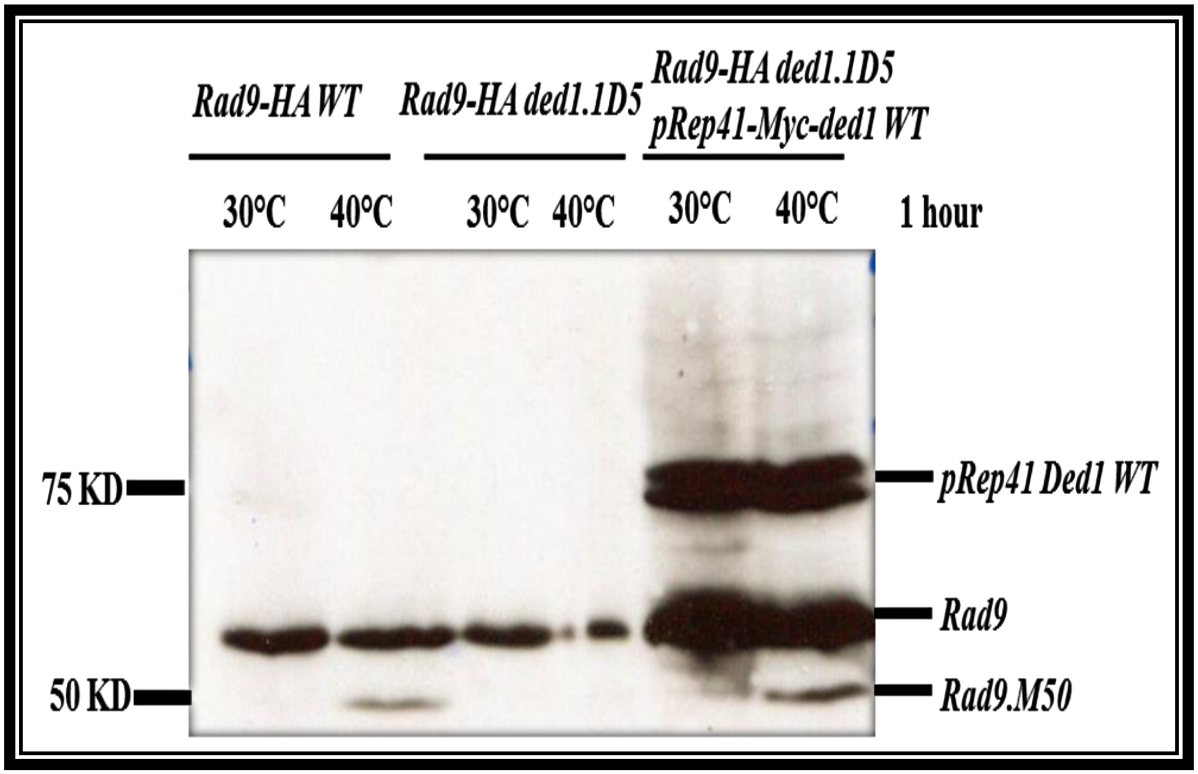


Figure 3- 2: The inducible Rad9 variant (Rad9-M50) requires the RNA helicase Ded1 in response to heat stress.

***S.pombe rad9-HA wild type* (WT), *rad9-HA ded1.1D5* and *rad9-HA ded1.1D5 + pREP41-Myc-Ded1WT* cells were grown at 30°C overnight before a sample was exposed to 30°C or 40 °C for one hour. Total protein extract was isolated and separated on a 10% SDS gel, transferred onto a nitrocellulose membrane and detected with anti-HA and anti-Myc antibodies.**

Knowing that the expression of Rad9 variant is not only induced by heat stress but also upon treatment of cells with the DNA alkylation agent methyl-methanesulfonate (MMS) (Janes, et.al. 2012; and this study), I investigated whether Ded1 is also required for the MMS induced up-regulation or whether it is only specific for heat stress. The same strains were exposed to 30°C either with or without 0.005% MMS for one hour (Figure 3-3). The variant was detectable in the wild type strain *rad9-HA WT* but strongly reduced in the *Rad9-HA ded1.1D5* strain at 30°C. The expression of the variant was restored in the strain that expresses the wild type Ded1 RNA helicase from the plasmid (*Rad9-HA ded1.1D5 pRep41-Myc-ded1*) indicating that Ded1 is also required for the induction of the variant in the response to MMS treatment.

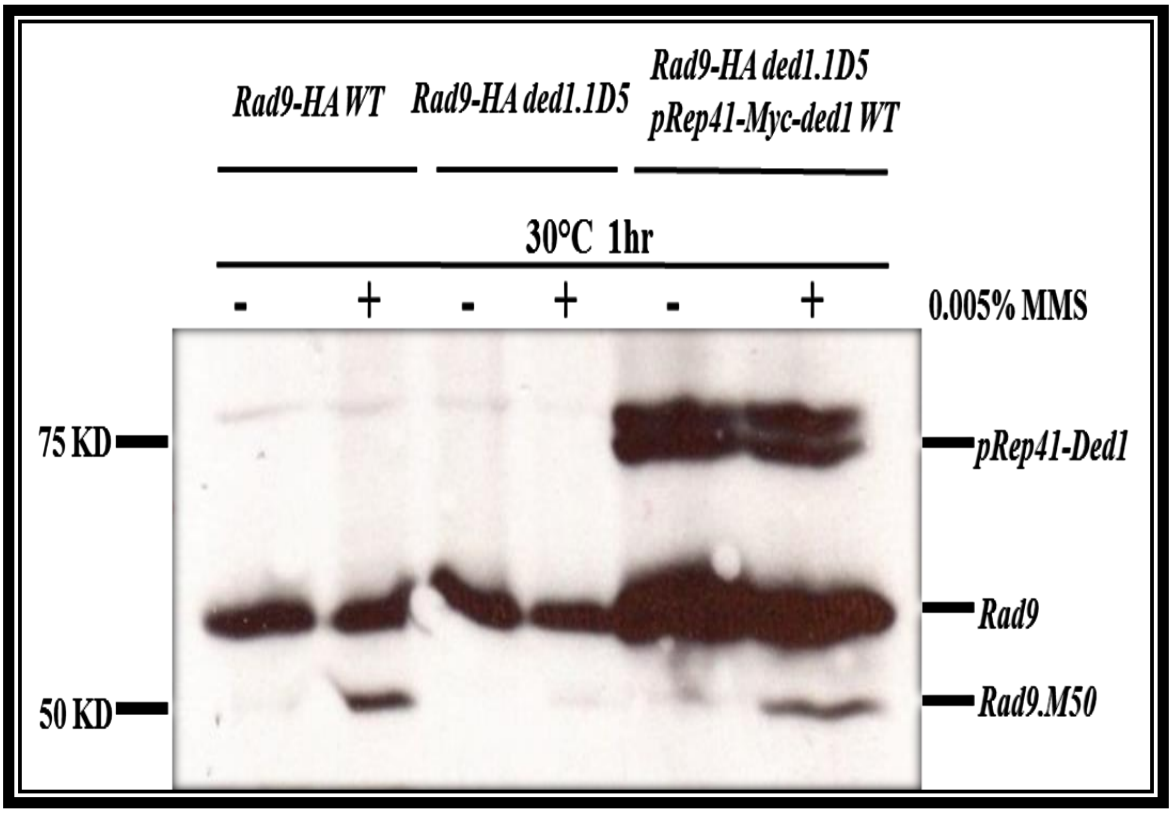


Figure 3- 3: The inducible Rad9 variant (M50) requires the RNA helicase Ded1 in response to MMS.

***S.pombe rad9-HA WT*, *rad9-HA WT ded1.1D5* and *ded1.1D5* + pREP41-Myc-Ded1cells were grown at 30°C overnight in YEA medium before a sample was exposed to either 30 ºC or 30 ºC+ 0.005% MMS for one hour. Total protein was isolated and separated on 10% SDS gel, transferred onto a nitrocellulose membrane and detected with anti-HA and anti-Myc antibodies.**

To test whether the absence of the Rad9 variant influences the temperature-sensitivity of the *ded1.1D5* mutant, the same strains including the *rad9* deletion strain (Δ*rad9*), which lacks full-length Rad9 and its variant, were exposed to 40°C to test cell survival. The indicated strains were grown in YEA liquid medium overnight before being diluted to $5x10^4$ cells/ml in YEA medium and exposed to 40°C. A sample was withdrawn every 20 minutes for 120 minutes.

The results show that the *ded1.1D5* cells were highly sensitive to heat stress indicating that Ded1 is essential for cell viability. The similar sensitivity observed for the *rad9-M50A ded1.1D5* strain shows that the absence of the variant has no impact on cell survival which is in line with the lack of heat sensitivity of the *rad9-M50A* single mutant to high temperature (Figure 3-4). While this observation suggests that Ded1 and Rad9-M50 act in the same pathway, the latter cannot be securely concluded since the *rad9-M50A* stain is not heat sensitive.

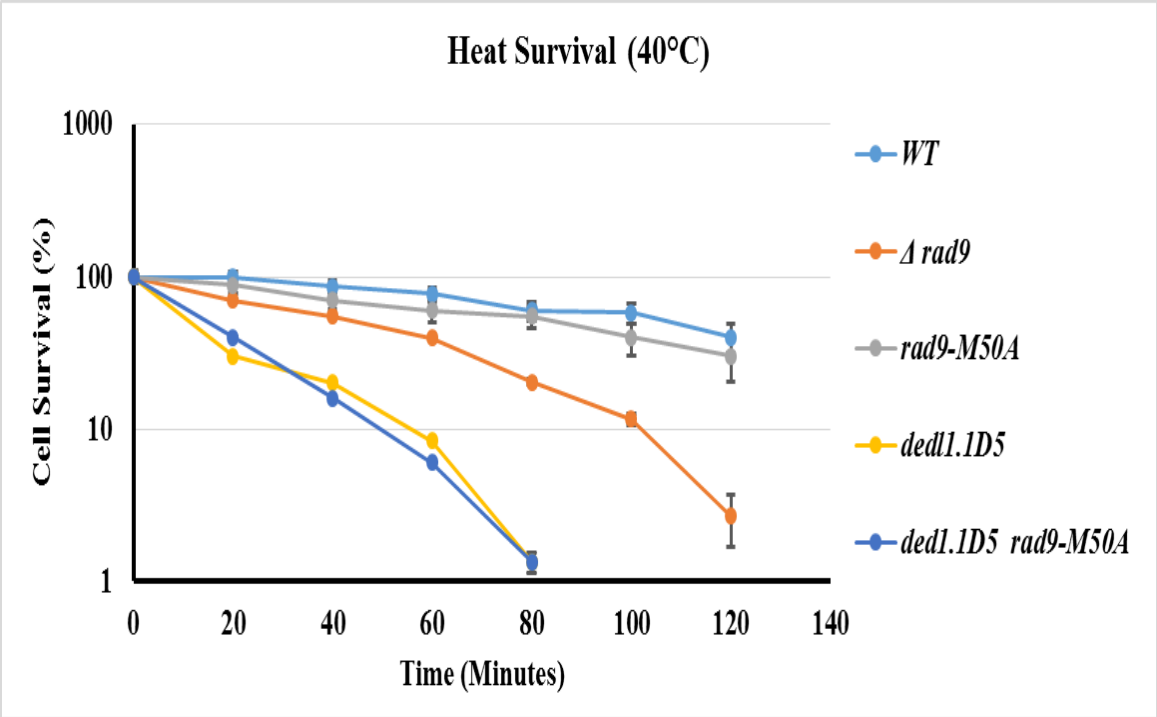


Figure 3- 4: Ded1 is required for cell survival in response to heat.

A 5x10^4 cells/ml cell dilution of wild-type (804), *Δrad9*, *rad9M50A*, *ded1.1D5* and *ded1.1D5 rad9M50A* cells was exposed to 40°C and a sample (75 µl) was withdrawn every 20 minutes for 120 minutes and plated on a YEA plate. Plates were incubated at 30°C for 4 days, and colonies were then counted.

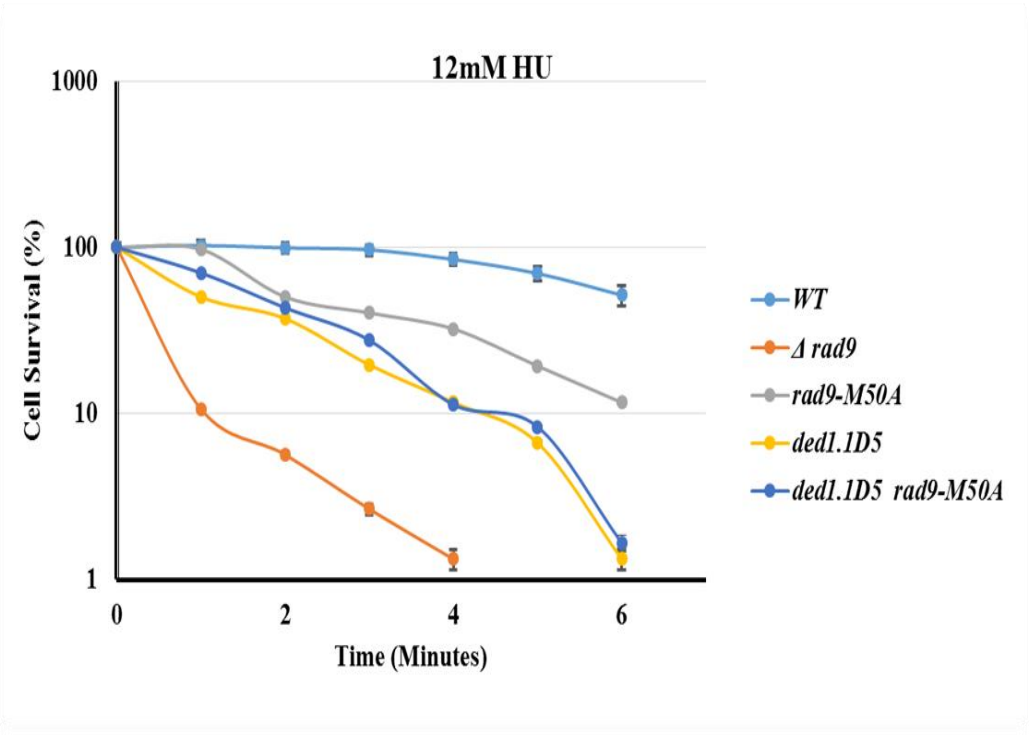
I extended this analysis to different types of DNA damage including the DNA replication inhibitor hydroxyurea (HU), MMS, UV light and the topoisomerase I inhibitor camptothecin (CPT) which breaks DNA replication forks. So far, Ded1 has not yet been implicated in the response to DNA damage (Hsing-Yin, et.al. 2002). DNA alkylation caused by MMS leads to adenine N3-methylation which results in cell cycle arrest in the response to replication stalling at lower MMS concentrations. Base excision repair is the mechanism to repair these DNA methylation adducts. In contrast, the exposure to high MMS concentrations can lead to DNA double strand breaks specifically in regions of adenine repeats (Lundin, et.al. 2005). Hydroxyurea leads to the stalling of DNA replication due to the inhibition of the enzyme

ribonucleotide reductase (RNR) which produces deoxyribonucleotides (Krakoff, et.al. 1968). Camptothecin (CPT) immobilises topoisomerase I at the DNA in front of DNA replication forks which results in DNA breaks when forks collide with this obstacle (Hsiang, et.al. 1985; Pommier, 2006).

The indicated strains were exposed to 12mM HU, 0.005% MMS, 40µM CPT or UV light, (Figures 3-5 & 3-6). Samples were taken every hour for 3 hours for cells treated with 0.005% MMS, while HU and CPT treated cells were taken every hour over a six hours period. Cells treated with UV light were exposed to the dosage of 0, 20, 40, 60 and 80 J/m^2 after being plated on YEA medium plates.

As expected, cells lacking Rad9 (*Δrad9*) showed a high sensitivity to all types of the DNA damage as the strain cannot produce the Rad9-Rad1-Hus1 checkpoint clamp (Figures 3-5 & 3-6). Interestingly, cells expressing the temperature sensitive *ded1.1D5* allele were HU and MMS sensitive even at 30°C which permits cell viability of the temperature-sensitive mutant, but resistant to UV light and CPT. This implies that the RNA helicase or proteins encoded by transcripts controlled by Ded1 affect cell survival when DNA replication forks stall (HU) or encounter methylated DNA (MMS). Unexpectedly, cells without the Rad9 variant (*rad9-M50A-HA*) were only HU sensitive but resistant to MMS although MMS increases the abundance of the variant. The *rad9M50A ded1.1D5* strain was as HU sensitive as either single mutant (Figure 3-5 A) strongly suggesting that Ded1 and Rad9-M50 act jointly in the same pathway when DNA replication forks stall. Given that the *rad9-M50A* strain was not sensitive to MMS, a similar conclusion cannot be drawn when DNA is methylated. This is intriguing since MMS up-regulates Rad9-M50 expression in a Ded1 dependent manner (Figure 3-3). Moreover, this implies that the inability to up-regulate Rad9-M50 is not the reason why the *ded1.1D5* strain is HU sensitive since HU does not induce the variant (Fletcher and Caspari, unpublished). It is more likely that Ded1 controls another event at stalled forks which also involves Rad9 variant. The latter variant is constitutively expressed in cycling cells at a low level which may be sufficient to deal with stalled DNA replication forks (Janes et al., 2012).

A.



B.

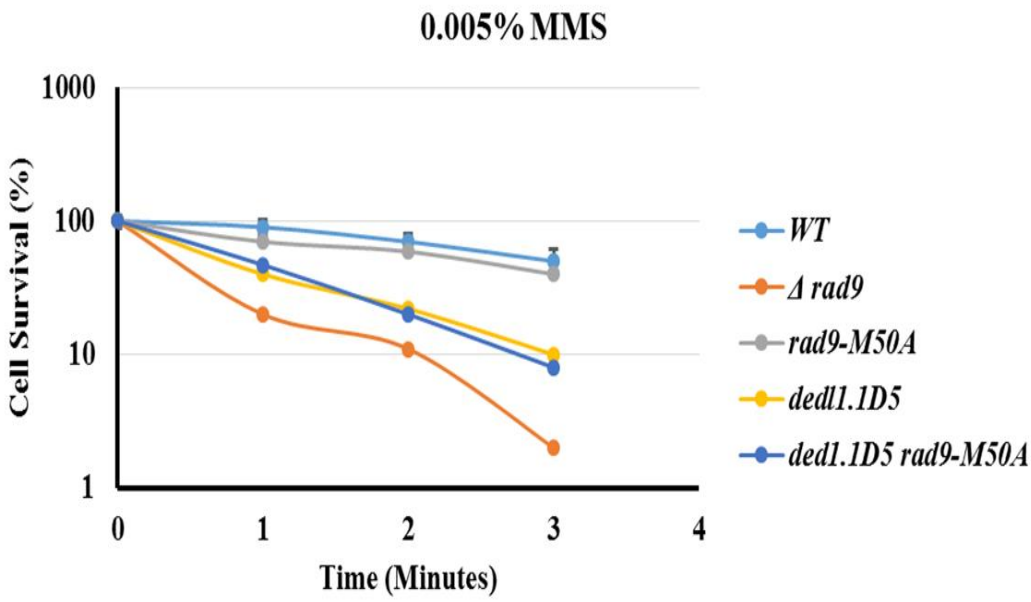


Figure 3- 5: The *ded1.1D5* mutant cells are HU and MMS sensitive while *rad9M50A* cells only HU sensitive.

A $5x10^4$ cells/ml cell dilution of wild-type, *Δrad9*, *rad9-M50A*, *ded1.1D5* and *ded1.1D5 rad9-M50A* cells was exposed to 12mM HU (A) or 0.005% MMS (B) for the indicated times. Cells (75µl) were plated on YEA agar plates and incubated at 30°C for 4 days, and colonies were then counted.

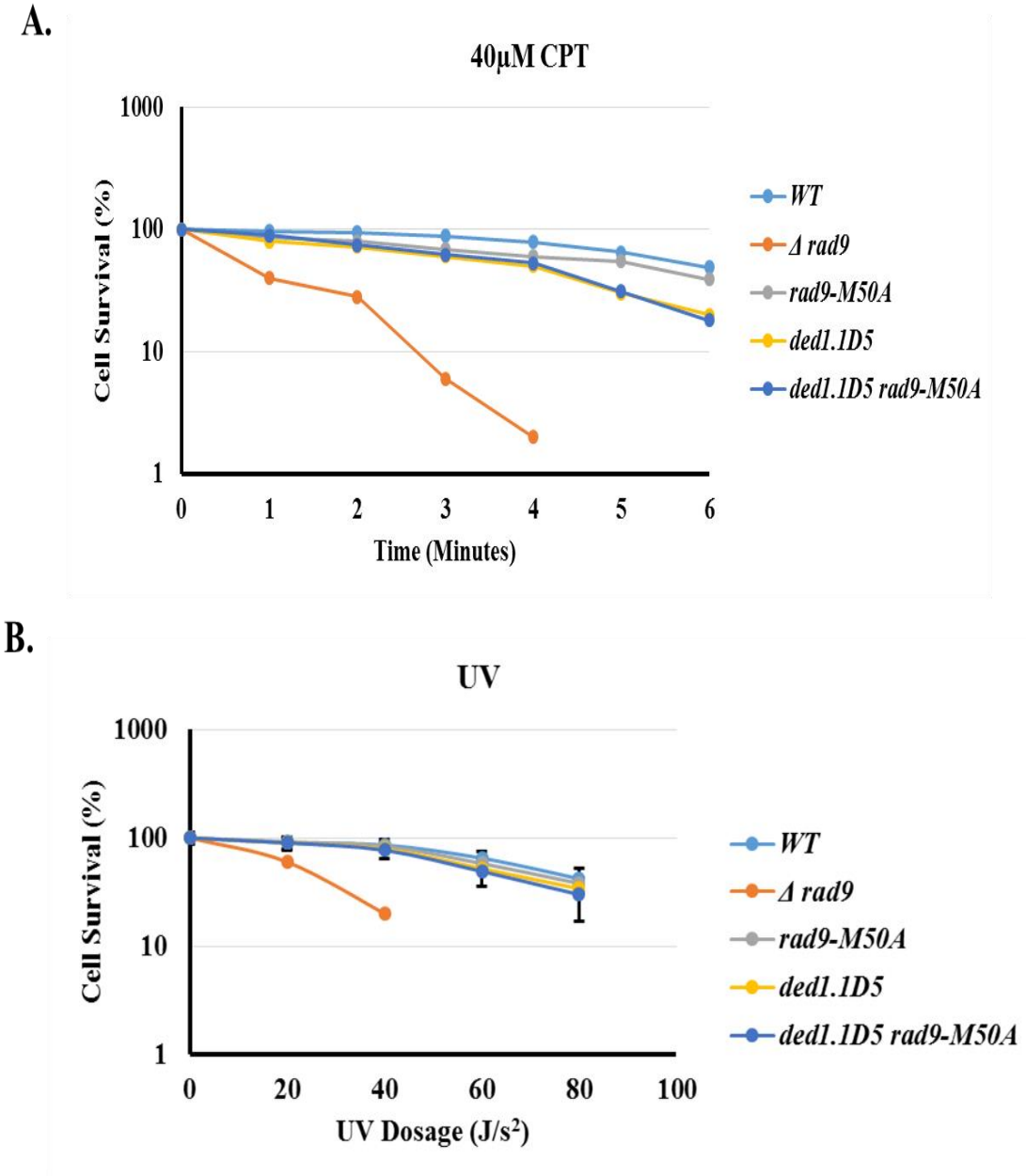


Figure 3- 6: *ded1.1D5* and *rad9-M50A* cells do not respond in the presence of the DNA replication inhibitor camptothecin (CPT) and UV light.

A $5x10^4$ cells/ml cell dilution of wild-type, *Δrad9*, *rad9-M50A*, *ded1.1D5* and *ded1.1D5 rad9-M50A* cells was exposed to 40µM CPT (A) or UV light (B). Cells (75 µl) were plated on YEA agar plates and colonies were counted after 4 days.

To test whether the requirements of Ded1 and Rad9 variant for the response to HU and MMS (Ded1 only) is limited to the acute exposure to high concentrations of the DNA damaging agents, the same strains were grown in the presence of lower drug concentrations to find out whether a chronic exposure reveals similar requirements. The strains were grown in YEA medium at 30°C prior to a serial dilution of 10^7, 10^6, 10^5 and 10^4 cells/ml. 5µl of cells were

spotted on YEA agar plates without drug or with the indicted concentration of drugs (Figure 3-7). The plates were then incubated at 30°C for 3 days.

