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The effect of mitochondrial deficiency on DNA damage response mutants of Saccharomyces cerevisiae

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I confirm that I am submitting this work with the agreement of my Supervisor.

Signed:

Date:

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ABSTRACT

The main aim of the project is to investigate the impact of mitochondrial processes on the survival of DNA damage response pathway mutants. This is useful to know as if it can be determined that a lack of functioning mitochondria affects the DNA damage response it could provide a convenient raison d'être for the Warburg effect. The Warburg effect being an altered metabolism seen in human tumours where cells have an increased uptake of glucose and a switch to glycolysis even when there is ample oxygen that would allow for the glucose to broken down for energy by oxidative phosphorylation. In the experiments *Saccharomyces cerevisiae* was utilised as a model. This was done because *Saccharomyces cerevisiae* can survive without its mitochondria, has protein homologues of the human DNA damage response proteins, and has a short generation time allowing for cultures to be performed relatively quickly.

There were two hypotheses that were tested. The first was that the removal of mitochondria directly impacts the function of the DNA damage response pathway lessening the impact of mutations in this pathway and thus promoting survival. The second, based on initial findings from spot tests, was that the removal of the mitochondria reduces the total amount of reactive oxygen species in the individual *Saccharomyces cerevisiae* cells and impairs apoptosis so that when proteins in DNA damage response pathway are mutated and generating reactive oxygen species the lower baseline amount of reactive oxygen species and the impaired apoptotic pathways prevent the death of the cell.

The first thing done in the project was the generation of *Saccharomyces cerevisiae* strains which lacked mitochondria, termed petite strains. This was done using ethidium bromide treatment. These strains were confirmed as petite using YPG plates as petite strains are unable to utilise glycerol as an energy source. Once confirmed as petite these strains were used in spot tests on plates containing drugs at various concentrations. These spot tests showed interesting improvements in survival on hydroxyurea containing media for *mec1-4* and *dun1Δ* strains when petite. Also of note was the fact that there was no improvement in survival for the petite *rad53-K227A* strain. This is interesting as Rad53 lies in between Mec1 and Dun1 in the DNA damage response pathway which threw doubt on the first hypothesis explanation that the survival of the DNA damage response mutants is due to the loss

of mitochondria affecting the functioning of DNA damage response pathway. The *mec1-4* and *dun1Δ* strains also showed differences in their temperature sensitivities when petite. Overall, the results of the spot tests and previous studies led to the development of the second hypothesis that the survival was based on levels of reactive oxygen species.

The next thing which was looked at was the status of Rad53 and Sml1 in the petite and non-petite *mec1-4* and wildtype strains when exposed to hydroxyurea. This was done by analysing Western blots for Rad53 and Sml1 from protein extracts. The results of these Western blots complicated the picture regarding the way in which the DNA damage response is impacted necessitating further research to find the reasons behind the apparent differences seen in the phosphorylation of Rad53 and Sml1.

The final experiment investigated whether there were significant differences in reactive oxygen species between untreated strains and hydroxyurea treated strains and between petite and non-petite strains. This experiment utilised the dye DCFDA which reacts with reactive oxygen species to form a fluorophore which was detected using a FACS machine. The results of this experiment suggested that there was no substantial difference in the levels of reactive oxygen species between the petite and non-petite *mec1-4* strains.