All strains grew normally at 30°C and at 2mM HU. The *Δrad9* strain was highly sensitive to both concentrations of MMS and to 4mM HU as the cells lack the DNA damage checkpoint clamp (Rad9-1-1). While *rad9M50A* cells failed to show a sensitivity under these conditions, *ded1.1D5* and the *ded1.1D5 rad9M50A* strains were mildly sensitive to 0.005% MMS (Figure 3-7). This sensitivity was independent of Rad9 variant which is consistent with the acute survival data. Taken together, these experiments reveal a novel MMS sensitivity of the *ded1.1D5* mutant at the permissive temperature of 30°C and a sensitivity to high HU concentrations (12mM) in a manner which is epistatic with the *rad9-M50A* strain that cannot express Rad9 variant.

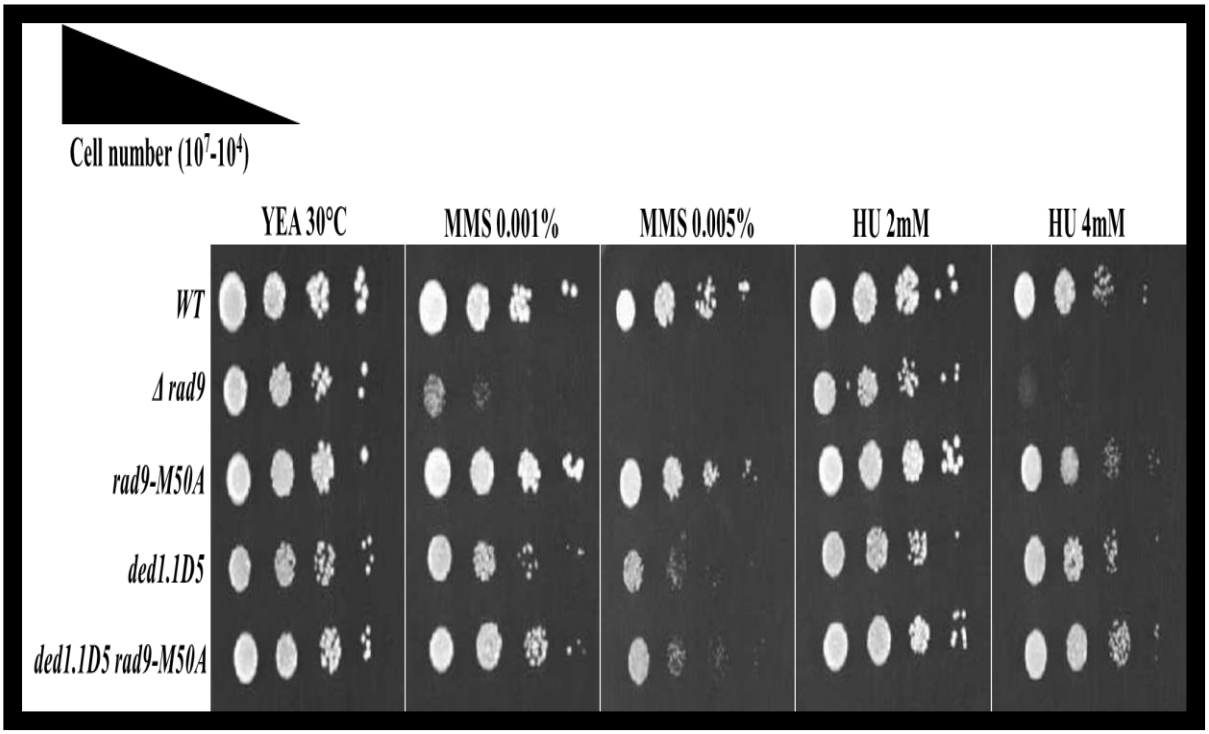


Figure 3- 7: *ded1.1D5* cells are MMS sensitive.

Serial dilutions (10^7-10^4 cells/ml) of the indicated strains were dropped (5 µl) onto rich medium agar plates without treatment or with 0.01%, 0.05% MMS, 2mM or 4mM HU. Plates were incubated for 3 days at 30°C.

3.2.2 Ded1 RNA helicase is required to induce a novel Cds1 variant and Cds1 variant M159 in the response to heat stress

Work by Jessica Fletcher in the group showed that the internal translation start site AUG-159 is, like AUG-50 in the *rad9* mRNA, used to induce the expression of an N-terminally truncated variant of Cds1 kinase. Cds1-M159 is up-regulated when the ribosome utilises the cryptic translation initiation at AUG159 at elevated temperature similar to the induction of Rad9 variant. Cds1 (Chk2) kinase is a DNA damage checkpoint protein which stabilises stalled DNA replication forks and arrests cell cycle progression. In fission yeast *S.pombe*, Cds1 is activated by Rad3 kinase in the response to a DNA replication arrest which results in a G2-M arrest when Cds1 phosphorylates Cdc25 phosphatase (Furnari, et al., 1979; Lindsay, et al., 1998; Ahn & Prives, 2002). The phosphorylation leads to the re-localization of Cdc25 phosphatase from nucleus to cytoplasm which result in a G2 arrest since the inhibitory tyrosine-15 phosphorylation of the main cell cycle regulator Cdc2 cannot be removed (Zeng, et.al. 1999). Cds1 also protects the stalled replication forks by excluding the endonuclease Mus81 from its vicinity (Kai, et.al. 2005). The function of the Cds1-M159 variant is as yet unknown. Given that the up-regulation of this variant is induced under the same heat stress condition as the Rad9 variant (Fletcher and Caspari, unpublished) and the Ded1 heat-inducible band (Paul, et.al. 2009), the *cds1-His-HA* allele was combined with the *ded1.1D5* mutation. As shown in Figure 3-8, heat stress (1h at 40°C) induces the Cds1-M159 variant (55kDa) as well as a shorter variant (40kDa). While the shorter variant was completely absent in the *ded1.1D5* background, the larger 55kDa variant starting at AUG 159 was only partly affected (Figure 3-8). This indicates that Ded1 RNA helicase is required for the heat inducible usage of at least two internal AUG codons in the *cds1* mRNA, the characterised AUG159 and one as yet uncharacterised AUG codon further down-stream of AUG159. This is consistent with the requirement of Ded1 to induce Rad9 variant at AUG 50 in the response to heat stress.

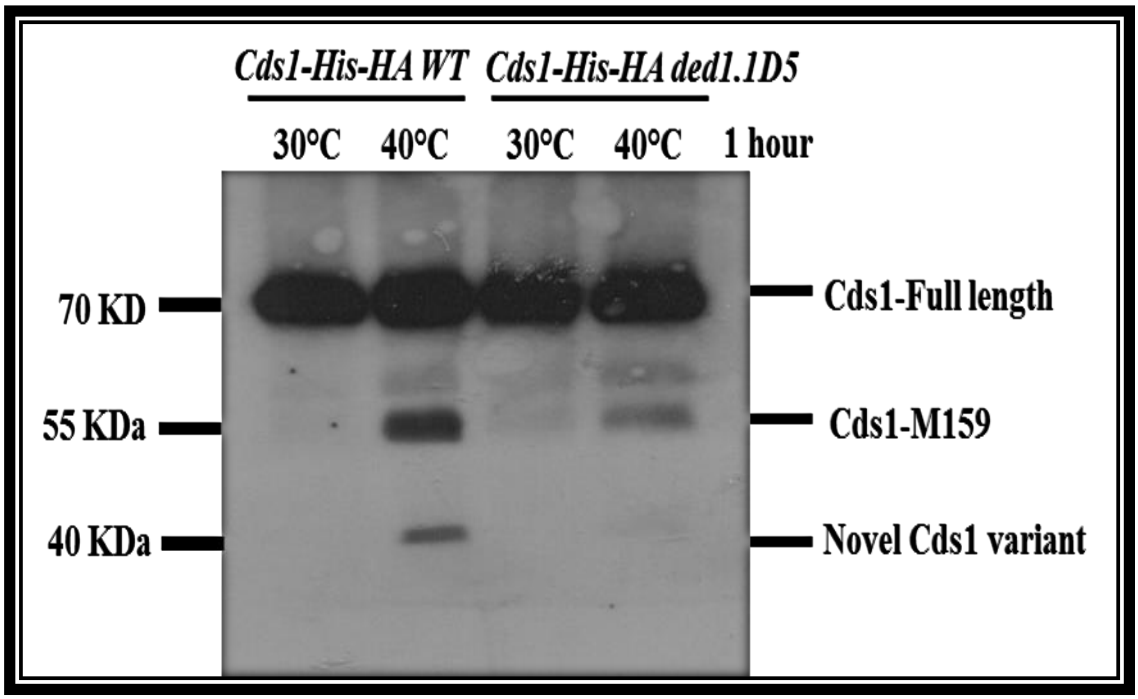


Figure 3- 8: Two variants of Cds1 kinase require the RNA helicase Ded1 to be induced in the response to heat stress.

***S.pombe cds1-His-HA wild type* (*WT*) and c*ds1-His-HA ded1.1D5* cells were grown at 30°C in YEA medium overnight before a sample was exposed to 30°C or 40°C for one hour. Total protein was isolated and separated on 10% SDS gel, transferred onto a nitrocellulose membrane and detected with anti-HA antibody.**

Given that Cds1 kinase is important for cell survival in the presence of HU (Lindsay et al., 1998) and that the *ded1.1D5* strain is HU sensitive (Figure 3-5 A), the temperature and HU sensitivity of a *ded1-1D5 cds1-M159A-HA* double mutant was investigated. The following strains including wild type (804), *ded1-1D5*, *cds1-M159A-HA* and *ded1-1D5 cds1-M159A-HA* were subjected to a drop test. The indicated strains were grown in YEA medium at 30°C prior to a serial dilution of 10^7, 10^6, 10^5 and 10^4 cells/ml. 5µl of cells were spotted on YEA agar plates without a drug or with 6mM HU. The plates were then incubated at 30°C and one YEA plate was incubated at 37°C for 3 days. As expected, cells with the temperature sensitive mutant allele of *ded1* were highly sensitive to 37°C independently of the *cds1-M159A* allele (Figure 3-9 A). In the presence of HU, cells lacking the larger 55kDa Cds1 variant (c*ds1-M159A-HA*) were as sensitive as the *ded1-1D5 cds1-M159A-HA* double mutant. Interestingly, loss of this variant rendered *ded1.1D5* more sensitive which indicate that the Cds1 variant and the RNA helicase act in parallel pathways when DNA replication forks stall in HU medium (Figure 3-9 B). This would suggest a role of Ded1 RNA helicase independently of Cds1 variant (M159) in the response to DNA replication fork arrest.

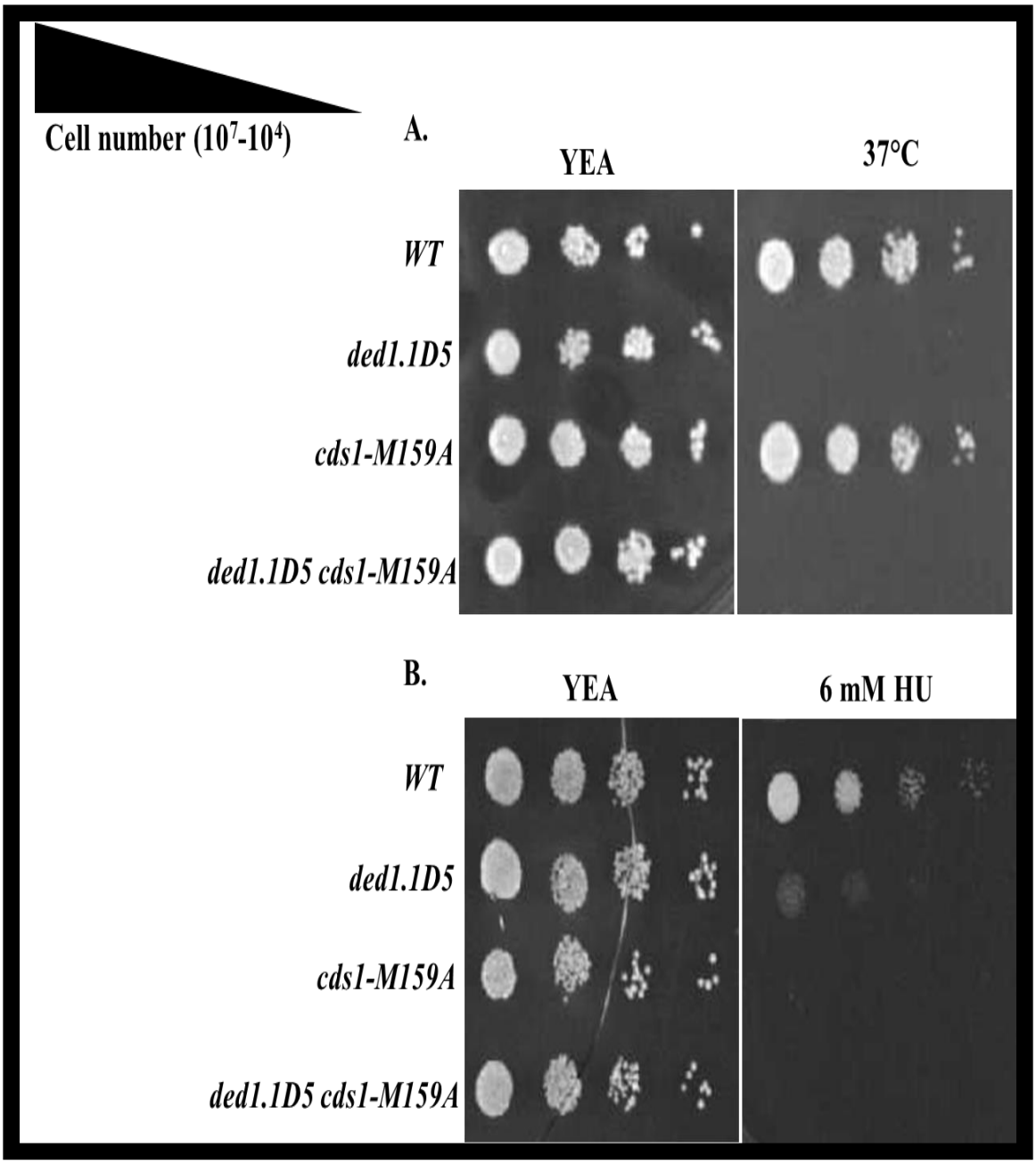


Figure 3- 9: Ded1 and Cds1 variant (M159) act in parallel pathways when DNA replication forks stall.

Serial dilutions (10^7-10^4 cells/ml) of the indicated strains were dropped (5 µl) onto rich medium agar plates without treatment or with 6mM HU. Plates without treatment or treated with HU were incubated at 30°C and one YEA plate was incubated at 37°C for 3 days.

3.3 Discussion

The variants of the DNA damage checkpoint proteins Rad9 and Cds1 are both up-regulated by heat stress (Janes, et al., 2012; Fletcher & Caspari, unpublished). Ded1 RNA helicase has been reported to have an essential role in facilitating ribosome scanning of mRNAs (Berthelot, et.al. 2004). This essential function of Ded1 supports the idea that the activity of the RNA helicase

leads to the initiation of cryptic AUG translation initiation sites by solving secondary structures in the mRNA. It also implies that the recognition of these cryptic sites is an active process rather than the passive consequence of elevated temperature (Figure 3-10).

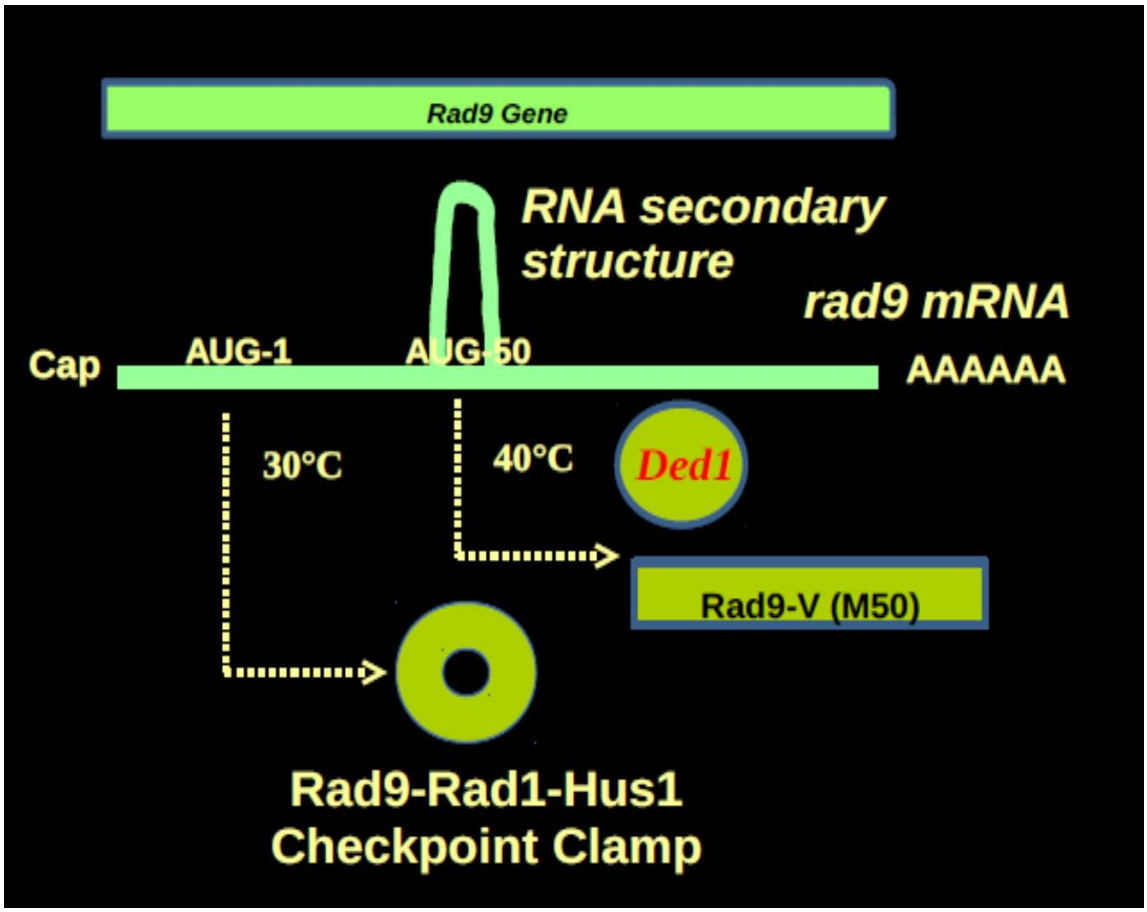


Figure 3- 10: The possible role of the RNA helicase Ded1 in the expression of Rad9 variant.

The heat inducible Rad9 variant requires Ded1 for its induction in response to heat and MMS as a secondary structure may prevent ribosomes from initiating translation from the cryptic AUG-50 codon.

This is further supported by the observation that heat and DNA alkylation can both increase expression of Rad9 variant (Figures 3-2 & 3-3). The latter processes are strongly reduced in the *ded1.1D5* mutant and restored upon the ectopic expression of wild type Ded1 RNA helicase from the *pRep41* plasmid (Figures 3-2, 3-3 & 3-8). The reduced induction at 30°C in the presence of MMS strongly indicates that the loss of Rad9 variant at elevated temperatures is a consequence of a drop in *ded1.1D5* activity and not a drop in cell viability. While Ded1 unwinds duplexes of mRNA to allow ribosomes to scan the mRNA to find translation start sites, it is currently unknown how this RNA helicase is activated under these conditions. It is

very intriguing in this context that a low mobility variant of Ded1 is induced in the response to heat stress (Liu, et.al. 2002). This variant of Ded1 is believed to be modified in a way that allows Ded1 to recognise the different secondary structures of mRNA or to recognise other unknown sites in mRNAs under heat stress conditions that may lead to the expression of Rad9 and Cds1 variants (Liu, et.al. 2002). Unfortunately, the nature of this post-translational modification of Ded1 is currently unknown.

The acute heat sensitivity of the *ded1.1D5* strain is consistent with the essential roles of the RNA helicase in translation. Although Rad9 and Cds1 variant are both heat inducible, cells which lack either variant (*rad9-M50A* and *cds1-M159A*) are not heat sensitive at 37°C or under acute conditions at 40°C (Figures 3-4 & 3-9; Janes et al., 2012). The missing relationship between sensitivity and inducability extends also to HU for the Rad9 variant. While the abundance of the variant does not change much in HU medium (Janes et al., 2012), *rad9-M50A* cells are sensitive to high HU concentrations (Figure 3-5 A). Interestingly, *ded1.1D5* cells are also HU sensitive and their sensitivity appears to be epistatic with the *rad9-M50A* allele (Figure 3-5 A). This result implies that Ded1 or one of the genes regulated by the RNA helicase is required to protect stalled DNA replication forks in a manner dependent on the Rad9 variant which does not require its up-regulation. Growing *S.pombe* cells express constitutively low level of Rad9 variant (Janes et al., 2012). Ded1 RNA helicases regulates a larger number of proteins although it is as yet unclear which one may be important in this context. One possible target of Ded1 could be the mRNAs of the small and large subunits (Suc22 and Cdc22) of the enzyme ribonucleotide reductase (RNR) which are up-regulated in S phase in the response to HU exposure (Sarabia, et.al. 1993) and heat stress (Harris, et.al. 1996). It is however still not clear why heat and MMS are both inducers of Rad9 variant. One possibility is that heat and MMS lead both to S phase arrests. Heat is known to stop DNA replication in human cells (Saladino & Ben; 1976, Corry, et.al. 1977; Bhuyan, 1979) and MMS does interfere with the same process (Friedberg, 2003). If this were to be correct, the way how heat and MMS arrest DNA replication must be different from HU as the RNR inhibitor does not increase Rad9 variant protein levels. One possibility is that heat and MMS arrests cells later in S phase compared to HU which blocks S phase right at the start. In this context, the novel MMS sensitivity of the *ded1.1D5* mutant (Figures 3-5B & 3-7) is interesting as it indicates a role of the RNA helicase or one of its targets at DNA replication forks which encounter alkylated DNA templates.

The relationship between the protein translation regulation and the fission yeast checkpoint response to DNA damage is still unclear. Interestingly, the DNA damage checkpoint (Chk1)

and the DEDA box helicase (Ded1) have been shown to interact physically *in vivo* although Ded1 is not modified in the response to DNA damage (Liu, et.al. 2002).

A link between checkpoints and translation was found in *Drosophila* where it is reported that during oogenesis the Gurken protein (one of the transforming growth factors) controls the pattern formation of the Dorsoventral protein (Ray & Schupbach, 1996). Expression of the Gurken protein needs the translation factor eIF4A (Vasa) which is considered to be related to DEAD box helicases but differs from Ded1 (Styhler, et.al. 1998). It has been shown that the generation of double-strand DNA breaks in mitosis arise due to the persistent modification of Vasa although the accumulation of Gurken was absent (Ghabrial & Schupbach, 1999). Interestingly, these events need the Mei-41 protein which is related to the checkpoint kinases ATM and ATR (Ghabrial & Schupbach, 1999). It has been proposed that the response of Vasa to the ATM and ATR checkpoint leads to decreased Gurken translation. In summary, this chapter reports that Ded1 RNA helicase is required for the up-regulation of the variants of Rad9 and Cds1 in the response to heat stress, and that Rad9 variant is also induced when DNA becomes alkylated by methylmethanesulfonate (MMS) in a manner dependent on Ded1 RNA helicase. Furthermore, cells expressing a mutated allele of *ded1* (*ded1.1D5*) are HU and MMS sensitive, and Ded1 acts jointly with Rad9 variant in the HU response. Finally, Ded1 is also involved in the expression of a shorter Cds1 variant from a translation initiation site down-stream of AUG-159.

This chapter reports thatDed1 RNA helicase is required for the up-regulation of the variants Rad9- M50 and Cds1-M159 in the response to heat stress. It also shows thatRad9 variant is induced when DNA becomes alkylated by methyl-methane-sulfonate (MMS). Interestingly, Ded1 and Rad9 variant act in the same pathway when DNA replication forks stall in HU medium and cells expressing a mutated allele of *ded1* (*ded1.1D5*) are HU and MMS sensitive.

Chapter 4: Dephosphorylation of the *S.pombe* DNA Damage Checkpoint Kinase Chk1 by Ppa2 and Ptc1 Phosphatases

4.1 Introduction

Although protein phosphatases play crucial roles in kinase signalling, the underlying mechanisms are complex and only poorly understood. Chk1 kinase prevents mitosis in response to DNA damage and heat stress in humans (Janes, et.al. 2012). Like many kinases, *S.pombe* Chk1 undergoes extensive phosphorylation by other kinases like ATR (Rad3) at serine 345 in presence of genomic lesions (Figure 4-1) (Walworth, et al., 1993). Little is however known how Chk1 activity is down-regulated when cells exit this arrest.

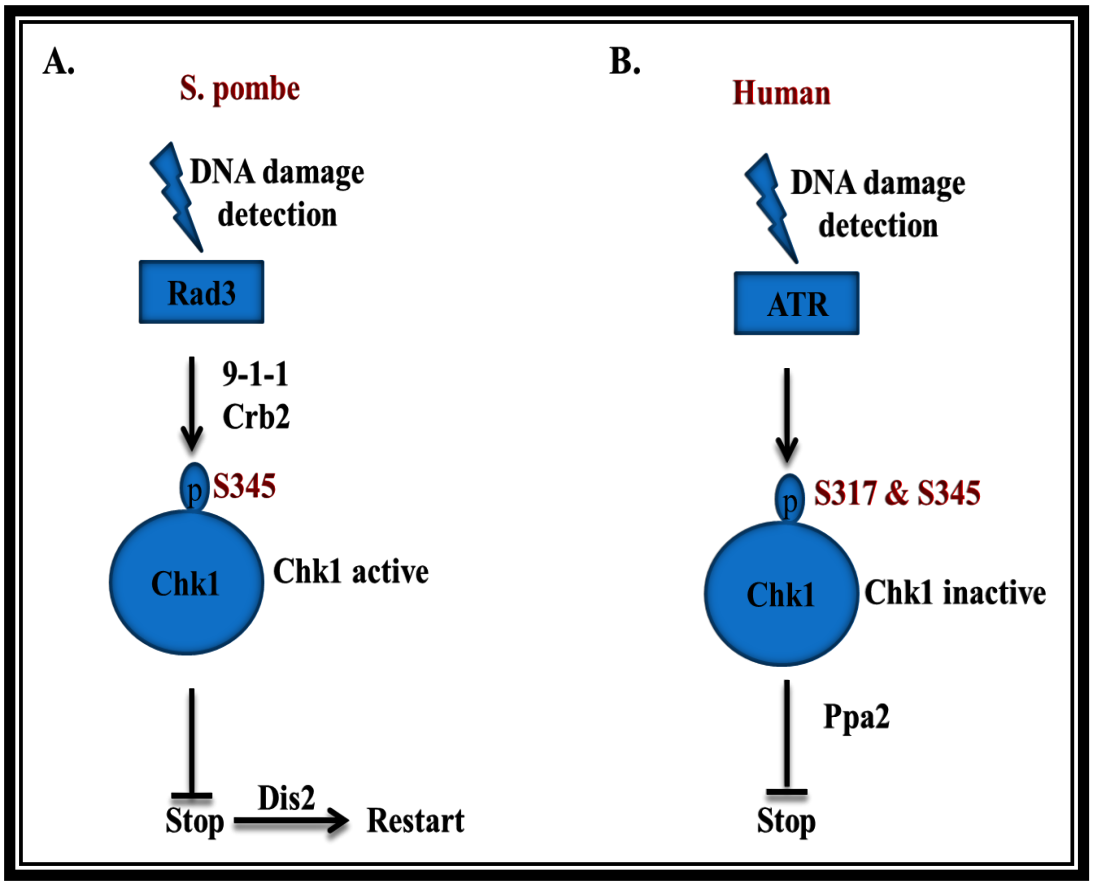


Figure 4- 1: Chk1 activation and inactivation by DNA damage.

(A) In the fission yeast *S.pombe*, Rad3 kinase is activated in the response to DNA damage leading to active Chk1 by phosphorylating serine-345 (S345) to initiate a G2-M arrest. Upon completion of the checkpoint signal, Chk1 is dephosphorylated by Dis2 phosphatase allowing cells to re-enter the cell cycle. (B) In human cells, Chk1 is phosphorylate at S317 and S345 by ATR (Rad3) kinase and dephosphorylated by Ppa2 phosphatase (Leung-Pineda, et al., 2006)

Dis2 phosphatase targets the nuclear fraction of *S.pombe* Chk1 and removes the phosphate from serine-345 thereby allowing fission yeast *S.pombe* cells to exit the Rad3-induced cell cycle arrest (Den & O'Connell, 2004). While Chk1 activity is not essential in lower eukaryotic cells like yeast, it is essential in higher eukaryotic cells. This implies additional roles of this kinase outside of the DNA damage response (Liu, et al., 2000). Ppa2 phosphatase has been shown to dephosphorylate human Chk1 *in vivo* which is normally phosphorylated at S317 and S345 by ATR (Rad3) kinase (Leung-Pineda, et al., 2006). Ptc1 is another Ser/Thr phosphatase which negatively regulates the MAPK pathway in the response to heat stress in the fission yeast *S.pombe* and human cells by removing the phosphorylation from Sty1 MAP kinase at threonine 171 (Shiozaki, et al., 1998). Janes, et al., 2012 discovered a novel role of Rad9 variant in the regulation of cell cycle progression under temperature stress conditions. Exposure of *S.pombe* cells to elevated temperatures leads to a prolonged G2 arrest and cells lacking Rad9 variant exit this arrest permanently (Janes et al., 2012). Intriguingly, heat stress leads to the rapid dephosphorylation of Chk1 at serine 345, an event which correlates with the up-regulation of Rad9 variant (Janes et al., 2012). This chapter addresses the question whether Rad9 variant is required for the heat-induced removal of the phosphate from serine 345 or whether the up-regulation of the variant is only coincidental. The results presented here clearly show that the heat-inducible variant of Rad9 is not required for the dephosphorylation of Chk1 but that Ptc1 and Ppa2 are required. I also discovered a genetic interaction between *rad9-M50A* (a strain which cannot produce Rad9 variant) and Ptc1 phosphatase.

4.2 Results

4.2.1 Ptc1 and Ppa2 phosphatases, but not Rad9 variant, are required for the de-phosphorylation of Chk1 at serine 345 at elevated temperatures.

Knowing that Rad3 phosphorylates Chk1 at S345 in response to DNA damage (Capasso et al., 2002; Kosoy and O'Connell, 2008) and that this phosphorylation of Chk1 is suppressed at elevated temperatures (Janes, et.al, 2012), an experiment was designed to test whether the heat-inducible Rad9-M50 variant is required for the removal of the phosphate from S345. To this end, the *rad9-HA* gene or its *rad9-M50A-HA* allele, which cannot induce the variant, was combined with the *chk1-Myc* gene to compare the dephosphorylation of Chk1 after cells were challenged with camptothecin (CPT) which leads to replicative DNA breaks and a strong phosphorylation of Chk1 at S345 (Walworth, et al., 1993). Cells were grown overnight at 30°C

in YEA liquid medium before being incubated with 40µM CPT for 3 hours at 30°C. Cells were then shifted to 40°C in the presence of CPT and a sample was withdrawn every 10 minutes for 30 minutes (Figure 4-2 A). In the presence of the heat-inducible Rad9 variant (*chk1-Myc rad9-HA*), the serine 345 phosphorylation, which results in the slower migration of the modified Chk1 kinase, disappeared after 20 minutes at 40°C. As previously reported (Janes et al., 2012), this correlated with the up-regulation of Rad9 variant (Figure 4-2 B). In the absence of Rad9 variant (*chk1-Myc rad9-M50A*), serine 345 was even faster de-phosphorylated as the shift band disappeared already at the 20min time point (Figure 4-2 C). This clearly shows that Rad9-M50 is not required for the dephosphorylation of Chk1. On the contrary, it suggests that Rad9 variant protects the serine 345 modification under heat stress conditions. The latter is interesting as loss of Rad9 variant shortens the heat-induced G2 arrest by approximately 20 min (Janes et al., 2012) which correlates with the premature loss of the serine 345 modification in its absence (Figure 4-2 C).

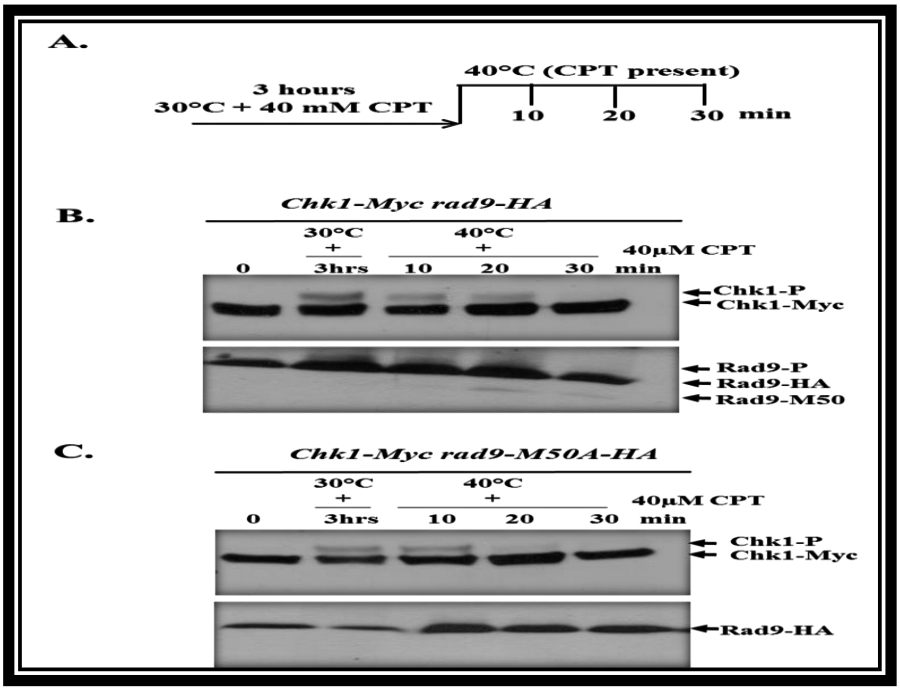


Figure 4- 2: De-phosphorylation of Chk1 in the presence and absence of Rad9-M50.

(A) Design of the experiment. To test whether Rad9M50 is required for the heat induced dephosphorylation of Chk1, Chk1 phosphorylation was induced by pre-incubating *chk1-Myc rad9-HA* and *chk1-Myc rad9-M50A* cells with 40µM CPT at 30°C for 3 hours. Cell were then shifted to 40°C and Chk1 phosphorylation at S345 was analysed in 10 min intervals for 30 min. The S345 phosphorylation results in a mobility shift (Walworth & Bernards, 1996). (B&C) *chk1-HAMycHis rad9-HA* and *chk1-Myc rad9-M50A* strains were grown overnight in YEA at 30°C prior to the pre-incubation with 40µM CPT for 3 hours at 30°C. Cells were then shifted to 40°C and sample were collected every 10 min for 30 min after the shift. Total protein was isolated and separated on a 8% SDS gel using a 100:1 acrylamide bis-acrylamide mixture, transferred onto a nitrocellulose membrane and detected with anti-HA antibody for Rad9-HA and anti-Myc antibody for Chk1-Myc.

Although research into the biological functions of Chk1 kinase is extensive, so far very little is known about its inactivation. The assembly of a large checkpoint complex at the damaged chromatin leads to Chk1 phosphorylation at serine-345 by ATR (Rad3) kinase and a G2-M arrest (Walworth, et al., 1993). How Chk1 activity is then down-regulated to allow cells to exit this arrest is not fully understood. Only the type-I serine/threonine phosphatase Dis2 has so far been implicated in this process (Den & O'Connell, 2004).

Given the rapid de-phosphorylation of Chk1 at serine 345 upon a heat shock, I wanted to test whether Dis2 or any of the other known phosphatases is involved in the process. Table 3-1 shows some of the known phosphatases and their biological functions. A good candidate is the cytoplasmic phosphatase Ppa2, which is strongly up-regulated at elevated temperatures and known to regulate cell cycle progression as well as the dephosphorylation of human Chk1 (Leung-Pineda, et al., 2006; Freeman, et al., 2010). Pyp1 and Ptc1 phosphatases have also been implicated in the response to heat stress by maintaining the MAPK kinase pathway in its active state upon their temperature dependent inactivation (Shiozaki, et.al. 1995; Nguyen & Shiozaki, 1999).

Table 4- 1: The phosphatases mentioned in this chapter and their biological functions. (Information available at http://www.pombase.org/)

Phosphatase	Functions	Reference
Ptc1	MAPK activity inactivation involved in osmosensory signalling pathway	Shiozaki, et.al. 1995
	Negatively regulate the heat stress activated MAPK cascade	Nguyen, et.al. 1999
	Serine/threonine dephosphorylation in response to heat stress	Shiozaki, et.al. 1994 Nguyen, et.al. 1999
Dis2	Mitotic spindle microtubules attachment to kinetochore	Buttrick, et al. 2011
	Mitotic DNA damage checkpoint	den Elzen, et.al. 2004
	Dephosphorylation of protein	Stone, et.al. 1993
Pyp1	Inactivation of MAPK activity involved in osmosensory signalling pathway	Nguyen, et.al. 1999
	Negative regulation of G2/M transition of mitotic cell cycle	Ottilie, et.al. 1992 Millar, et.al. 1992
	Peptidyl-tyrosine dephosphorylation	Hannig, et.al. 1993
	Peptidyl-tyrosine dephosphorylation involved in inactivation of protein kinase activity	Shiozaki, et.al.1995 Nguyen, et.al. 1999
Ppa2	Negative regulation of G2/M transition of mitotic cell cycle	Kinoshita, et.al. 1993
	Protein dephosphorylation	Kinoshita, et al. 1990
	Signal transduction	GO_REF:0000051

To this end, I constructed *chk1-Myc* strains in which either *ppa2*, *dis2*, *ptc1* or *pyp1* are deleted to check whether any of these phosphatase are involved in the dephosphorylation of Chk1 after heat shock. *The chk1-Myc WT*, *chk1-Myc Δppa2*, *chk1-Myc Δptc1*, *chk1-Myc Δpyp1* and *chk1-*

Myc Δdis2 strains were grown overnight at 30°C in YEA liquid medium before their treatment with 40µM CPT at 30°C for 3 hours and their subsequent shift to 40°C in the presence of CPT. Sample were withdrawn every 10 minutes for 30 minutes and total protein extracts were analysed (Figure 4-3). Interestingly, cells lacking either *ppa2* or *ptc1* showed a slight reduction in the reversal of the phosphorylation of Chk1 compared to the *chk1-myc WT* strain. Interestingly, deletion of *dis2* had no impact on the heat-inducible dephosphorylation despite its involvement in this process at 30°C (den Elzen, et.al. 2004). The partial requirement of Ppa2 is in line with its ability to dephosphorylate Chk1 in human cells as well as with its up-regulation at elevated temperatures (Leung-Pineda, et al., 2006). Since Chk1 was dephosphorylated in all strains, none of these phosphatases can be responsible on their own for this event. The other important point to consider is that CPT was present throughout the experiment. This implies that the DNA damage response upstream of Chk1 must also be inactivated for Chk1 S345 to be dephosphorylated. Heat stress is therefore expected to inactivate the Rad3 dependent modification of Chk1 at S345 and to activate more than one phosphatase to remove this modification. Why this is important for cells remains to be explored.

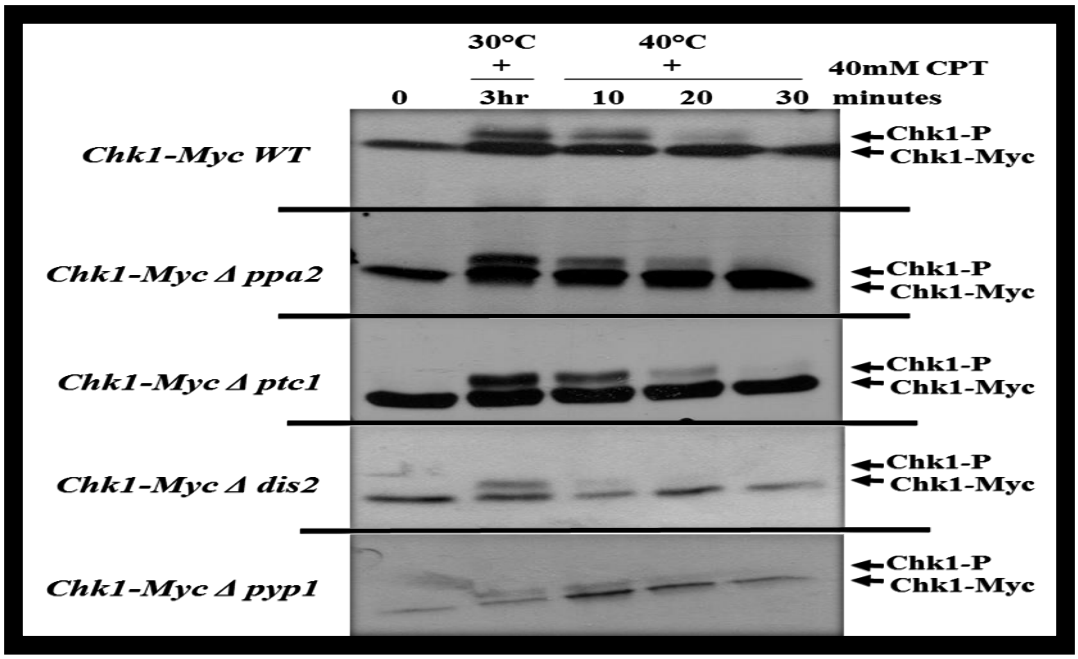


Figure 4- 3: De-phosphorylation of Chk1 at S345 in the absence of Ppa2, Ptc1, Dis2 and Pyp1 phosphatases.

The indicated strains were grown overnight in YEA at 30°C prior to pre-incubation with 40µM CPT for 3 hours at 30°C and then shifted to 40°C in the presence of CPT. Samples were taken every 10 minutes for 30 minutes after the shift. Total protein was isolated and separated on a 8% SDS gel using a 100:1 acrylamide bis-acrylamide mixture, transferred onto a nitrocellulose membrane and detected with anti-Myc antibody.

4.2.2 Rad9 variant may act as an adaptor for Pyp1, Dis2 or Ptc1 phosphatases

The finding that Ppa2 and Ptc1 have both a role in the dephosphorylation of Chk1 lead me to extend the analysis of the requirement of the phosphatases for the response to heat and DNA damage. Table 4-1 summarise what is known about the three phosphatases Ptc1, Dis2, and Pyp1 and their substrates in the response to heat stress.

Table 4- 2: The role of the phosphatases to heat stress and their substrates.

Phosphatase	Response to heat	Substrate	Reference
Ptc1	Highly sensitive	Sty1-T171	Shiozaki, et al., 1994
Dis2	Sensitive	Chk1-S345	Den, et.al, 2004
Pyp1	Highly resistant	Sty1-Y173	Shiozaki, et al., 1995

In order to investigate whether any of these phosphatases is required for the survival of elevated temperatures, the phosphatase deletion strains were subjected to a heat survival assay at 40°C. To this end, *WT*, *Δptc1*, *Δpyp1* and *Δdis2* strains were grown in YEA liquid medium overnight before diluted to 10^7 cells/ml and exposed to 40°C. Samples were withdrawn every 20 minutes for 120 minutes and plated on YEA plates to score the number of colony forming cells after 4 days at 30°C (Figure 4-4). The results confirmed what is already known about these phosphatases such that the *ptc1* deletion cells are highly heat sensitive, while the *pyp1* deletion cells are more resistant than wild type cells (Shiozaki, et.al. 1995; Nguyen, et.al. 1999). What was however new, is a requirement of Dis2 phosphatase for heat survival.

To test whether any of the phosphatase deletions genetically interacts with cells unable to produce Rad9 variant, the following strains were constructed: *rad9-M50A Δptc1*, *rad9-M50A Δdis2* and *rad9-M50A Δpyp1* and subjected to survival tests.

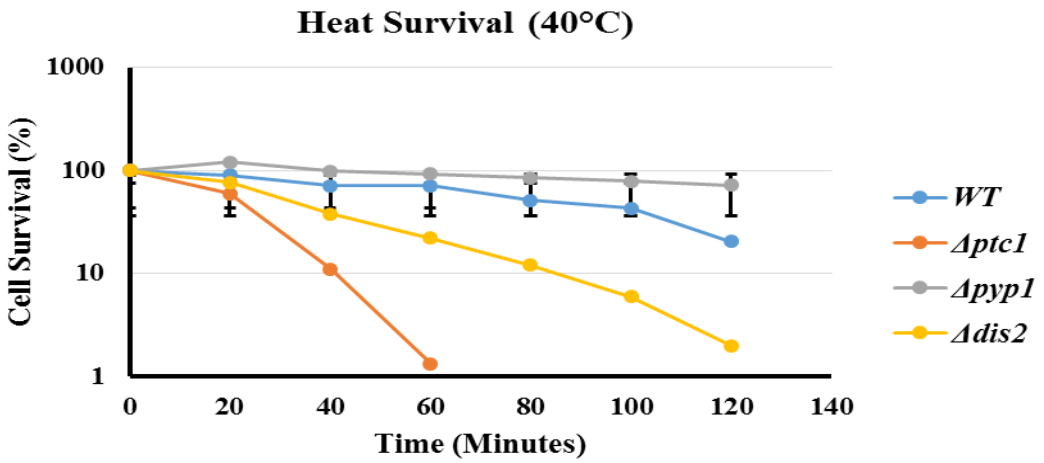


Figure 4- 4: The Phosphatases Dis2 and Ptc1 are required for heat survival.

Aliquots of cell dilutions (5x10^4 cells/ml) of wild-type, *Δptc1*, *Δpyp1* and *Δdis2* strains were exposed to 40°C and a sample (75 µl) was withdrawn every 20 minutes for 120 minutes. Plates were incubated at 30°C for 4 days, and colonies were then counted.

4.2.2.1 Loss of Rad9 variant suppresses the MMS, HU and Heat sensitivity of cells without Ptc1 Phosphatase

Ptc1 phosphatase has been implicated in the regulation of the MAP kinase pathway at elevated temperatures by dephosphorylating Sty1 MAPK at threonine 173 (Shiozaki, et al., 1994). To find out whether Ptc1 and Rad9 variant genetically interact, wild type (804), *rad9-M50A, Δptc1* and *rad9-M50A Δptc1* strains were grown in YEA liquid medium overnight, diluted to 5x10^4 cells/ml and exposed to 40°C. A sample (75µl) was withdrawn every 20 minutes for 120 minutes and plated on a YEA plate to determine the number of surviving cells (Figure 4-5). The strain lacking the Rad9 variant (r*ad9-M50A*) was resistant to heat stress while cells deficient in *ptc1* were highly sensitive as previously published (Shiozaki, et al., 1994). Interestingly, the combination of both mutations (*rad9-M50A Δptc1*) supressed the heat sensitivity of the *ptc1* deletion (Figure 4-5). This indicates that the Rad9 variant and Ptc1 act in the same pathway and that Rad9 variant can complement loss of the phosphatase.

Since Ptc1 regulates the MAP kinase Sty1, it is likely that Rad9 variant targets the same heat response pathway. I extended this analysis by treating the same strains with DNA damaging agents to see if this genetic interaction is specific to heat or whether it also extends to DNA damage. The strains were grown in YEA medium at 30°C prior to a serial dilution of 10^7, 10^6, 10^5 and 10^4 cells/ml. 5µl of cells were then spotted on YEA agar plates without drug or with

the indicted concentration of drugs (Figure 4-6). Interestingly, loss of Rad9 variant strongly suppressed the MMS sensitivity, weakly the HU sensitivity but not the CPT sensitivity of *ptc1* deletion cells. This finding confirms the close link between Rad9 variant and Ptc1 phosphatase and shows that this functional link is not limited to heat stress. Interestingly, all three treatments, heat, MMS and HU interfere with DNA replication in a way which does not break the DNA (Saladino & Ben; 1976, Corry, et.al. 1977; Bhuyan, 1979; Friedberg, 2003). Whether these responses are linked with the Sty1 MAP kinase remains to be seen.

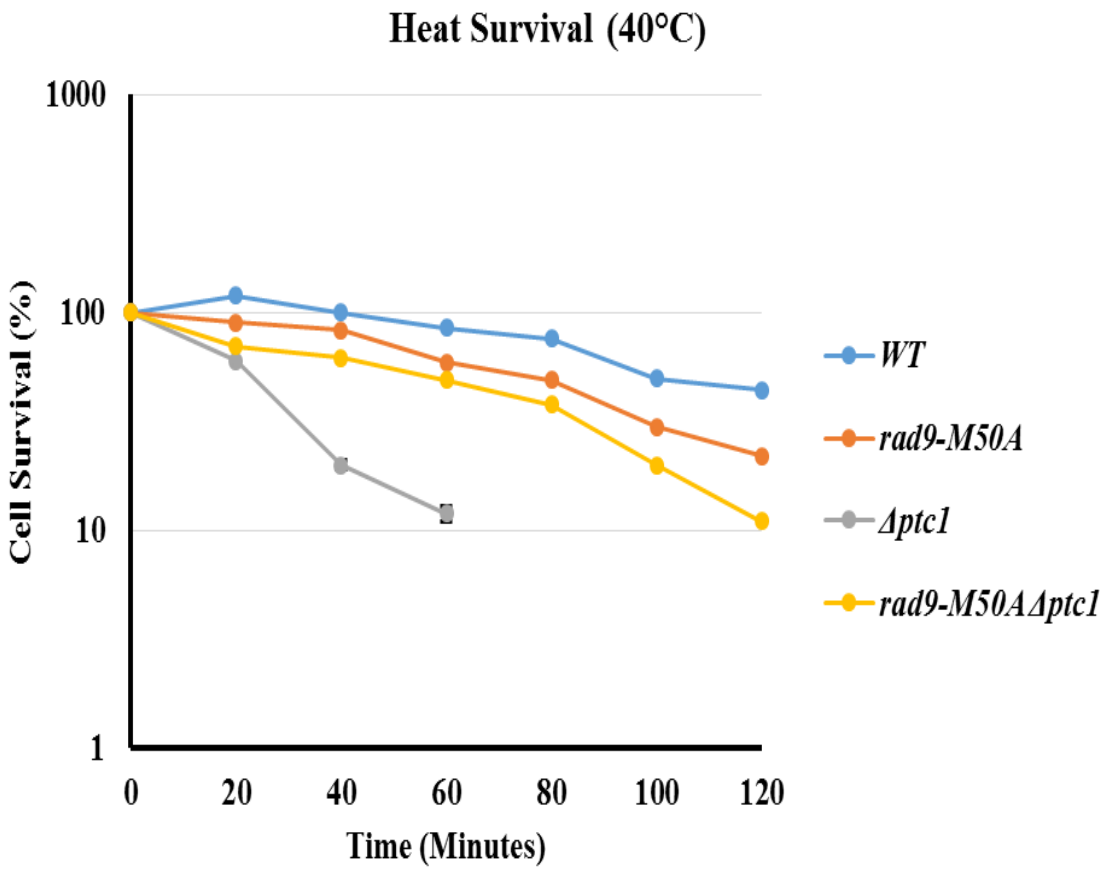


Figure 4- 5: Loss of Rad9 variant supresses the heat sensitivity of *Δptc1* cells.