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LIST OF ABBREVIATIONS

Acyl-CoA: Acyl coenzyme A

ADP: Adenine diphosphate

AMP: Adenosine monophosphate

APS: Ammonium persulphate

ATP: Adenine triphosphate

Atg1: Autophagy-related 1

ATM: Ataxia-telangiectasia mutated serine/threonine kinase

ATR: Ataxia-telangiectasia and Rad53-related protein

ATRIP: ATR interacting protein

AZC: L-Azetidine-2-carboxylic acid

Bcl-2: B-cell lymphoma 2

Bis-Tris: Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane

Chk2: Checkpoint kinase 2

DCFDA: Dichlorodihydrofluorescein diacetate

Ddc2: DNA damage checkpoint signaling 2

DNA: Deoxyribonucleic acid

dNTPs: Deoxyribonucleotide triphosphates

Dun1: DNA-damage uninducible

FACS: Fluorescence-activated cell sorting

Ggc1: Guanosine diphosphate/ guanosine triphosphate carrier 1

H_2O_2: Hydrogen peroxide

Hst3: Homolog of Sir2 3

HU: Hydroxyurea

Lys7: Lysine requiring 7

MCT1: Monocarboxylate transporter 1

MCT4: Monocarboxylate transporter 4

Mec1: Mitosis entry checkpoint 1

MHR1: Mitochondrial homologous recombination protein 1

MMS: Methyl methanesulphonate

mTORC1: Mammalian target of rapamycin complex 1

NAC: N-acetyl-L-cysteine

NAD: Nicotinamide adenine dinucleotide

NADPH: Dihydronicotinamide-adenine dinucleotide phosphate

Ntg1: Endonuclease 3-like glycosylase

p53: Tumor suppressor p53

Pol3: DNA polymerase III

Rad53: Radiation sensitive 53

Rfx1: Regulatory factor X 1

RNR: Ribonucleotide reductase complex

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulphate

Sir2: NAD-dependent deacetylase sirtuin 2

Sml1: Suppressor of Mec1 lethality

Snf1: Sucrose non-fermenting 1

Sod1: Superoxide dismutase 1

S phase: Synthesis phase

TCA: Trichloroacetic acid

TBS: Tris buffered saline

TBST: Tris buffered saline with tween 20

TCS2: Tuberous Sclerosis Complex 2

Tel1: Telomere maintenance 1

TEMED: Tetramethylethylenediamine

Tris: Tris(hydroxymethyl)aminomethane

Tris HCL: Tris (hydroxymethyl)aminomethane hydrochloride

YPD: Yeast peptone dextrose

YPG: Yeast peptone glycerol

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1. INTRODUCTION

The following introduction will first cover the human cancer related proteins ATM and ATR and their homologues in *Saccharomyces cerevisiae* before going on to discuss how these proteins are linked to oxidative stress in cells. After this the current knowledge on mitochondria and the Warburg effect is presented before discussing the reasons for using *Saccharomyces cerevisiae* as a model organism. This is followed by a summary of all of the drugs which were used during the experiments and their known effects upon cells with a focus on hydroxyurea. The introduction ends with a presentation of the aims of the project, the hypotheses and how they were investigated, and what these investigations appeared to show.

1.1. ATM, ATR and their yeast homologues

In humans the proteins ATM and ATR have roles in the repair of DNA damage, checkpoint responses induced by DNA damage and other replication stresses, and in the normal functioning of meiosis (Savitsky et al. 1995; Harper & Elledge, 2007; Jeggo et al. 2016).

The autosomal recessive disorder ataxia-telangiectasia is caused by mutations in ATM and affects multiple systems in the human body including DNA repair mechanisms and the reproductive system (Savitsky et al. 1995). One of the symptoms of ataxia-telangiectasia is a higher incidence of cancer and somatic mutations of ATM are associated with cancer (Choi et al. 2016). It is because of this that research has been undertaken to determine the functions of ATM and ATR (Savitsky et al. 1995).

ATM and ATR are evolutionarily conserved (Falck et al. 2005; Blackford & Jackson, 2017). In the yeast *Saccharomyces cerevisiae*, the homologues of ATR and ATM are the serine-threonine kinases Mec1 and Tel1 which have overlapping functions (Harper & Elledge, 2007; Jeggo et al. 2016).

Mec1 is considered to be the homologue of ATR because it has greater structural similarity and it forms a complex with a protein called Ddc2 which is similar to the complex ATR forms with ATRIP (Wang et al. 2017). This complex is composed of two Mec1 proteins and two Ddc2 proteins (Wang et al. 2017). Tel1 is considered to be the homologue of ATM (Cassani et al. 2019). The removal of Mec1 or ATR with

no other modifications results in inviability. In *Saccharomyces cerevisiae* this is due to the suppression of the following pathway; Mec1 phosphorylates Rad53 in response to single strand and double strand breaks in DNA, the phosphorylated Rad53 then phosphorylates the protein Dun1 which then phosphorylates Sml1 causing it to be broken down (Zhou & Rothstein, 2002). Sml1 is an inhibitor of ribonucleotide reductase which catalyses the rate limiting step in the production of dNTPs necessary for DNA repair and thus if it is not removed the cell cycle cannot progress and DNA damage cannot be repaired (Danielsson et al. 2008) There are, however, problems associated with complete removal of Sml1 as the high dNTP levels cause a greater number of mutations to occur in the genome under normal conditions and at sufficiently high levels the ability of the cell to enter S phase is inhibited (Andreson et al. 2010).