Aliquots of cell dilutions ($5x10^4$ cells/ml) of wild-type, *rad9-M50A*, *Δptc1* and *rad9-M50A Δptc1* cells were exposed to 40°C and a sample was taken every 20 minutes for 120 minutes and plated on YEA plates at 30°C. Plates were incubated at 30°C for 4 days, and then colonies were counted.

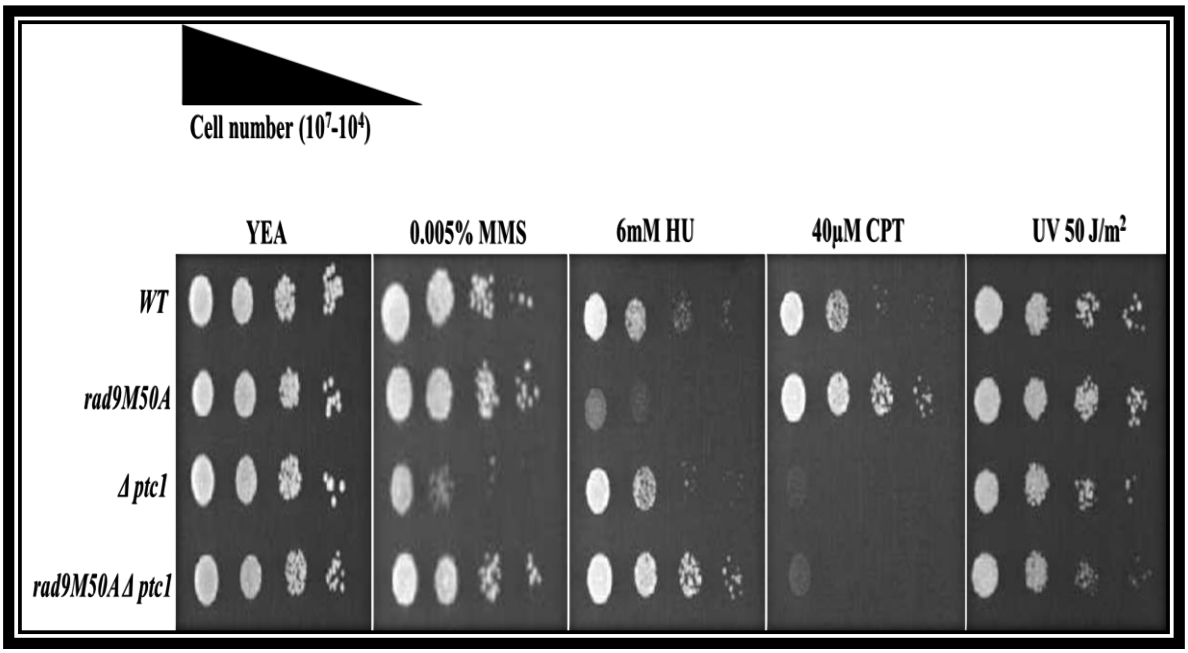


Figure 4- 6: Loss of Rad9 variant suppresses the MMS and HU, but not the CPT sensitivity of *ptc1* deletion cells.

Serial dilutions (10^7-10^4 cells/ml) of the indicated strains were dropped (5 µl) onto rich medium agar plates without treatment or with 0.005% MMS, 6mM HU, 40µM CPT or 50J/m^2 UV. Plates were incubated for 3 days at 30°C.

4.2.2.2 Combination of *rad9-M50A* with *Δpyp1* or *Δdis2* increases the sensitivity of the double mutants

Since Ptc1 regulates the Sty1 MAPK kinase by de-phosphorylating threonine 171 (Nguyen & Shiozaki, 1999), I extended the analysis to Pyp1. The two phosphatases Pyp1 and Pyp2 have been implicated in the down-regulation of Sty1 by dephosphorylating tyrosine 173 with Pyp1 being the main phosphatase (Shiozaki, et al., 1995). Heat stress inactivates Pyp1 (and probably Pyp2) by an unknown mechanism that results in the increased activity of Sty1 promoting cell survival at elevated temperatures (Samejim, et al., 1997; Shiozaki, et al., 1998; Nguyen & Shiozaki, 1999). How the dephosphorylation of threonine 171 by Ptc1 regulates Sty1 is not yet clear. I also included Dis2 in the analysis given its dephosphorylation of Chk1 during the normal recovery from the DNA checkpoint arrest (Den & O'Connell, 2004). Unexpectedly, the acute heat survival test revealed that both, the *rad9-M50A Δdis2* and the *rad9-M50A Δpyp1* double mutants were more sensitive than the corresponding single mutants (Figure 4-7). This was particularly impressive for the *rad9-M50A Δpyp1* strain as the *Δpyp1* single mutant is very heat resistant. The heat resistance in the absence of Pyp1 is attributed to the hyper-activation of the Sty1 MAPK kinase since the activating tyrosine 173 phosphorylation is expected to

remain for longer at the kinase. Tyrosine 173 is modified by the upstream MAP kinase Wis1 (Millar, et.al. 1995; Shiozaki & Russell, 1995b). This implies that Rad9 variant either removes the phosphorylation of Y173 or prevents the up-regulation of Pyp2, the back-up phosphatase, as this would prematurely terminate the activity of the MAP kinase in *pyp1* deletion cells. An alternative explanation would be provided if the absence of Rad9 variant would result in the permanent activation of Sty1 in *pyp1* deletion cells as this could result in an irreversible G2 arrest and cell death.

Why *dis2* deletion cells are heat sensitive is not yet clear and it is very interesting that loss of Rad9 variant increases this sensitivity. It is however clear that Dis2 and Pyp1 act in parallel pathways to Rad9 variant which functions probably upstream of Ptc1.

This genetic relationship extended also to DNA replication problems caused by HU and MMS, but not to replicative DNA breaks caused by CPT (Figure 4-8). While the *dis2* single deletion is slightly MMS and HU sensitive, the single *pyp1* deletion is resistant to both treatments.

Taken together, this genetic analysis places, Rad9 variant and Ptc1 in the same pathway whereas Pyp1 and Dis2 act in a parallel pathway. Importantly, both pathways become important when DNA replication is stopped at elevated temperatures, in the presence of alkylated templates (MMS) or when the nucleotide pool in depleted (HU). They are not important when DNA replication forks break in the presence of the topoisomerase I inhibitor CPT.

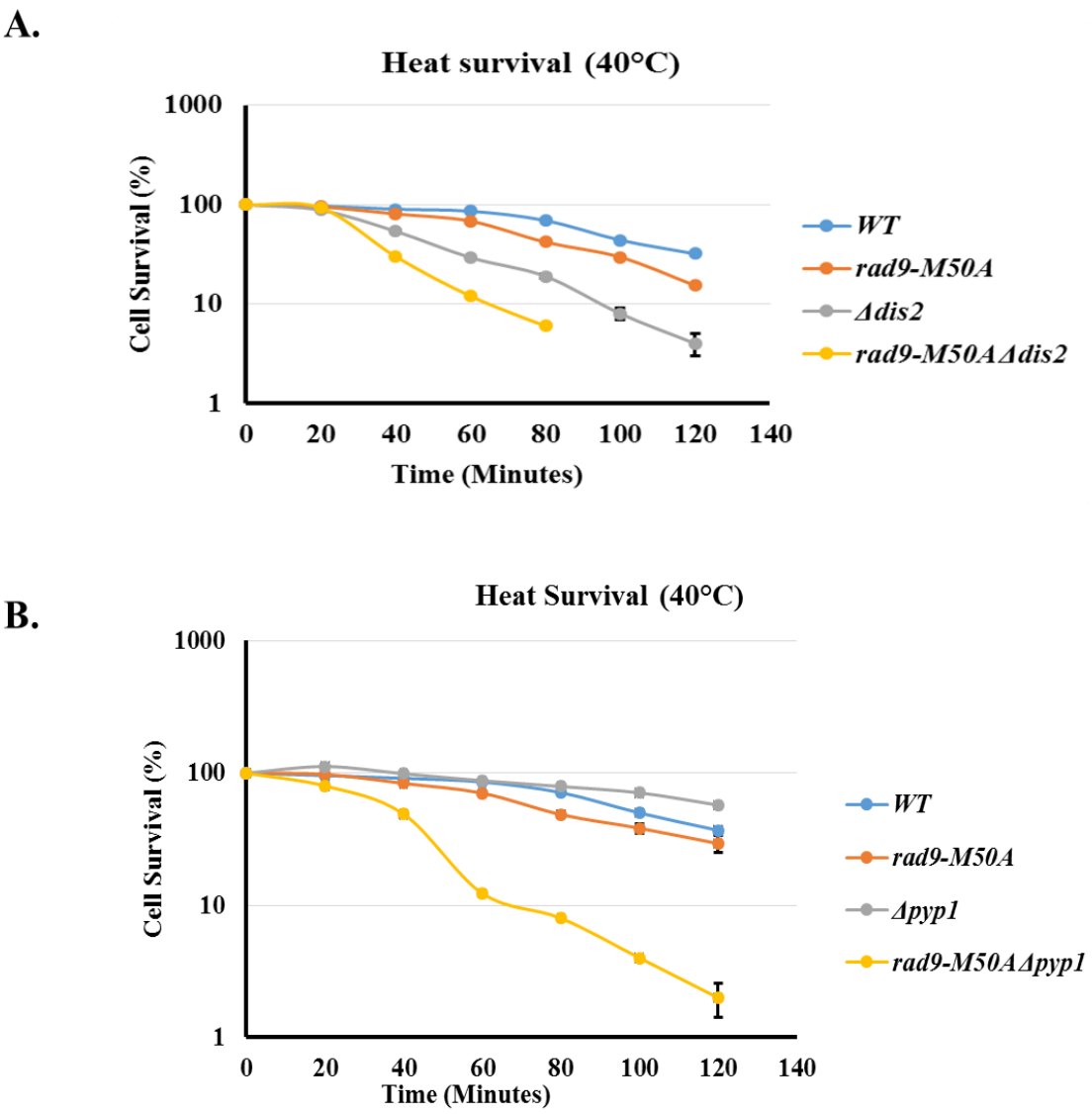


Figure 4- 7 : Loss of Rad9 variant increases the heat sensitivity of either *dis2* or *pyp1* deletion strains.

(A&B) Aliquots of cell dilutions ($5x10^4$ cells/ml) of the indicated strains were exposed to 40°C and samples (75 µl) were taken every 20 minutes for 120 minutes, and spread on YEA plates. Plates were incubated at 30°C for 4 days, and then cells were counted.

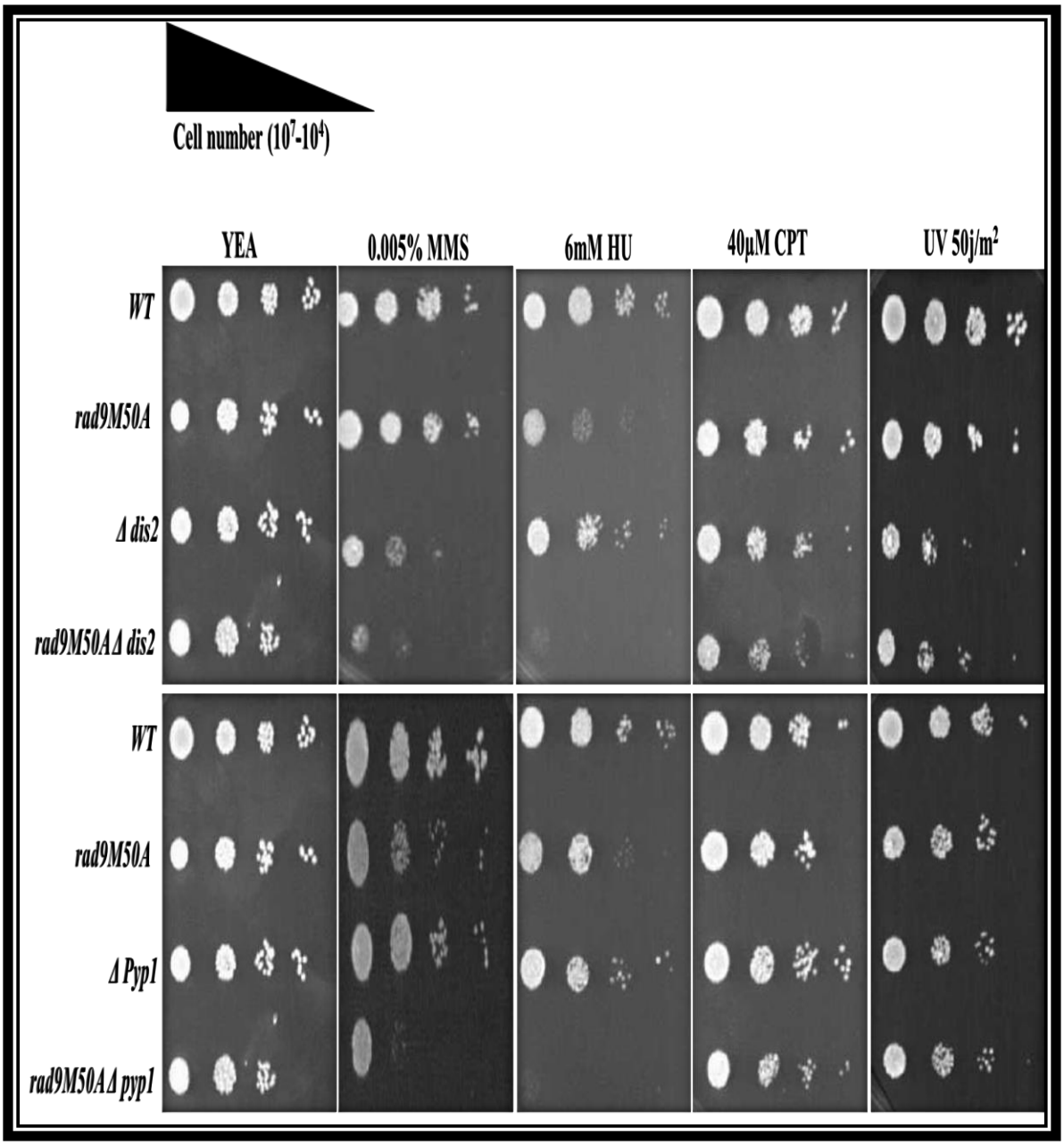


Figure 4- 8: Loss of Rad9 variant increases the MMS and HU sensitivity of *dis2* and *pyp1* deletion strains.

Serial dilutions (10^7-10^4 cells/ml) of the indicated strains were dropped (5 µl) onto rich medium agar plates without treatment or with 0.005% MMS, 6mM HU, 40µM CPT or 50J/m^2 UV. Plates were incubated for 3 days at 30°C.

4.2.3 Discussion

Chk1 phosphorylation at serine 345 by Rad3 kinase occurs in the response to a wide range of DNA damage agents (Walworth & Bernards, 1996; Wang, et.al. 1998). Rad3 kinase associates with Chk1 and phosphorylates the kinase when over-expressed in fission yeast cells (Martinho, et.al. 1998). As previously reported, Chk1 S345 is dephosphorylated by Dis2 during the

recovery from a G2 arrest at 30°C caused by DNA damage (Den & O'Connell, 2004), while human Chk1 is dephosphorylated by Ppa2 (Leung-Pineda, et al., 2006). I show here that in the fission yeast *S. pombe*, Chk1 is dephosphorylated by Ppa2 and maybe Ptc1, but not by the Rad9 variant, at elevated temperatures. As shown in Figure 4-2, Chk1 S345 phosphorylation was reversed within 20 minutes either in the presence or absence of the Rad9 variant. This shows that the induction of Rad9 variant and Chk1 dephosphorylation are only coincidental (Janes, et.al 2012). The results presented here clearly show that Ppa2 and maybe Ptc1 dephosphorylate Chk1 at S345, but not Dis2, at high temperatures (Figure 4-3). Ppa2 was reported to have a role in cell cycle regulation and to dephosphorylate human Chk1 (Leung-Pineda, et al., 2006)**.** The finding that Ptc1 is also involved in Chk1 dephosphorylation is more surprising as Ptc1 was reported to dephosphorylate Sty1 MAPK at T171 in the response to elevated temperatures (Shiozaki, et al., 1995; Nguyen & Shiozaki, 1999). Why S345 needs to be dephosphorylated at high temperatures although the DNA damaging drug (CPT) is still present is not yet clear. Maybe Rad3 activation changes under heat stress conditions like the activation of ATM and ATR in human cells, and activation of Chk1 is no longer required since heat can efficiently arrest the cell cycle (Furusawa, et.al. 2012) (Figure 4-9).

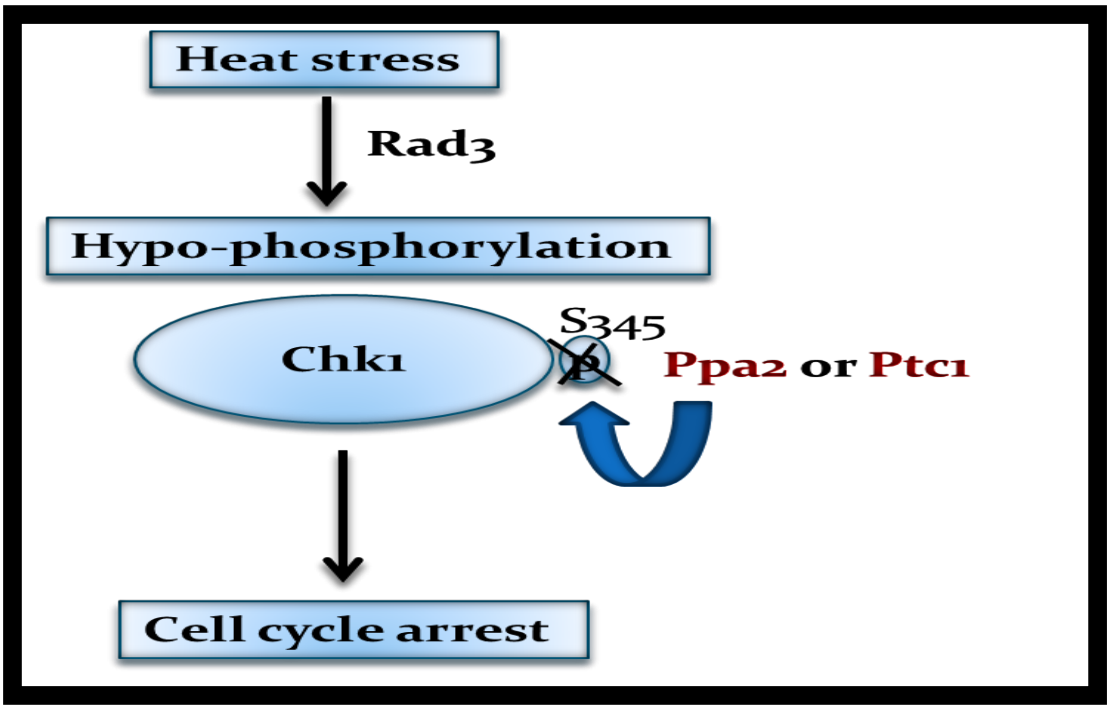


Figure 4- 9: *S.pombe* Chk1 dephosphorylation by Ppa2 or Ptc1 phosphatases.

A novel finding presented in this chapter is the genetic link between Rad9 variant and Ptc1 phosphatase in the response to heat, MMS and HU. Since loss of the Rad9 variant rescues *ptc1* deletion cells (Figures 4-5 & 4-6), both proteins are likely to act in the same pathway. In the response to heat, the activation of Sty1 is attenuated when Ptc1 dephosphorylate the MAP kinase at Thr171 (Nguyen & Shiozaki, 1999). Under heat stress conditions Rad9 variant is upregulated and this may block the access of Ptc1 to Sty1 kinase. Since no report indicated so far a link between MMS or HU and Sty1, Ptc1 could well have more targets other than Sty1. For example, Ptc1 has been found to interact genetically with the DNA damage response protein Crb2/53BP1 (Das, et al., 2013). Chk1 binds to Crb2 in order for Rad3 to phosphorylate Chk1 at S345. So there is a possibility here that Ptc1 dephosphorylates Crb2 and this could explain the MMS and HU sensitivities of the *ptc1* deletion strain.

Moreover, my data show that Pyp1 and Dis2 act in parallel pathways as the double mutants with *rad9-M50A* are more sensitive to heat, MMS and HU (Figures 4-7, 4-8). Since Dis2 dephosphorylates Chk1 S345 to terminate the DNA damage-induced G2 arrest at 30°C, Rad9 variant clearly acts independently of this Chk1 regulation by Dis2.

In conclusion, the data support a model (Figure 4-10) where heat increases the expression of Rad9 variant and down-regulates Chk1 phosphorylation at S345 through Ppa2 and probably Ptc1 phosphatase. Rad3 kinase activity may change so that Chk1 S345 phosphorylation is no longer required, and Ppa2 and Ptc1 gain access to the modified S345. At the same time the Sty1 MAP kinase would be activated by the inhibition of Ptc1 and Pyp1. Given the epigenetic link between Rad9 variant and Ptc1, the variant may act jointly with Ptc1 on Sty1 as it is not involved in Chk1 dephosphorylation. This may help to explain why heat seems to have opposite effects on Ptc1 as the phosphatase actively removes S345 from Chk1 but is prevented from performing the same function on T171 of Sty1. The results also indicate that Dis2 and Pyp1 act in parallel to the Rad9 variant in the response to heat stress, HU and MMS.

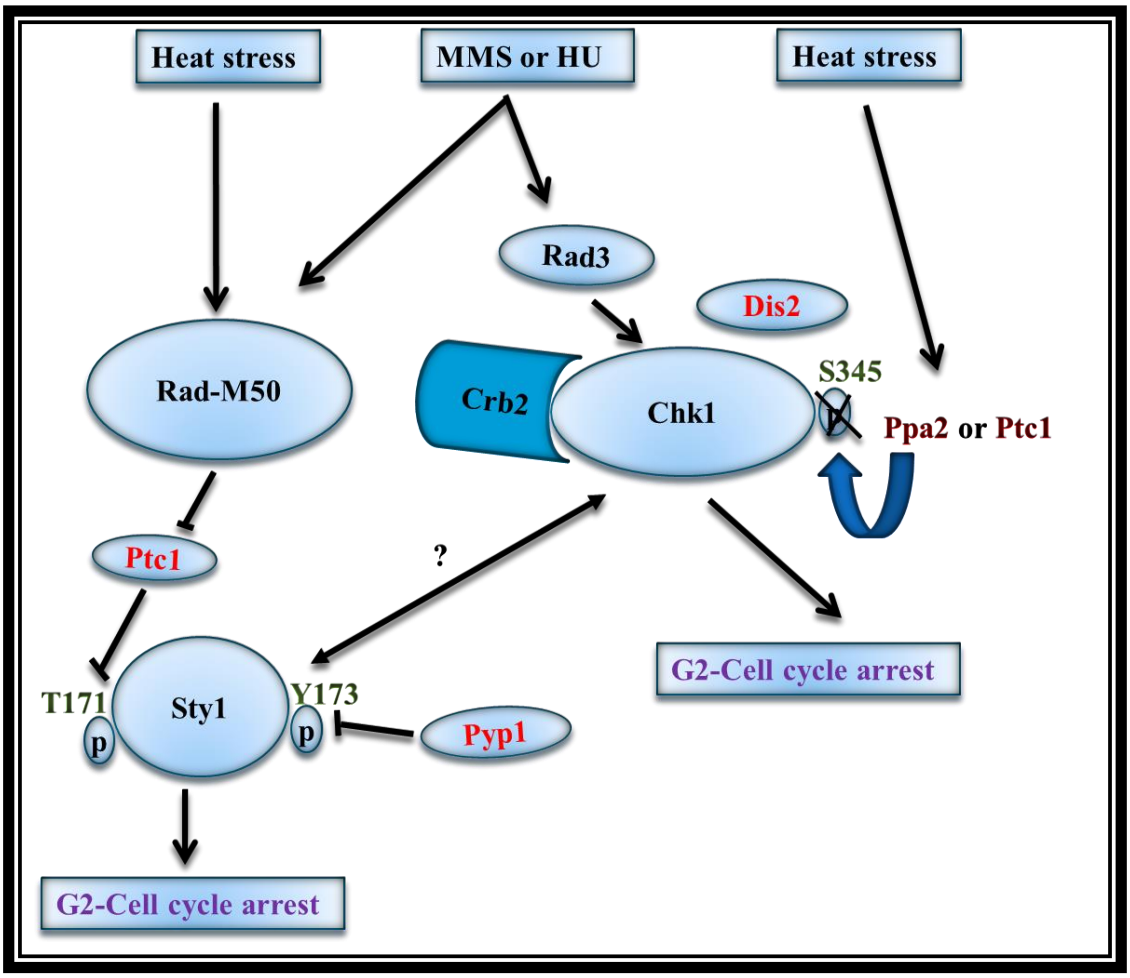


Figure 4- 10**: A diagram illustrating the outcome of this chapter.**

Chapter 5: The Rad9 Variant of the DNA Damage Checkpoint Protein Rad9 Regulates the MAP Kinase pathway in the Response to Heat Stress

5.1 Introduction

The fission yeast (*S.pombe*) Sty1/Spc1 MAP Kinase (MAPK) pathway has been shown to have a key role in the response to a wide range of stress responses including heat stress, UV light, oxidative stress and osmotic stress (Millar, et.al. 1995; Shiozaki & Russell, 1995; Degols, et.al. 1996; Degols & Russell, 1997; Shieh, et.al. 1997). Sty1 is phosphorylated at threonine 171 and tyrosine 173 by the MAP Kinase Kinase Wis1 which in turn is phosphorylated and activated by the two MAPKKKs Wis4 and Win1 (Samejima, et.al. 1997; Shieh, et.al. 1997; Shiozaki, et.al. 1997; Samejima, et.al. 1998). Sty1 MAPK is closely related to HOG1 in the budding yeast *S.cerevisiae* and p38 and Erk1/2 kinases in human (Toone & Jones, 1998). Although Wis1 plays a key role in Sty1 activation in the response to most stress conditions, heat stress appears to be very different in that it modulates Sty1 phosphorylation mainly by inactivation of the tyrosine phosphatase Pyp1 which removes the activating tyrosine 173 modification (Degols, et.al. 1996; Samejima et al. 1997). Phosphorylation of threonine 171 is removed by the phosphatase Ptc1 (Nguyen & Shiozaki, 1999), although the role of this modification is less well understood. Recent evidence suggests that threonine-mono-phosphorylated Sty1 has a limited biological activity in the context of transcriptional regulations (Vázquez, et.al. 2015). The two minor phosphatases Pyp2 and Ptc3 act as back-up systems for Pyp1 and Ptc1, respectively (Nguyen & Shiozaki, 1999).

Atf1 is one of the two transcription factors acting down-stream of Sty1 in the presence of UV-induced DNA damage and under oxidative stress conditions (Shiozaki & Russell, 1996; Wilkinson, et.al. 1996; Toone, et.al. 1996, Takeda, et.al. 1995; Nguyen, et.al. 2000). Activated Atf1 associates with Pcr1 to form a nuclear transcription complex that activates a large number of stress-response genes (Gaits, et.al. 1998). In the presence of oxidative and osmotic stress, Wis1 kinase is activated by phosphorylation at Ser-469 and Thr-473 by the MAP kinase kinase kinases Win1 and Wis4 (Samejima, et.al. 1997, 1998; Shieh, et.al. 1997; Shiozaki, et.al. 1997).

The results reported so far suggest a novel regulation of Sty1 kinase by the heat-inducible variant of the DNA damage checkpoint protein Rad9. Rad9 variant (Rad9.M50) is induced when ribosomes utilize the cryptic translation initiation site AUG-50 within the *rad9* gene

specifically at elevated temperatures (Janes, et.al. 2012) and in the response to methyl-methanesulfonate (MMS) which methylates the N3 position of adenine (Chapter 3). My genetic and biochemical data imply that Rad9 variant regulates the phosphorylation status of Sty1 kinase activity by modulating the activity of Ptc1 and maybe Pyp1 phosphatases independently of the MAPK kinase Wis1.

5.2 Results

5.2.1 Loss of Rad9M50 rescues the heat, hydrogen peroxide (H_2O_2) and UV sensitivity of *wis1* deletion cells

In the fission yeast *S. pombe*, Wis1 MAPK kinase is the upstream activator of the Sty1 MAP kinase in the response to a wide range of environmental stresses including heat, hydrogen peroxide (H_2O_2) and UV (Millar, et.al. 1995; Shiozaki & Russell, 1995; Kato, et.al. 1996). The genetic relationship between the Rad9 variant and Wis1 was analysed because of two reasons. First, the genetic link between Rad9 variant and the phosphatase Ptc1 (Chapter 4), which modulates Sty1 activity through the dephosphorylation of threonine 171, indicates a possible role of the variant in the regulation of Sty1. Secondly, Wis1 phosphorylates T171 which is dephosphorylated by Ptc1 phosphatase. I first tested the effect of the absence of Rad9 variant on the deletion of *wis*1 by constructing a *Δwis1 rad9M50A* strain. Subsequently, wild type *WT* (804), *Δwis1*, *rad9M50A* and *Δwis1 rad9M50A* cells were subjected to a drop test to investigate the response to elevated temperatures (37°C), H_2O_2 and UV light. The strains were grown overnight in YEA medium at 30°C prior to a serial dilution of 10^7, 10^6, 10^5 and 10^4 cells/ml. 5µl of these cell dilutions were spotted on YEA agar plates without drug or with 1mM H_2O_2 or exposed to UV radiation (254nm) at 10, 20. 40 and 60 J/m^2 or 37°C.

Consistent with previous publications, cells lacking *wis1* are highly sensitive to hydrogen peroxide, heat and high doses of UV light (Figure 5-1). Interestingly, when I combined the deletion of *wis1* with the *rad9M50A* mutant, which cannot make the Rad9 variant, *Δwis1* cells survived significantly better in the presence of heat stress, H_2O_2 and at high doses of UV light (40 and 60 J/m^2). This unexpected result implies a role of Rad9 variant in the regulation of the MAPK pathway down-stream of Wis1 kinase since loss of the variant compensates for the absence of Wis1. Given the critical importance of Wis1 for the phosphorylation of Sty1 at T171 and Y173, it is unlikely that Rad9 variant acts through its link with Ptc1 phosphatase because both sites need to be modified before they can be dephosphorylated and this modifications

normally requires Wis1. It is therefore more likely that loss of Rad9 variant allows another dual-specific kinase to access Sty1 by-passing Wis1.

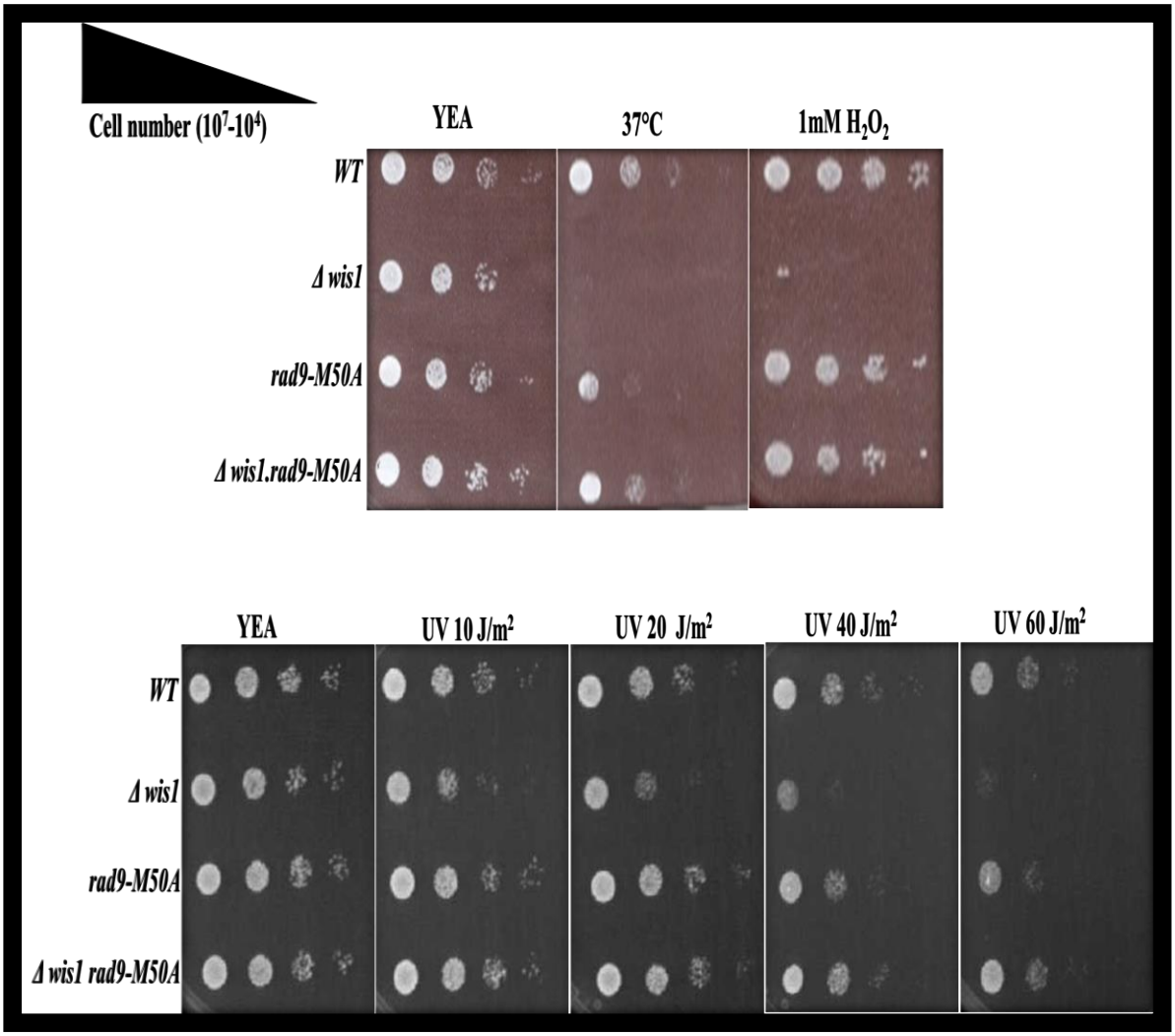


Figure 5- *1*: **Loss of Rad9 variant renders *wis1* deletion cells more resistant to heat, H_2O_2 and UV stresses.**

The indicated strains were grown overnight at 30°C, counted and then serially diluted to 10^7, 10^6, 10^5 and 10^4 cells/ml. 5µl were dropped onto YEA agar plates without drug or with the indicated concentration of H_2O_2 or were exposed to the indicated doses of UV light . One plate without treatment was incubated at 37°C while the rest of the YEA agar plates were incubated at 30°C for 3 days.

An acute survival test was then conducted with the same strains. The cells were grown overnight at 30°C in YEA medium prior to the exposure of 40°C, 1mM H_2O_2 or UV (254nm). The results were consistent with the drop test as loss of Rad9 variant strongly reduced the heat, hydrogen peroxide and ultra violet light sensitivity of the *Δwis1 rad9M50A* double mutant (Figure 5-2).

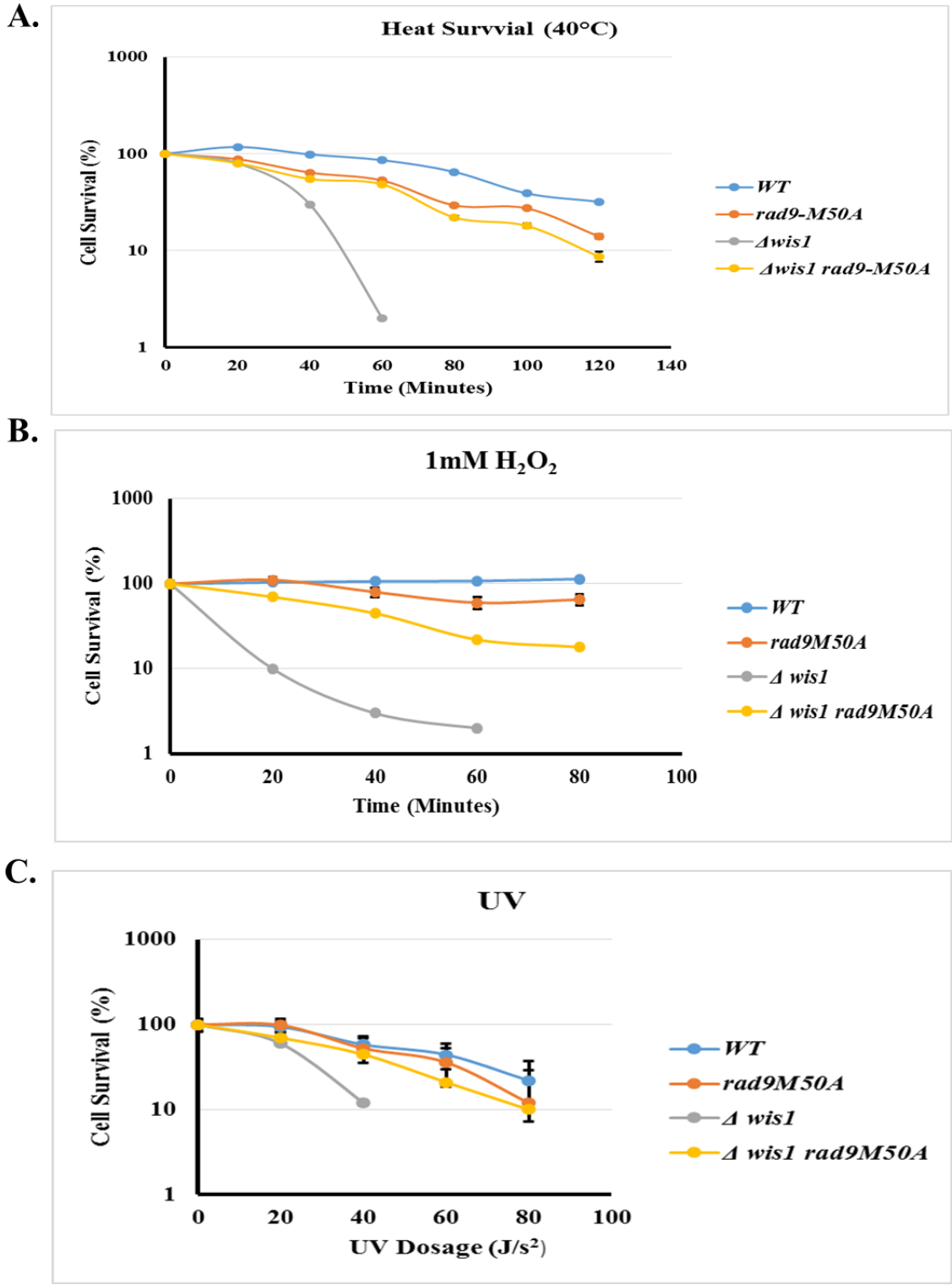


Figure 5- *2*: **Loss of Rad9 variant saves the *wis1* deletion sensitivity to heat, H_2O_2 and UV stresses.**

The indicated strains were grown at 30°C to less than 10^7 cells/ml. Aliquots of $5x10^4$ cells were transferred into 10ml of rich medium and incubated at 40°C or treated and incubated at 30°C with 1mM H_2O_2. 75µl samples of each strain were spread on rich medium agar plates every 20 minutes for 120 minutes at 40°C (A) and every 20 minutes for 80 minutes at 30°C with cells treated with 1mM H_2O_2 (B) or exposed to 20, 40, 60 and 80 J/m^2 UV after plated and dried on YEA agar plates (C). YEA agar plates were incubated at 30°C for 4 days and then counted. Cell numbers can exceed 100% when resistant cells continue to grow in the experiment. This can, for example be seen for the wild type strain in panel B.

To test whether this rescue is specific to loss of Rad9 variant, I used a strain deficient in the *hus1* gene which encodes a second subunit of the 9-1-1 checkpoint clamp in combination with the *wis1* deletion (*Δwis1Δhus1*). As shown in Figures 5-3 and 5-4, loss of Hus1 and indirectly loss of the 9-1-1 ring failed to rescue the heat sensitivity of the *wis1* deletion strain. This supports the conclusion that the rescue is specific to Rad9 variant which acts outside of the 9-1-1 ring (Janes et al., 2012)

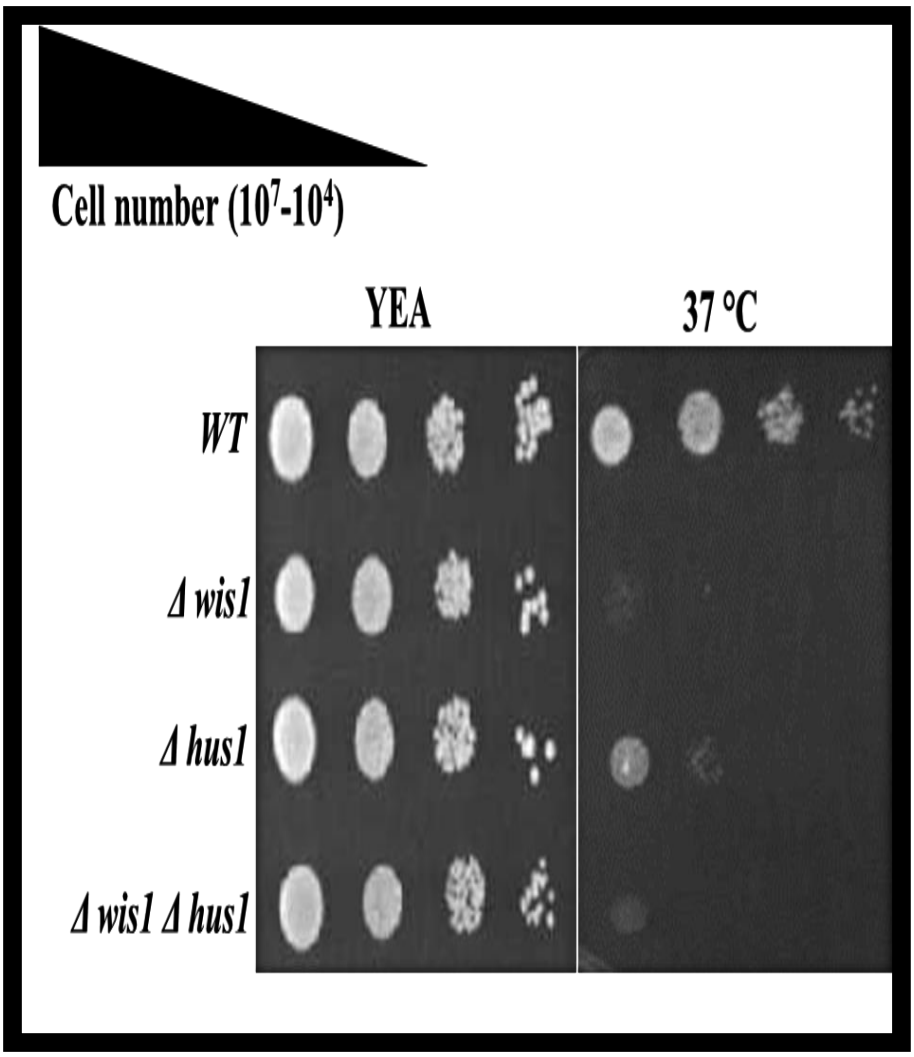


Figure 5- *3***: Loss of *hus1* does not rescue *wis1* deletion cells in the response to heat stress.**

The indicated strains were grown overnight at 30°C, counted and then serial diluted to 10^7, 10^6, 10^5 and 10^4cells/ml. 5µl were dropped into YEA agar plates. One plate was incubated at 37°C while the other plate was incubated at 30°C for 3 days.

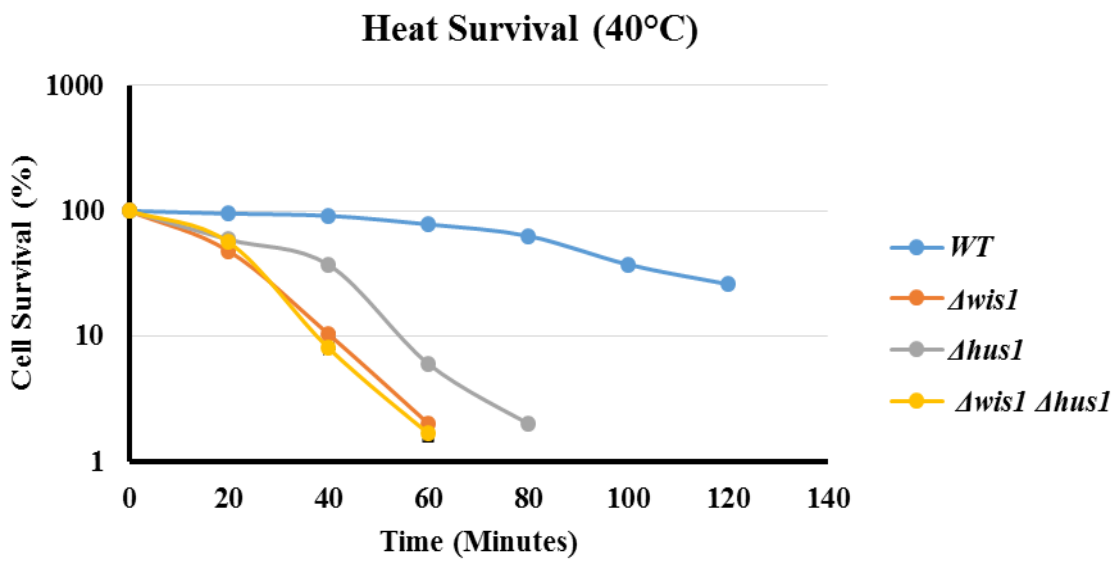


Figure 5- *4*: The rescue of the *wis1* deletion strain is dependent on Rad9-M50.

The indicated strains were grown at 30°C to less than 10^7 cells/ml. Aliquots of $5x10^4$ cells were incubated at 40°C. 75µl aliquots of each strain were spread on rich medium agar plates every 20 minutes for 120 minutes and incubated at 30°C for 4 days and colonies were then counted.

5.2.2 The suppression of the *wis1* deletion by loss of Rad9 variant is dependent on Sty1

The rescue of the *wis1* deletion strain was much unexpected since Wis1 phosphorylates the downstream MAP kinase Sty1 at Thr171 and Tyr173 (Nguyen & Shiozaki, 1999). To find out whether the rescue caused by loss of the variant in *wis1* deletion cells is dependent on the downstream kinase Sty1, the deletion of *sty1* was combined with the *rad9-M50A* mutant allele. The drop test was then repeated with the following strains: wild type (804), *Δsty1*, *rad9-M50A* and *Δsty1 rad9-M50A*. Loss of *sty1* left cells highly sensitive to heat and H_2O_2 in line with what has already been published (Millar, et.al. 1995; Shiozaki & Russell, 1995; Kato, et.al. 1996) (Figure 5-5). However, combining the *sty1* deletion with the *rad9-M50A* mutation neither increased the sensitivity nor saved the cells. This result indicates that Rad9 variant by-passes Wis1 and somehow regulates the MAPK pathway through Sty1 without the requirement of the upstream MAPK kinase.

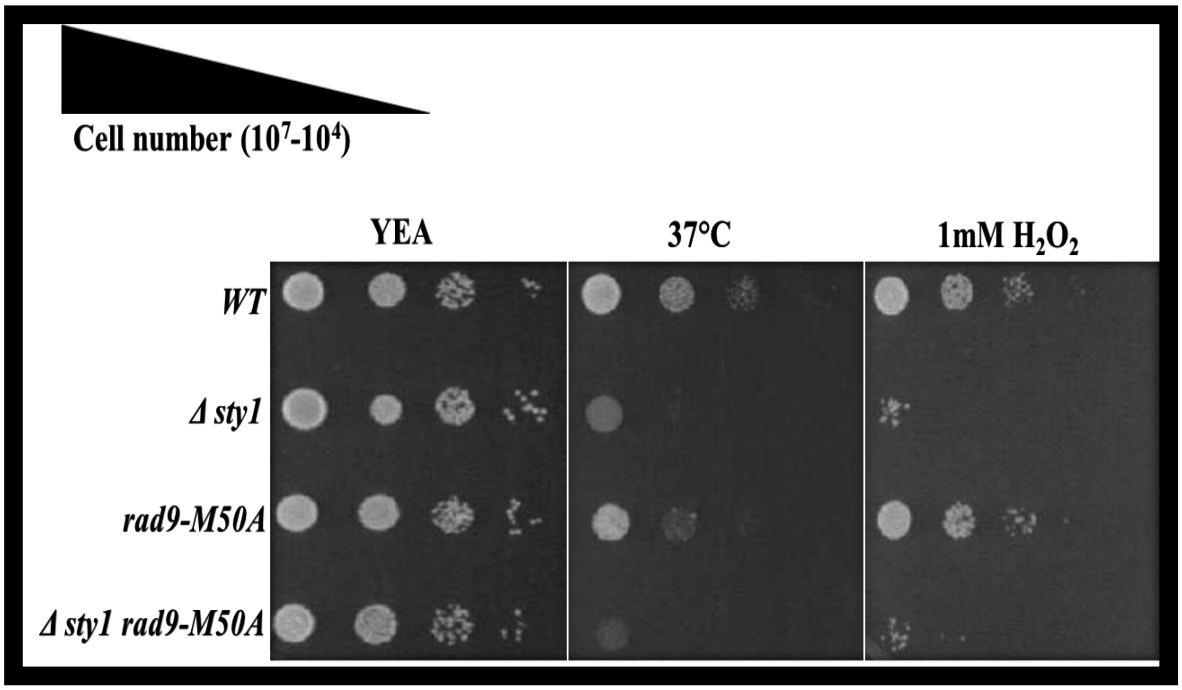


Figure 5- *5***: Loss of Rad9 variant does not rescue the *sty1* deletion strain**.

The indicated strains were grown overnight at 30°C, counted and then serially diluted to 10^7, 10^6, 10^5 and 10^4/ml. 5µl aliquots were dropped onto YEA agar plates without drug or with indicated concentration of H_2O_2. One plate without treatment was incubated at 37°C while the rest of the YEA plates were incubated at 30°C for 3 days.

The same strains were also subjected to heat stress and hydrogen peroxide in an acute survival test where the cells were grown overnight at 30°C, then counted and exposed to either 40°C or 1mM H_2O_2 and a sample was plated every 20 minutes for the indicated times (Figure 5-6). This experiment confirmed the drop test where cells deficient for *sty1* could not survive either in the absent or presence of the Rad9 variant (*sty1 rad9M50A*). This suggests that loss of the variant gives another kinase access to Sty1 to modify T171 and/or Y173 when Wis1 is not present. It is worth pointing out here that hydrogen peroxide does not induce expression of the variant, unlike heat stress, although loss of the variant suppresses the sensitivity of *wis1* deletion cells (Janes et al., 2012). This could be explained by the low basal level of Rad9 variant that may be sufficient to restore Sty1 phosphorylation.

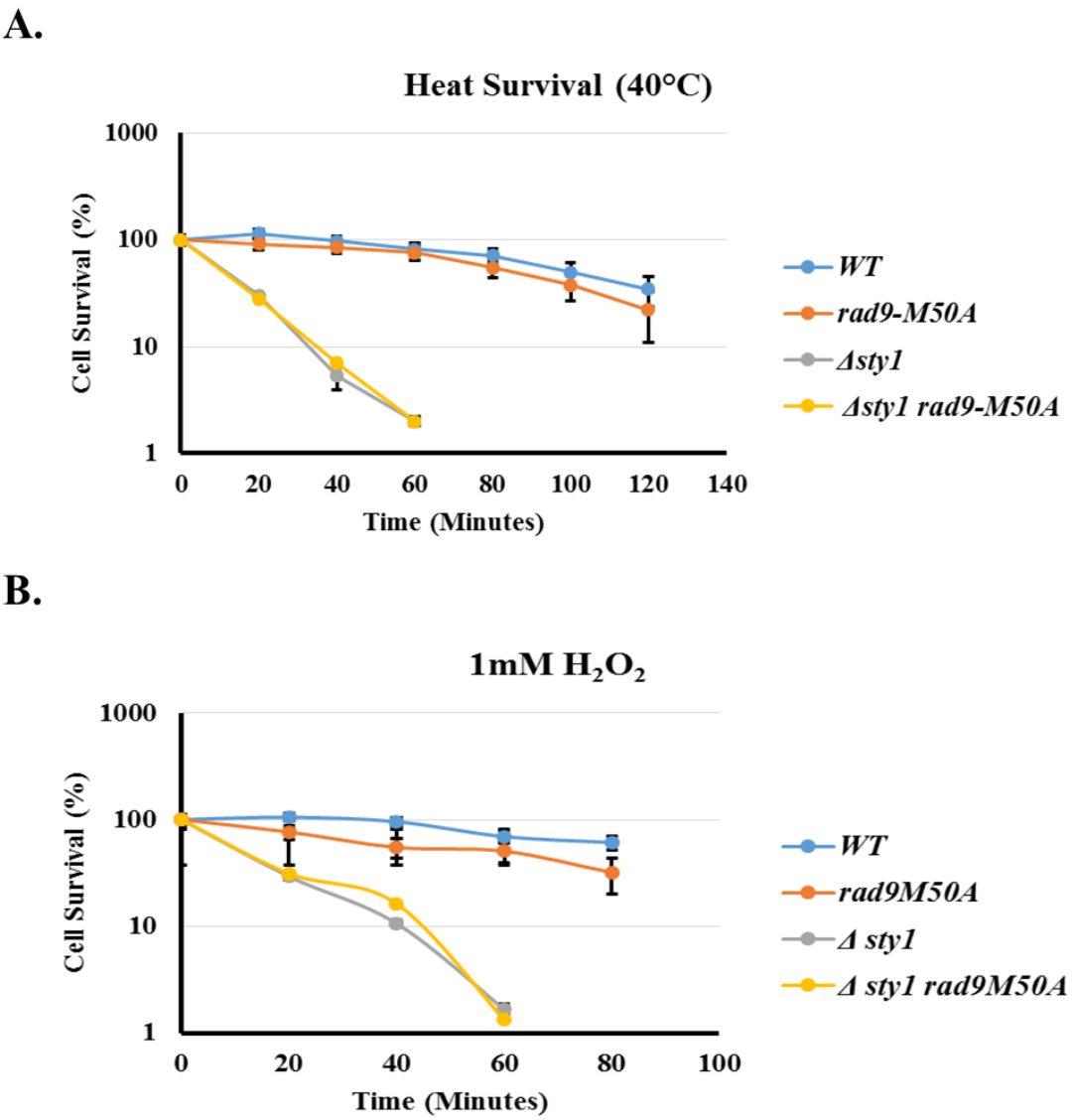


Figure 5- *6*: **Loss of Rad9 variant does not rescue *sty1* deletion cells in response to heat and H_2O_2.**

The indicated strains were grown at 30°C to less than 10^7 cells/ml. Aliquots of $5x10^4$ cells were transferred into 10ml of rich medium and incubated at 40°C or treated and incubated at 30°C with 1mM H_2O_2. 75µl samples of each strain were spread on rich medium agar plates every 20 minutes for 120 minutes at 40°C (A) and every 20 minutes for 80 minutes at 30°C with cells treated with 1mM H_2O_2 (B) . YEA agar plates were incubated at 30°C for 4 days and then counted.

5.2.3 Loss of Rad9 variant restores the phosphorylation of Sty1 in a *wis1* deletion background

Taken together, these observations imply that Sty1 is phosphorylated at Thr 171 and Tyr 173 in the absence of both, the Rad9 variant and the upstream kinase Wis1. To test this idea, a

phospho-specific p38 antibody was used which detects the dual phosphorylation of Thr 171 and Tyr 173 of Sty1. This phospho-specific antibody was raised against the mammalian p38 phosphorylated kinase (Thr 180 and Tyr 182) and cross-reacts with the highly conserved section of Sty1 containing Thr 171 and Tyr 173 (Shiozaki and Russell 1997; Gaits et al. 1998).

To investigate whether the loss of Rad9-M50 impacts on Sty1 phosphorylation in the response to heat stress, the following strains *rad9-HA-Wt*, *rad9-M50A*, *Δwis1*, *Δsty1*, *Δwis1 rad9-M50A* and *Δsty1 rad9M50A* were grown overnight at 30°C prior to a shift to 40°C. Samples were withdrawn every 20 minutes for one hour and subjected to total protein extraction. As expected and in line with previous publications (Degols et al. 1996; Kato et al. 1996; Shiozaki & Russell 1996, Shiozaki, et.al. 1999) no phosphorylation of Sty1 was detected in the absence of either *wis1* or *sty1* (Figure 5-7, first two rows). In Rad9 wild type cells, Sty1 phosphorylation increased transiently after 20 minutes at 40°C before declining again. Interestingly, in the absence of Rad9 variant (*rad9-M50A*) Sty1 phosphorylation remained high for the first 40 minutes before declining again (Figure 5-7, rows 3&4). Consistent with the survival experiments, loss of the Rad9 variant restored the phosphorylation of Sty1 at an even stronger level than in wild type cells, but only in the *wis1* deletion background and not in *sty1* deletion cells (Figure 5-7, last two rows). This confirms that loss of Rad9 variant can bypass the requirement of Wis1 for the dual phosphorylation of Sty1.

Next, I wanted to check whether this reactivation of Sty1 in *wis1 rad9-M50A* cells is specific to heat or whether it also extends to oxidative stress. The same strains were grown overnight in YEA medium at 30°C prior to the treatment with 1mM H_2O_2 at 30°C. A sample was withdrawn at 0, 20, 40, 60 minutes and subjected to total protein extract. No phosphorylation was detected in cells which lack either *wis1* or *sty1* (Figure 5-8, first two rows). In Rad9 wild type cells, an increase in Sty1 phosphorylation was detected after 20 minutes in the presence of oxidative stress before its decline. Interestingly, in the absence of Rad9 variant (*rad9-M50A*), the highly phosphorylated form of Sty1 was detect for the first 40 minutes before declining again (Figure 5-8, rows 3&4). In the line with the H_2O_2 survival data, the phosphorylation of Sty1 was restored in the absence of Rad9 variant at an even stronger level compared to wild type cells again only in the *wis1* deletion background but not in *sty1* deletion cells (Figure 5-8, last two rows). This led to the conclusion that loss of Rad9 variant activates the dual phosphorylation of Sty1 in the response to oxidative stress and heat independently of Wis1 kinase.

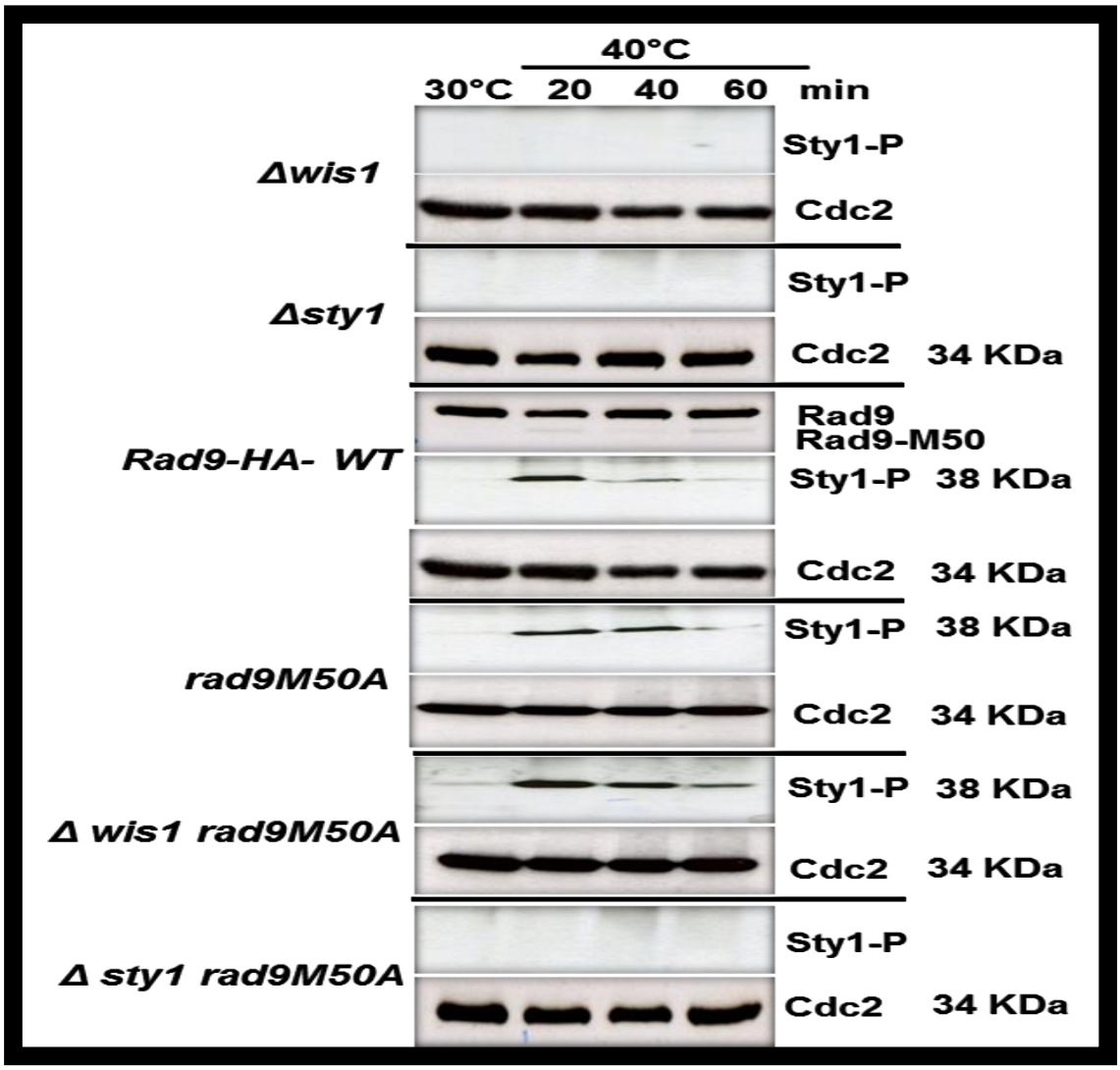


Figure 5- *7*: Loss of Rad9 variant restores the dual phosphorylation of Sty1 in *wis1* deletion cells but not in *sty1* deletion cells in the response to elevated temperature.

The indicated strains were grown at 30°C in YEA medium to 10^7 cells/ml. $5x10^8$ aliquot samples were incubated at 40°C in YEA medium and withdrawn at the indicated times. Total protein extracts were separated on a 10% SDS-PAGE and incubated with an anti-HA antibody to detect Rad9-HA, the anti-p38-phospho antibody to detect Sty1 phosphorylation and an anti-Cdc2 antibody to detect Cdc2 as a loading control.

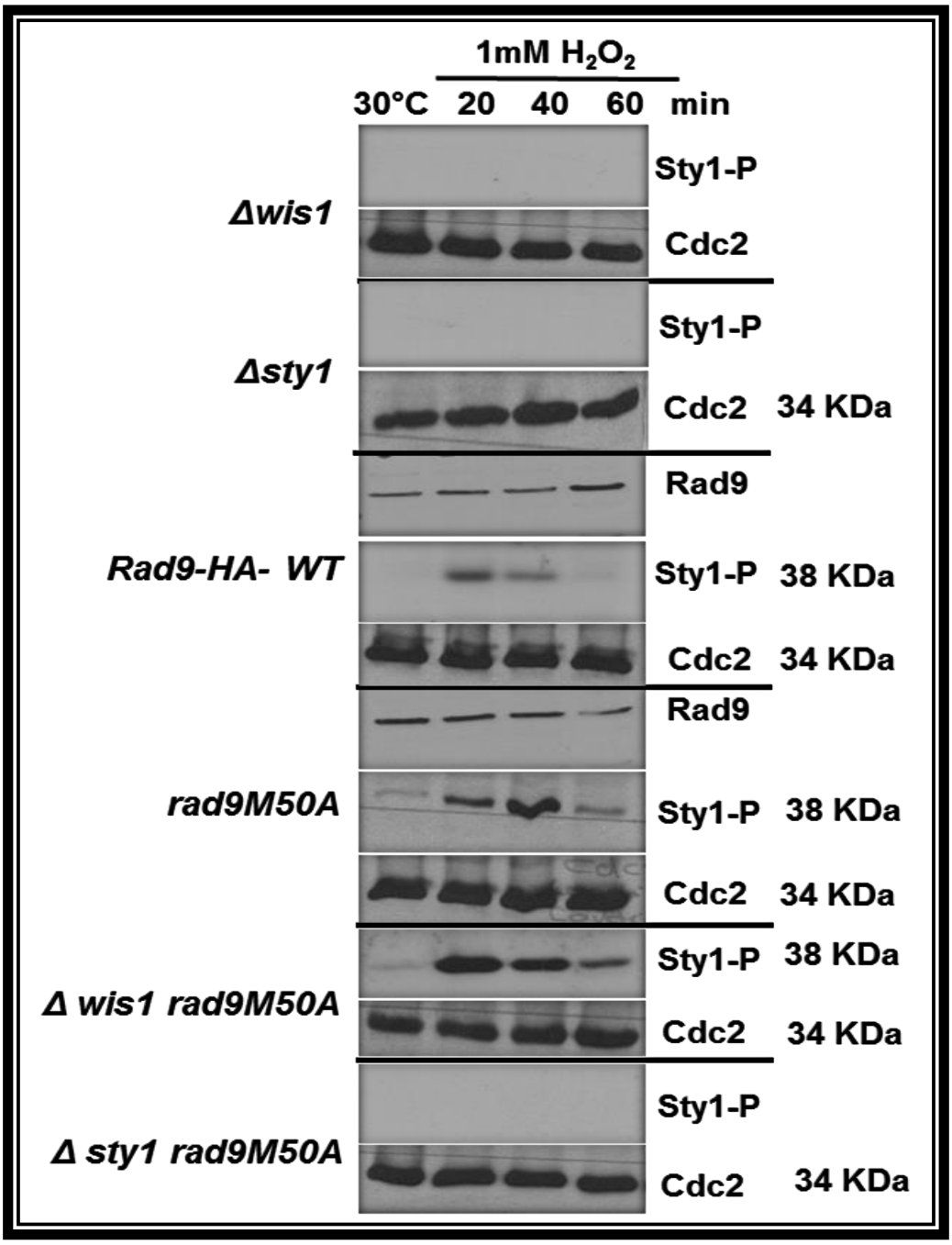


Figure 5- 8: Loss of Rad9 variant restores the dual phosphorylation of Sty1 in *wis1* deletion cells but not in *sty1* deletion cells in response to oxidative stress.

The indicated strains were grown at 30°C in YEA medium to 10^7 cells/ml. 5x10^8 aliquot samples were treated with 1mM H_2O_2 in YEA medium and withdrawn at the indicated times at 30°C. Total protein extracts were separated on 10% SDS-PAGE and incubated with an anti-HA antibody to detect Rad9-HA, the anti-p38-phospho antibody to detect Sty1 phosphorylation and an anti-Cdc2 antibody to detect Cdc2 as a loading control.

5.2.4 Loss of Rad9 variant partly restores Atf1 phosphorylation in the absence of Wis1

The Atf1 transcription factor is the main target of Sty1 and is phosphorylated at least 11 sites by the kinase *in vitro* and *in vitro* (Wilkinson, et.al. 1996; Gaits, et.al. 1998; Lawrence, et.al. 2007).

Since loss of Rad9 variant restores the dual phosphorylation of Sty1 in *wis1* deletion cells, I next asked whether this would extend to Atf1 in the absence of *wis1* under heat stress condition. To this end, the same strains (*rad9-HA-WT, rad9-M50A*, *Δwis1*, *Δsty1*. *Δwis1 rad9-M50A* and *Δsty1 rad9M50A)* were grown in YEA medium at 30°C overnight prior to a shift to 40°C. Sample were withdrawn every 20 minutes for one hour and subjected total protein extraction. Atf1 was visualised with an anti-Atf1 antibody. The mobility of the transcription factor was reduced in wild type cells at the 20 min time point after the temperature shift. This was in line with the dual phosphorylation of Sty1 at the same time point (Figure 5-9, first row). In line with the Sty1 modification, the phosphorylation-induced mobility shift of Atf1 lasted for 40 min in the absence of Rad9 variant (*rad9-M50A*) (Figure 5-9. Second row). In *wis1* deletion cells, Atf1 levels were lower than in wild-type cells and no shift was observed (Figure 5-9, third row). Loss of Rad9 variant in a *wis1* deletion background partly restored the mobility shift (Figure 5-9, last row) strongly suggesting that Sty1 is re-activated in this strain.

Given that the mobility shift was not very clear, I resorted to the use of Phos-tag SDS PAGE electrophoresis. Inclusion of this modified acrylamide reduces the mobility of phosphorylated protein in the presence of manganese ions (Kinoshita, et.al. 2006). A repeat of the earlier experiment using 8% Phos-tag SDS PAGE showed that Atf1 phosphorylation was dependent on Wis1 and Sty1 (Figure 5-10, first tow rows) as no slower migrating (phosphorylated) bands were detected. The main band of the unphosphorylated Atf1 transcription factor is around 60kDa. Interestingly, a few minor, slower migrating bands were visible in the absence of the MAP kinases implying that other kinases can also phosphorylate Atf1. In Rad9 wild type cells, Atf1 was highly phosphorylated after 20 minutes at 40°C before the reduced mobility decreased again (Figure 5-10, third row). Loss of Rad9 variant prolonged the phosphorylation of Atf1 again by 20 min in both, *rad9-M50A* single mutant and the *Δwis1.rad9-M50A* double mutant at 40°C (Figure 5-10, fourth & fifth rows). This result confirms that loss of Rad9 variant by-passes the requirement of Wis1 for the dual phosphorylation of Sty1 at Thr 171 and Tyr 173, and that this allows Sty1 to phosphorylate Atf1 under heat stress conditions. Consistent with

this conclusion, loss of Rad9 variant did not re-activate Atf1 phosphorylation in a *sty1* deletion background (*Δsty1 rad9.M50A*) (Figure 5-10, last row).

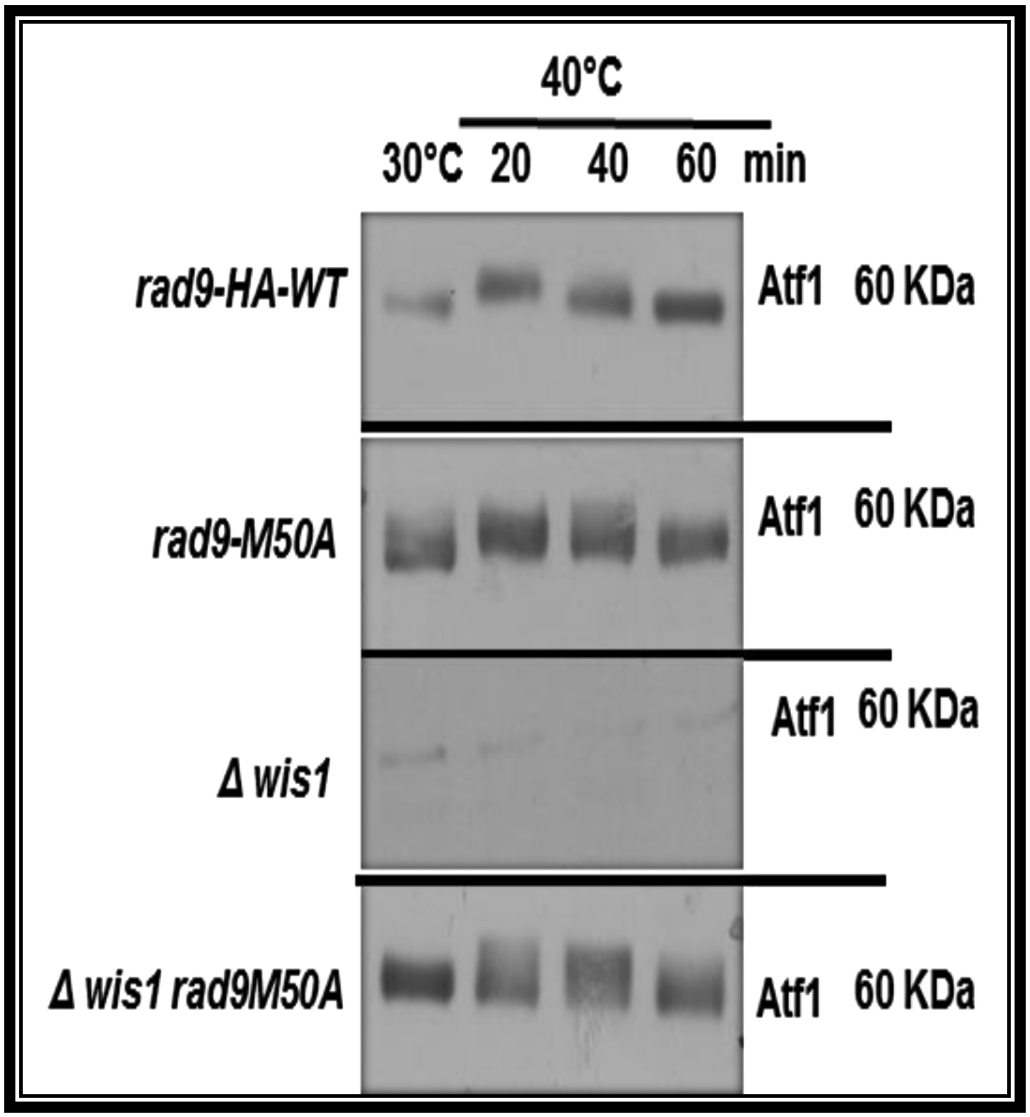


Figure 5- *9***: Atf1 phosphorylation in the response to heat stress.**

The indicated strains were grown in rich medium at 30°C to a cell number below 10^7 cells/ml. Aliquots (5×10^8 cells) were withdrawn and incubated in rich medium at 40°C for the indicated times. Total protein extracts were separated on a 8% SDS PAGE gel and detected with an anti-Atf1 antibody.

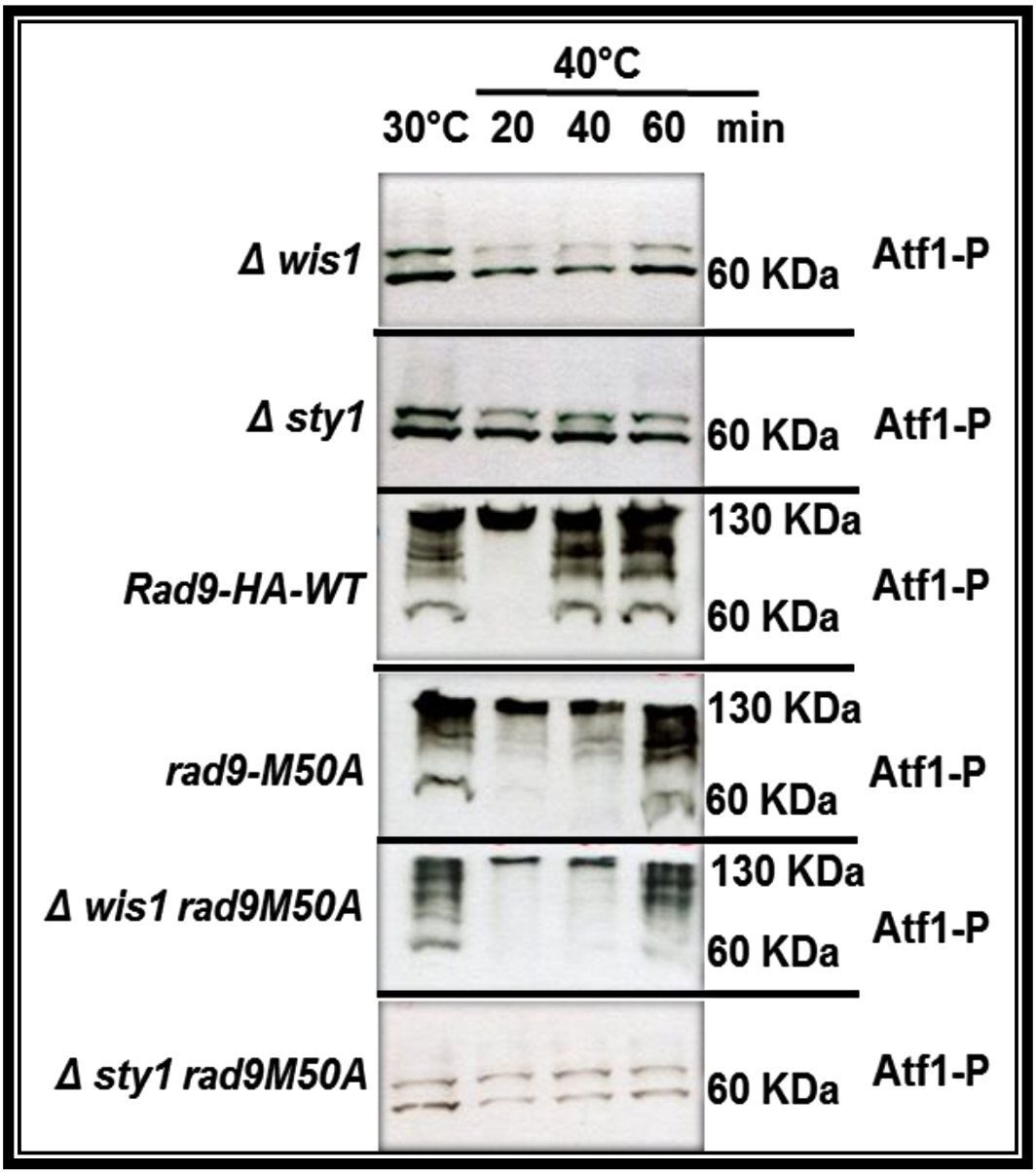


Figure 5- *10***: Loss of Rad9 variant prolongs the phosphorylation of Atf1 in *wis1* deletion but not in *sty1*deletion cells in the response to heat stress.**

Indicated strains were grown in rich medium at 30°C to a cell number below 10^7 cells/ml. Aliquots (5×10^8 cells) were withdrawn and incubated in rich medium at 40°C for the indicated times. Total protein extracts were separated on by 8% Phospho-tag SDS PAGE gel and detected with an anti-Atf1 antibody.

The same strains were grown in YEA medium at 30°C overnight and then treated with 1mM H_2O_2 at 30°C to find out whether the re-activation of ATF1 phosphorylation is specific to heat stress. Two slower migrating Atf1 bands were detected in strains either lacking Sty1 or Wis1 but no hyper-phosphorylation occurred (Figure 5-11, first two rows). As in the response to heat, Atf1 was highly phosphorylated at the 20 min time point in Rad9 wild type cells (Figure 5-11, third row), and for 40 min in the strains either lacking *rad9* variant (*rad9-M50A*) or in the

Δwis1.rad9-M50A double mutant (Figure 5-11, fourth & fifth rows). This indicates that loss of Rad9 variant by-passes the requirement of Wis1 for the dual phosphorylation of Sty1 at Thr 171 and Tyr 173, and that this allows Sty1 to phosphorylate Atf1 also under oxidative stress conditions. Consistent with this conclusion, loss of Rad9 variant did not re-activate Atf1 phosphorylation in a *sty1* deletion background (*Δsty1 rad9.M50A*) (Figure 5-11, last row).

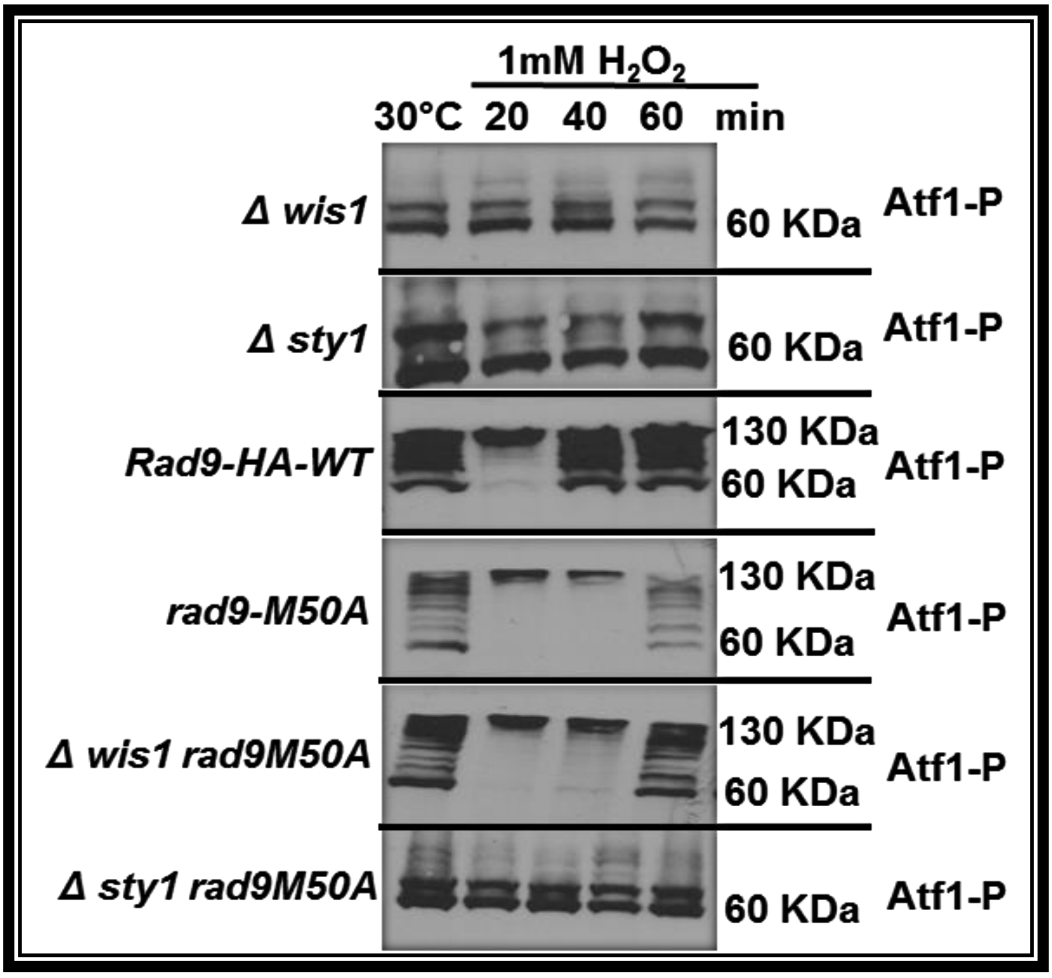


Figure 5- 11: Loss of Rad9 variant prolongs the phosphorylation of Atf1 in *wis1* deletion but not *sty1* deletion cells in response to oxidative stress.

Indicated strains were grown in rich medium at 30°C to a cell number below 10^7 cells/ml. Aliquots (5×10^8 cells) were withdrawn and incubated in rich medium with 1mM H_2O_2 for the indicated times at 30°C. Total protein extracts were separated on by 8% Phospho-tag SDS PAGE gel and detected with an anti-Atf1 antibody.

5.2.5 Loss of Ptc1 prolongs and increases activation of Sty1 upon heat stress

After a heat shock, the activation of Sty1 is attenuated by Ptc1 upon dephosphorylation of threonine 171 (Nguyen & Shiozaki, 1999). In Chapter 4, I report that Rad9 variant and Ptc1 phosphatase act in the same pathway. To obtain further evidence for this conclusion, I tested the phosphorylation of Sty1 in the absence of either Ptc1, Rad9 variant (*rad9-M50A*) or both proteins (*rad9-M50A* double mutant). All strains were grown in YEA medium at 30°C overnight before incubation at 40°C for one hour. Sample were taken every 20 minutes for 60 minutes and subjected to total protein extraction. The phosphorylation of Sty1 peaked at the 20 min time point in wild type cells and was no longer detectable after 40 min (Figure 5-12, first row). In contrast, loss of either Rad9 variant or Ptc1 resulted in a prolonged phosphorylation of Sty1 from 20 min to 60 min (Figure 5-12, second & third rows). Consistent with the epistatic relationship between both proteins, the *ptc1 rad9-M50A* double mutant showed a similarly extended modification of Sty1 although the initial phosphorylation was delayed by 20min (i.e. peak at 40 min instead of 20 min; Figure 5-12, last row). Given that Ptc1 dephosphorylates Sty1 at Thr-171 (Nguyen & Shiozaki, 1999), the prolonged modification of the MAP kinase could either indicate that Rad9 variant dephosphorylates Sty1 T171 or that a kinase becomes more active in the absence of the variant which can modify T171.

I next tested the phosphorylation of Atf1 in the same set of strains under the same conditions. As shown in Figure 5-13, the phosphorylation of Atf1 was high after 20 min in wild type cells before it declined rapidly. As in the case of the Sty1-T171 phosphorylation, either loss of Rad9 variant or loss of Ptc1 prolonged Atf1 hyper-phosphorylation by at least 20 min. The *ptc1 rad9-M50A* double mutant showed again a very similar phenotype. This was also consistent with the phosphorylation of Atf1 which was high for 40 min in the absence of either Rad9 variant or Ptc1 phosphatase (Figure 5-13). In summary, these data place Rad9 variant and Ptc1 phosphatase on the same regulatory circuit which controls T171 phosphorylation of Sty1 and indirectly Atf1 phosphorylation at elevated temperatures.

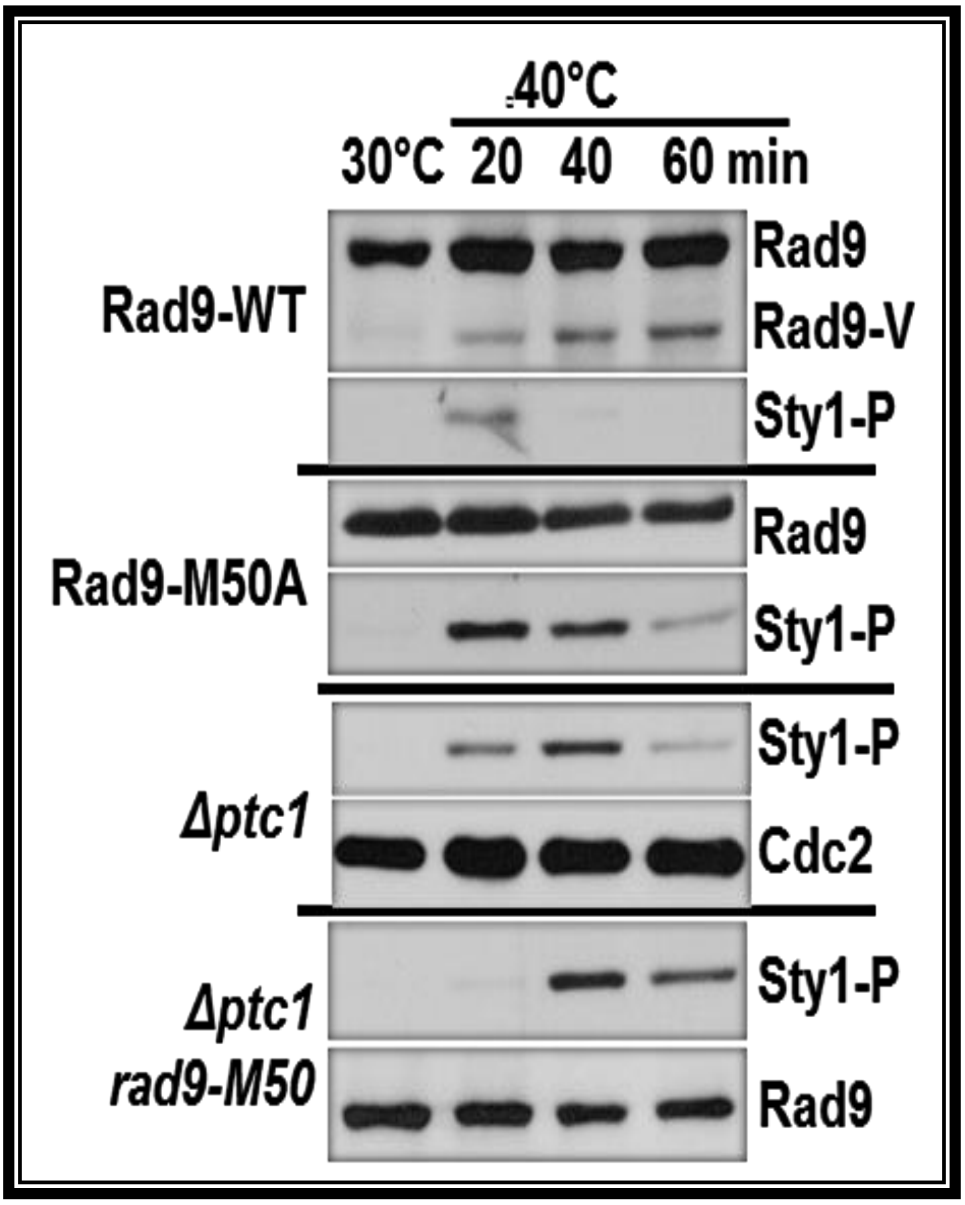


Figure 5- 12: Loss of Rad9 variant and Ptc1 phosphatase activates Sty1 in the response to heat stress.

The indicated strains were grown at 30°C in YEA medium to 10^7 cells/ml. $5x10^8$ aliquot samples were incubated at 40°C in YEA medium and withdrawn at the indicated times. Total protein extracts were separated on a 10% SDS-PAGE and incubated with an anti-HA antibody to detect Rad9-HA, the anti-p38-phospho antibody to detect Sty1 phosphorylation and an anti-Cdc2 antibody to detect Cdc2 as a loading control.

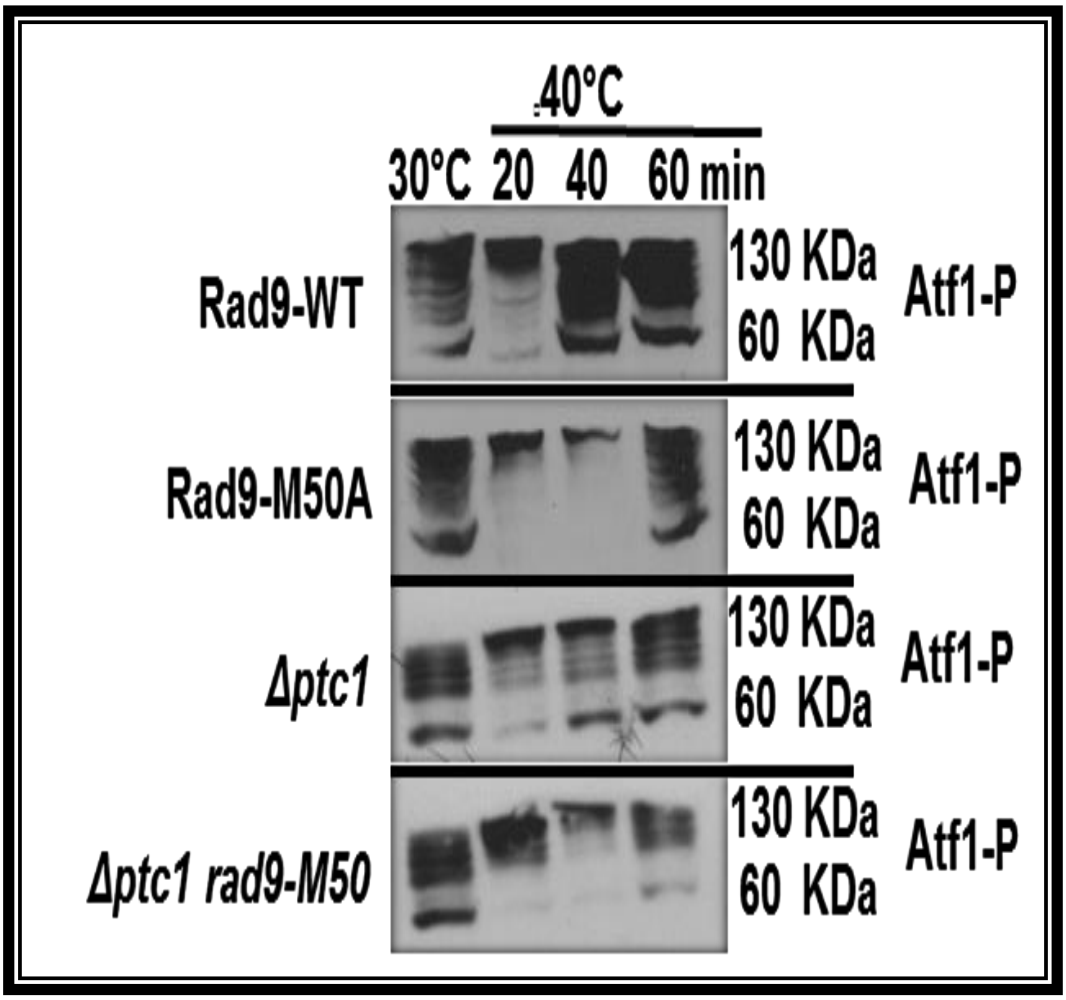


Figure 5- 13: Loss of Rad9 variant and Ptc1 phosphatase prolongs Atf1 phosphorylation at elevated temperatures.

The indicated strains were grown in rich medium at 30°C to a cell number below 10^7 cells/ml. Aliquots (5×10^8 cells) were withdrawn and incubated in rich medium at 40°C for the indicated times. Total protein extracts were separated on by 8% Phospho-tag SDS PAGE gel and detected with an anti-Atf1 antibody.

5.3 Discussion

A variety of stress conditions including heat, oxidative reagents and UV light activate the Wis1-Sty1MAPK pathway (Millar, et.al. 1995; Shiozaki & Russell, 1995; Degols, et.al. 1996; Degols & Russell, 1997; Shieh, et.al. 1997). Wis1 phosphorylates Sty1 at T171 and Y173 which in turn leads to the hyper-modification of the transcription factor Atf1 (Millar, et.al. 1995; Shiozaki & Russell, 1995a; Degols, et.al. 1996; Degols & Russell, 1997). The findings reported here reveal now that Rad9 variant can regulate the phosphorylation status of Sty1 independently of Wis1 (Figures 5-1, 5-2, 5-5 & 5-6). Loss of the variant prolongs Sty1 phosphorylation at high temperatures for up to 40 min in the absence of Wis1 (Figure 5-12)

which implies that the variant either dephosphorylates Sty1 or promotes its phosphorylation. This raises however the interesting question which alternative kinase could modify Sty1 in the absence of the variant in wis1 defective cells? Possible dual-specific kinases are Cki1 (SPBC1347.06c), Gsk3 (SPAC1687.15), Hhp1 (SPBC3H7.15), Hhp2 (SPAC23C4.12), Lkh1 (SPAC1D4.11c), Mik1 (SPBC660.14), Pek1 (SPBC543.07) or Wee1 (SPCC18B5.03). As explained later in the General Discussion, the most likely candidates are the DNA damage response kinases Wee1 and Mik1 (Lundgren, et.al. 1991), and the Casein Kinases (Hhp1, Hhp2) as they are also involved in the stress response (Dhillon & Hoekstra, 1994).

$Hhp1^{Ck1}$ Caseine kinase I is one of the dual-specific kinases that phosphorylates threonine and serine residues in yeast but not in human cells (Tuazon & Traugh, 1991; Flotow & Roach, 1991). $Hhp1^{Ck1}$ recognises substrates that contain Ser/Thr (PO4)-Xaa-Xaa- Ser/Thr motifs where the N-terminal serine or threonine has been already modified by a priming kinase (Flotow et.al. 1990). If Hhp1 (or Hhp2) is the dual-specific kinase which can substitute for Wis1 in the absence of Rad9 variant, it will need a priming kinase to recognise Sty1. This makes it less likely that either Hhp1 or Hhp2 act in the absence of Rad9 variant on Sty1.

Another good candidate and a well stablished dual-specific kinase is Wee1 since this kinase modifies threonine 14 and tyrosine 15 in Cdc2 kinase to regulate cell cycle progression (Den, et.al. 1995). Wee1 also physically interacts with Chk1 and is modified by Chk1 (O´Connell, et.al. 1997), and also associates with the tyrosine phosphate Pyp1 (Hannig, et.al. 1993).

The third good candidate is Mph1/TTK1 kinase which has a key role in the spindle assembly checkpoint by activating the Bub and Mad pathways (Hardwick, et.al. 1996; He, et.al.1998). Interestingly, Mph1 has a positive genetic interaction with Pyp1 phosphatase (Ryan, et.al. 2012). Table 5-1 summarises what is known about these kinases in the fission yeast *S. pombe*.

The re-activation of the Sty1-Atf1 pathway at elevated temperatures and under oxidative stress conditions by deleting Rad9 variant would explain why the *rad9-M50A* mutant is not heat sensitive although its expression is up-regulated (Janes et al., 2012).

Table 5- 1: The roles of Wee1, Hhp1 and Mph1 kinases in *S.pombe*.

Kinases	Functions	Reference
Hhp1	protein serine/threonine kinase activity	Hoekstra, et al. 1994 Petronczki, et.al. 2006
	peptidyl-serine autophosphorylation	Hoekstra, et.al. 1994
	peptidyl-threonine autophosphorylation	Hoekstra, et.al. 1994
	regulation of DNA repair	Dhillon, et.al. 1994
	homologous chromosome segregation	Petronczki, et.al. 2006
	signal transduction	GO_REF:0000051
Mph1	protein serine/threonine kinase activity	Shepperd, et.al. 2012
	peptidyl-threonine phosphorylation	Shepperd, et.al. 2012
	Bub1-Bub3 complex localization to kinetochore	Shepperd, et.al. 2012
	cellular protein localization with mad3 protein	Millband, et.al. 2002
	Meiosis & mitotic spindle assembly checkpoint	He, et.al. 1998 Kovacikova, et.al. 2013
	signal transduction involved in mitotic spindle assembly checkpoint	Shepperd, et.al. 2012
Wee1	protein serine/threonine kinase activity	Parker, et.al. 1992 Hannig, et.al. 1993
	mitotic DNA damage checkpoint	Rowley, et.al. 1992
	negative regulation of G2/M transition of mitotic cell cycle	Russell, et.al. 1987
	negative regulation of protein kinase activity by regulation of Cdc2 protein phosphorylation	Parker, et.al. 1992
	signal transduction involved in mitotic DNA damage checkpoint	Synnes, et.al. 2002

Chapter 6: General Discussion & Wider Impact of the Findings

This work revealed several novel findings about the cellular roles of the alternative translation product Rad9 variant in the fission yeast *Schizosaccharomyces pombe*. First, expression of Rad9 variant is induced in the response to methyl-methane-sulfonate (MMS) which alkylates DNA and not only at elevated temperatures as previously reported (Janes et al., 2012). Secondly, the Ded1 RNA helicase is required for the activation of the internal translational start site AUG-50 in the response to heat and MMS which produces Rad9 variant. The RNA helicase is also involved in the induction of the heat-inducible variant of Cds1 kinase (Chapter 3). Thirdly, the two phosphatases Ppa2 and Ptc1, but not Rad9varinat, dephosphorylate Chk1 at serine 345 in the response to heat stress (Chapter 4). Fourthly, loss of Rad9 variant rescues the heat sensitivity of a *ptc1* phosphatase mutant but increases the sensitivity of *dis2* and *pyp1* phosphatase mutants. This implies that Rad9 variant acts in the same heat response pathway as Ptc1 (Chapter 4). And finally, loss of Rad9 variant prolongs the activation of the MAP kinase Sty1/Spc1 in the presence of heat and oxidative stress in a manner independent of the upstream MAPK kinase Wis1 (Chapter 5).

Rad9 is a highly conserved protein that acts as a subunit of the Rad9-Rad1-Hus1 DNA clamp (Dore, et.al. 2009) which is loaded by Rad17 onto damaged DNA (O'Connell, et.al. 2000). Janes et.al. (2012) reported a new variant of Rad9 in the fission yeast *S.pombe* expression of which is induced by heat stress when ribosomes utilise the cryptic translation initiation codon methionine-50 (AUG50). The results presented here show that induction occurs also in the presence of the DNA alkylating agent methyl-methane-sulfonate (MMS) (Figure 3-1). This raises the intriguing question of why elevated temperatures and DNA alkylation induce both the variant? The common trigger could be DNA single-stranded breaks which accumulate during S phase at high temperatures (Velichko, et.al. 2012) or occur when the replication template is alkylated (Lundin, et.al. 2005). Since both treatments also block DNA replication (Groth, et.al. 2000), a general S phase arrest may provide an alternative signal that Rad9 variant acts in S phase which is supported by the mild sensitivity of the *rad9-M50A* mutant, which cannot synthesise the variant, to the high concentrations of the DNA replication inhibitor hydroxyurea (HU) (Figure 4-6).

The first interesting finding of the project is the dependency of Rad9 induction on the DEAD box RNA helicase Ded1 in the response to heat stress and MMS (Figure 6-1). The cryptic translational start site, AUG-50, may be part of a secondary structure in the mRNA which is resolved by Ded1 when the temperature changes from 30°C to 40°C, or when the DNA is alkylated (Figures 3-2 & 3-3). How Ded1 is activated under both conditions is as yet unclear. Ded1 is a well-known RNA helicase and RNA helicases are able to flatten out RNA secondary structures (Chuang, et.al. 1997; de la Cruz, et.al. 1997). This activity may indicate that ribosomes normally do not initiate translation at the internal AUG-50 codon since it is hidden in a secondary structure at low temperatures (Hinnebusch, 2014). This conclusion is consistent with the observation that Ded1 is an important translation initiation factor able to scan messenger RNA molecules for secondary structures (Chuang, et.al. 1997; de la Cruz, et.al. 1997). Ded1 was shown to be linked with the 43S ribosome to allow the scanning of mRNAs with long 5-UTRs structures (Berthelot, et.al. 2004; Firczuk, et.al. 2013). The requirement of Ded1 for this process is not limited to the internal initiation site AUG-50 in the *rad9* mRNA as the RNA helicase is also necessary to up-regulate the heat-inducible protein variant of Cds1 kinase (Figure 3-8; Fletcher and Caspari, unpublished). This implies that Ded1 can activate a larger number of protein variants in the response to heat and MMS.

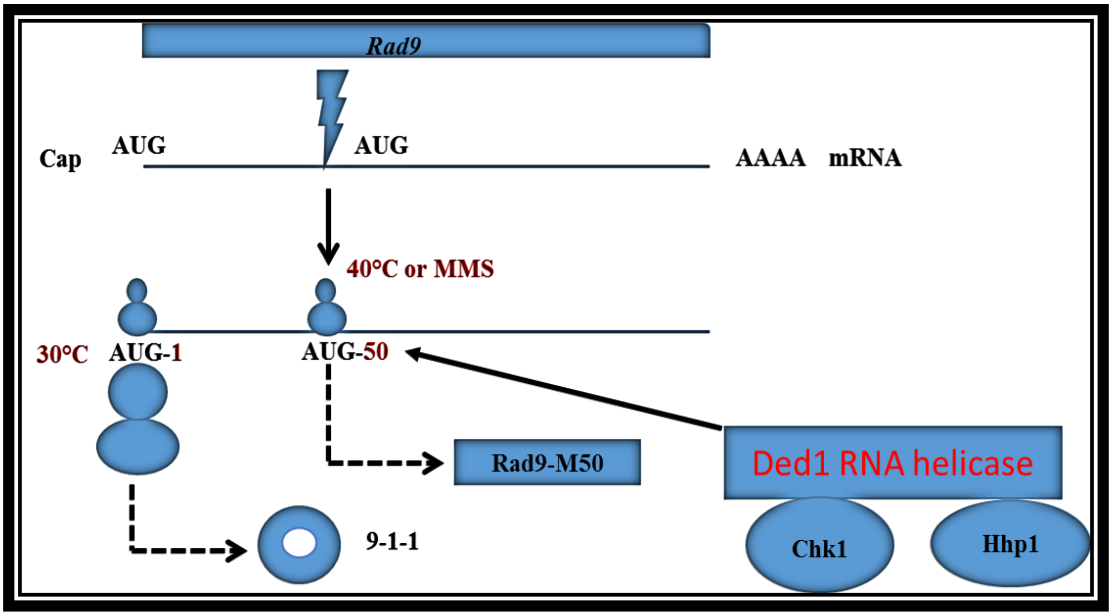


Figure 6- 1 : The *S.pombe* Ded1 RNA helicase is required for the induction of Rad9 variant in the response to heat and MMS treatment.

How Ded1 is regulated is not well understood. Casein kinase1 (Hhp1) may be involved in the transmission of the heat signal to Ded1 as Hhp1 physically interacts with Ded1 in yeast (Johnson, et.al. 2013). The human Ded1 orthologoue DDX3 and the Hhp1 orthologoue Casein Kinase 1 (CK1) were also shown to form a complex in the context of Wnt-β-catenin signalling (Cruciat, et.al. 2013). Interestingly, fission yeast cells without Hhp1 (*hhp1* deletion cells) are MMS sensitive (Dhillon & Hoekstra, 1994) which implies that Hhp1 may activate Ded1 also in the presence of alkylated DNA. Another DNA damage response protein which associates with Ded1 is Chk1 kinase (Liu, et.al. 2002). Given that the Rad3-Chk1 DNA damage signalling pathway is activated by MMS (Sommariva, et.al. 2005). Chk1 could also translate the MMS signal to Ded1 (Figure 6-1). Ded1 has not been shown to be modified in the response to DNA damage but it is phosphorylated at elevated temperatures (Liu, et.al. 2002). The genetic data shown in Figure 3-5 indicate that Ded1 may have a role in the response to DNA replication problems caused by hydroxyurea (HU) and MMS as the temperature-sensitive *ded1.1D5* mutant is sensitive to both drugs. This is interesting since the induction of Rad9 variant could also be linked to DNA replication problems. A DNA replication role of Ded1 is supported by the findings that the RNA helicase regulates the expression of the large (Cdc22) and small (Suc22) subunit of ribonucleotide reductase, the tetrameric enzyme that provides cells with dNTPs in the response to HU exposure (Sarabia, et.al. 1993) and heat stress (Harris, et.al. 1996).

6.1 Dephosphorylation of Chk1 at elevated temperatures is independent of Rad9 variant

The report by Janes and colleagues (2012) implied a role of Rad9 variant in the dephosphorylation of Chk1 at serine 345 at elevated temperatures as its induction correlated with the loss of the S345 modification. My work shows now that the heat-induced dephosphorylation of Chk1 is independent of Rad9 variant but requires the two serine/threonine phosphatases Ppa2 and Ptc1. Chk1 is phosphorylated by Rad3/ATR kinase at serine 345 in the response to DNA damage which can be detected as a mobility shift (Walworth, et al., 1993). It is well established that this modification activates Chk1 which results in a G2 cell cycle arrest (Capasso, et.al. 2002; Zou & Elledge, 2003). Under normal growth conditions (e.g. 30°C), the phosphorylation of Chk1 at S345 is removed by Dis2 phosphatase once the phosphorylation is no longer protected from dephosphorylation through its binding to the 14-3-3 proteins, Rad24 or Rad25 proteins and/or to Crb2/53BP1 (Heiko, 2003; Den & O'Connell,

2004; Dunaway, et.al. 2005). This Dis2-dependent dephosphorylation of Chk1 S345 coincides with the re-entry into the cell cycle after the damage-induced G2-M arrest is abolished (Den & O'Connell, 2004). The recovery signal which activates Dis2 is still unknown. Interestingly, Dis2 was not required under heat stress conditions where its activity was provided by the two phosphatases Ppa2 and Ptc1 (Figure 4-3). In both mutant strains, the DNA-induced S345 modification is removed but more slowly compared to a *dis2* deletion strain. This suggests a model (Figure 7-3) in which Dis2 acts on Chk1 at low temperatures whereas Ptc1 and Ppa2 act at elevated temperatures. Why a temperature shift from 30°C to 40° leads to the dephosphorylation of Chk1-S345 within 20 minutes is still unclear (Figures 4-2 & 4-3; Janes, et.al. 2012). One possible explanation could be that heat takes over as an effective trigger in stopping the cell cycle (Nitta, et.al. 1997) which would allow cells to switch of the DNA damage-induced arrest. Alternatively, activated Chk1 may be disadvantages for cells under heat arrest conditions as it interferes with important survival responses. Interestingly Ppa2 was previously shown to dephosphorylate Chk1 in human cells to maintain Chk1 in a low-activity state during an unperturbed cell cycle (Leung-Pineda, et.al. 2006). My genetic data show that Rad9 variant acts jointly with Ptc1 but not with Pyp1 (Figures 4-5 & 4-6). This may explain why induction of Rad9 variant coincides with the dephosphorylation of Chk1 (Janes et al., 2012). Since Ptc1 was previously shown to interact with Crb2 to which the activated Chk1 kinase binds (Das, et al., 2013), the scaffold protein Crb2 may be the missing link connecting the DNA damage-induced S345 phosphorylation with its heat-induced dephosphorylation (Figure 6-2). This further supported by the ability of Rad9 to bind to Crb2 via its phosphorylated tail domain which is also present in the Rad9 variant (Furuya, et.al. 2004). As discussed later this switch may be linked with an alternative regulation of the MAP kinase Sty1/Spc1.

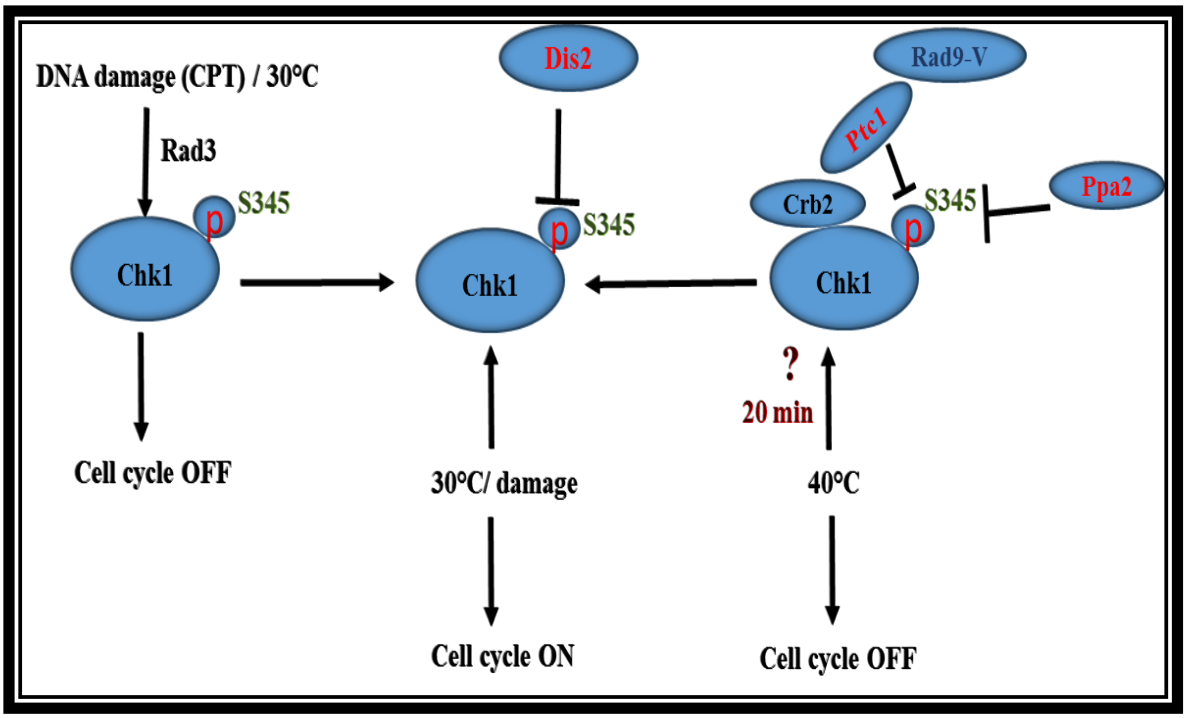


Figure 6- 2: The dephosphorylation of Chkl in the response to DNA damage and elevated temperatures.

6.2 Rad9 variant may shield the MAP kinase Sty1/Spc1 from an alternative dual-specific kinase other than Wis1

Since heat stress activates the MAP kinase pathway in yeast and human cells (Shiozaki & Russell, 1995; Dorion & Landry, 2002), I investigated the requirement of Rad9 variant for the regulation of the Wis1-Sty1-Atf1 MAP kinase pathway. Environmental factors including UV light, osmotic conditions, heavy metal, oxidative reagents and heat stress all promote the phosphorylation of the MAP kinase Sty1/Spc1 at Threonine 171 and Tyrosine 173 by the up-stream kinaseWis1 (Shiozaki & Russell, 1995; Nguyen & Shiozaki, 1999). Once activated, Sty1 modifies the transcription factor ATF1 to up-regulate genes aiding with cell survival (Shiozaki & Russell, 1996; Wilkinson, et.al. 1996). It is noteworthy that Ptc1 removes the modification from Sty1 at T171 to down-regulate the MAP kinase pathway (Aaron & Kazuhiro, 1999) and dephosphorylates Chk1 at S345 at elevated temperatures. Given the epigenetic link between Rad9 variant and Ptc1, both proteins may act jointly on Sty1 and Chk1.

Sty1 and Chk1 both converge on Cdc25 phosphatase to regulate mitotic progression in the response to DNA damage via Rad3 and in the presence of environmental stress via the MAP kinase Sty1 (Alao & Sunnerhagen, 2008; Sundaram, et.al. 2008). How the DNA damage and

environmental stress signals are integrated between both pathways is still enigmatic but the link between Rad9 variant and Ptc1 phosphatase may help to explore this important point. Since both pathways are major blocks of cancerous cell growth, a deeper insight into their interaction would be crucial to understand how normal cells transform into malignant tumours. So far only a few cross-over points between the DNA damage checkpoint and the MAP kinase system are known. For example, a functional interaction between the MAP kinases ERK1 and ERK2, and the DNA checkpoint kinase Chk2/Cds1 has been reported in large cell lymphomas (Dai, et.al. 2011).

As shown in Figure 5-7, loss of Rad9 variant prolongs the dual phosphorylation of Sty1 at T171 and Y173 at 40°C by approximately 20 minutes. Importantly, this prolonged phosphorylation is independent of the canonical upstream MAPK kinase Wis1. That Sty1 is genuinely activated under these conditions is supported by the 20 min extension of the heat-induced hyperphosphorylation of Atf1 at 40°C in cells without Rad9 variant, again independently of Wis1 (Figure 5-10). This Wis1-independent activation of Sty1-Atf1 signalling is not specific to heat stress as it also occurs in the response to oxidative stress (Figures 5-8 & 5-11). It is now very intriguing that loss of the phosphatase Ptc1 also extends the phosphorylation of Sty1 at T171 and Y173 (Figure 5-12) and prolongs the activation of Atf1 (Figure 5-13) at 40°C in a manner which is not further affected by loss of Rad9 variant. This is not only in line with the epigenetic relationship between Rad9 variant and Ptc1, but also implies that both proteins jointly regulate the MAP kinase. While Ptc1 dephosphorylates Sty1 kinase (Nguyen & Shiozaki, 1999), Rad9 variant may allow an alternative kinase to phosphorylate Sty1. The latter is suggested since Wis1 can be absent but Sty1 is still modified at T171 and Y173 in cells without Rad9 variant. This can only be explained if an alternative kinase gains access to Sty1 (Figure 6-3).

The number of alternative dual-specific kinases is small in the fission yeast genome and possible candidates are Hhp1, Hhp2, Wee1 and Mps1/Mph1/TTK1 kinase. Hhp1 and the closely related kinase Hhp2 are linked to the heat response (Hoekstra, et.al. 1994) and may regulate Ded1 RNA helicase, *wee1* deletion cells are heat sensitive (Aligue, et.al. 1994), and the mitotic kinase Mph1 co-fractionates with Rad9 variant (Caspari, unpublished data).

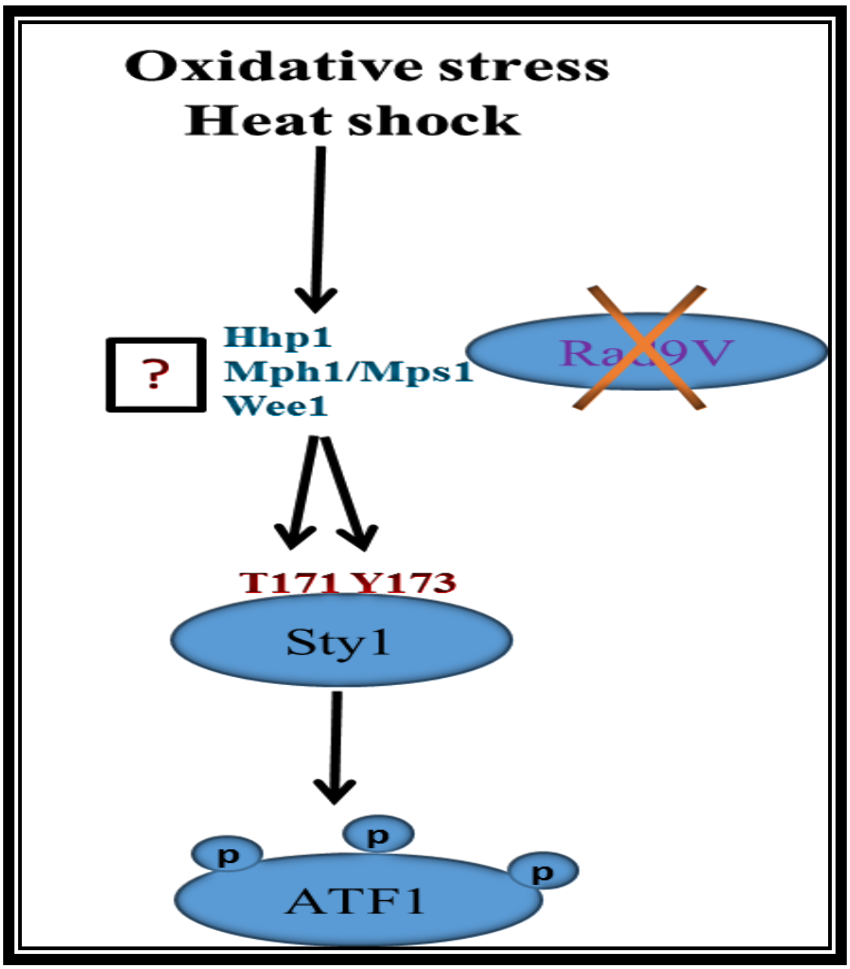


Figure 6- 3: Loss of Rad9 variant gives access to another dual-specific kinase (Hhp1, Mph1/Mps1 or Wee1) to phosphorylate Sty1 at T171 and Y173.

Another possible candidate could be the Wee1-related kinase Mik1 which regulates cell cycle progression specifically in S phase in the response to DNA damage including MMS (Lundgren, et.al. 1991; Christensen, et.al. 2000). Interestingly, like Wee1, Mik1 activity is dependent on the heat shock protein Hsp90 (Goes & Martin, 2001). Interestingly, tyrosine 15 in Cdc2 kinase which is the canonical target of Wee1 and Mik1 sits next to a threonine residue (T14-Y15) as in the case of Sty1 (T171-Y173) although no third amino acid separates the two in the case of Cdc2. Furthermore, Wee1 phosphorylates T14 and Y15 in Cdc2 (Den, et.al. 1995). It could therefore be possible for Wee1 or Mik1 to modify Sty1 in the absence of both Wis1 and Rad9 variant. The latter would indicate that the variant normally shields Sty1 from these alternative kinases.

An alternative explanation to the postulated involvement of Wee1, Mik1 and Mph1 could be provided by the other MAP kinase pathways in fission yeast. Byr1 and Pek1 are two additional MAP kinase kinases which, like Wis1, target MAP kinases. Although Byr1 acts in the sexual

differentiation pathway (Henkel, et.al. 2001) and Pek1 functions in the cell integrity pathway (Madrid, et.al. 2006), loss of Radf9 variant may allow another MAPKK to phosphorylate Sty1.

6.3 Wider Impact of the Findings

The most intriguing observation resulting from this work is the dual phosphorylation of the MAP kinase Sty1 in the absence of its up-stream partner Wis1 when Rad9 variant has been removed from cells. There is currently no similar observation in the literature. The reason why this could have a high impact is the importance of the MAP kinase pathway and the DNA damage checkpoint system for the suppression of cancerous growth. The human paralogue of Sty1 is p38, a MAP kinase which directly associates with the main tumour suppressor p53 (Chen, et.al. 2014). The second important connection between both pathways is the ability of heat to re-program the DNA damage response. For example, the detection of DNA breaks by the ATM-Chk2 pathway is strongly impaired at elevated temperatures, a feature which is used to sensitise tumours to radiotherapy (Turner & Caspari, 2014). The ability of Rad9 variant to regulate the MAP kinase pathway at elevated temperatures could be important in this context as full-length Rad9 is a key factor involved in the detection of broken chromosomes.

The MAP kinase pathway is a major barrier for cancerous cell growth. Inactivation of the p38 pathway promotes cancer formation in mice as it prevents cell death (Han & Sun, 2007). Conversely, persistent activation of p38 blocks uncontrolled cell growth but can render cancer cells drug resistant (Lidsky, et.al. 2014). Human p38 is also involved in cardiovascular disorders (Martin, et.al. 2015), respiratory disorders including Astma (Banerjee, et.al. 2012) and chronic pain (Lin, et.al. 2014). This illustrates how important it is to understand the regulation of p38 and its fission yeast paralogue Sty1. The role of elevated temperature in the regulation of p38 and Sty1 is important for two reasons. First, some cancers have slightly higher local temperatures compared to the surrounding tissue. For example, the temperature of bladder and breast tumours is up to 2°C higher (Lawson & Chughtai, 1963; Stefanadis, et.al. 2001). The second reason is concerned with the use of elevated temperatures to treat tumours (Rao, et.al. 2016). Under both conditions, temperature-induced changes to the MAP kinase pathway and to Rad9 may have direct implications for the outcome of the treatment or the growth of cancer cells. Future work is however required to study the response of human Rad9 to temperature stress. There are two human Rad9 genes, *Rad9A* and *Rad9B*, of which Rad9A

encodes three protein coding variants and Rad9B four protein variants (Ensembl database, accessed 07 December 2016) (Dufault, et.al. 2003). Which of the variants is most closely related to the fission Rad9 variant Rad9.M50 is not clear yet, but variant RAD9B-001 (ENST00000409246.5) shares an N-terminal deletion with Rad9.M50.

Further research could aim to test whether Wee1 or Mps1 kinases are able to interact with Sty1 and to phosphorylate T171 and Y173 in the absence of Rad9 variant. It would also be interesting to find out whether the variant binds to another kinase and where in the cell the variant is?

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