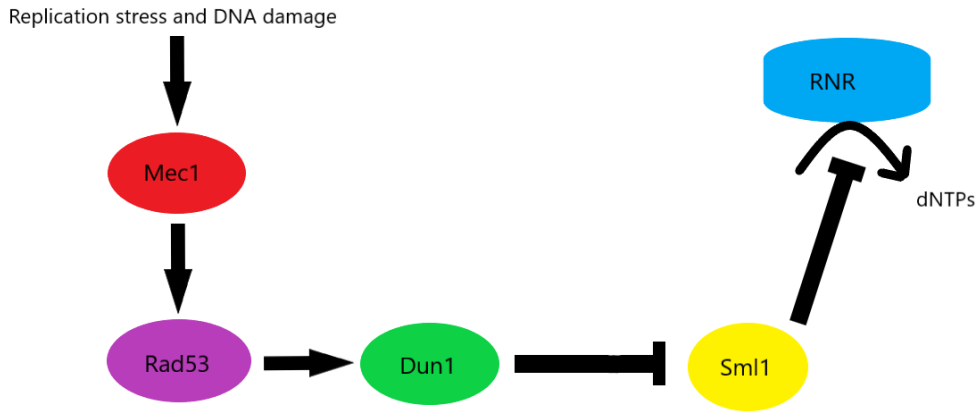

Figure 1. Depiction of the Mec1 to RNR pathway. Mec1 phosphorylates Rad53 which in turn phosphorylates Dun1. Dun1 then phosphorylates Sml1 causing it to be broken down which relieves the inhibition of the dNTP production of RNR by Sml1.

The reason for using *dun1Δ* mutants in the experiments was to determine whether the changes in growth seen in the Mec1 mutants were due to Dun1 not being activated or were due to the other downstream effects of Mec1. Dun1 is a paralog of the human protein Chk2 (Harper & Elledge, 2007). As mentioned above Dun1 phosphorylates Sml1 when activated but this is far from Dun1's only role. Dun1 also

phosphorylates a number of transcription factors including Rfx1 which represses transcription of genes encoding subunits of the ribonucleotide reductase (Yam et al. 2020).

The *mec1-4* mutant is caused by a missense allele which is characterised by its sensitivity to a number of different factors including increased temperature, hydroxyurea, and methyl methanesulphonate (Corcoles-Saez et al. 2018). The response to an increased temperature is protein aggregation, DNA double-strand break formation, and permanent replication fork stalling leading to cell death (Cha & Kleckner, 2002; Corcoles-Saez et al. 2018). This occurs, however, alongside a relatively small reduction in the abundance of dNTP which suggests that the reason for lethality in Mec1 mutants is not related to the production of dNTPs by the ribonucleotide reductase (Earp et al. 2015; Corcoles-Saez et al. 2018).

The *rad53-K227A* mutant has a single amino acid substitution at position 227 of the Rad53 protein from lysine to alanine which causes the protein to have no kinase activity (Fay et al. 1997).

1.2. ATM and oxidative stress

Hydrogen peroxide, a reactive oxygen species, catalyses the formation of a disulphide ATM dimer proving a link between the DNA damage response and oxidative stress (Finkel, 2011).

The reactive oxygen species damage to DNA can activate the ATM mediated DNA damage response in humans with ATM also activating p53-mediated transcription of autophagy genes and the tumour suppressor TCS2 (Alexander et al. 2010; Filomeni et al. 2015). ATM has been shown to activate the tumour suppressor TSC2 through the liver kinase B1-AMP-activated protein kinase pathway in the presence of high levels of reactive oxygen species which suppresses mTORC1 which induces autophagy (Alexander et al. 2010). The AMP-activated protein kinase has a *Saccharomyces cerevisiae* homologue Snf1 and so a similar pathway may exist in *Saccharomyces cerevisiae* and so before the experiments it was speculated that the growth of Tel1 and Mec1 dead mutants may be less affected by mitochondrial removal having already removed one of the ways in which reactive oxygen species can activate autophagy (Yi et al. 2017).

Lack of functioning ATM has been shown to increase oxidative stress in mouse models with associated changes in antioxidant and protective enzymes and potential imbalances in signalling pathways (Gage et al. 2001; Barzilai et al. 2002). This is thought to be due to elevated reactive oxygen species but also decreased cysteine levels caused by impaired transport and increased utilisation in the production of glutathione (Kamsler et al. 2001; Barzilai et al. 2002). ATM-deficient cells also display spontaneous apoptosis while also having impaired apoptosis activation in response to DNA damage (Elson et al. 1996; Barzilai et al. 2002).

ATM deficiency also results in a build-up of unrepaired DNA damage (Barzilai et al. 2002; Choi & Chung, 2020). Double strand breaks in the DNA lead to continuous activation of poly-ADP ribose polymerase which depletes the cellular pool of NAD (Barzilai et al. 2002; Choi & Chung, 2020). In otherwise normal cells this may increase oxidative stress as the reduced form of NAD acts as an antioxidant (Kirsch & de Groot, 2001; Barzilai et al. 2002). However, the conversion of oxidised NAD to reduced NAD is connected to the Krebs cycle and thus requires the cell to be utilising its mitochondria normally and so the removal of mitochondria or the Warburg effect would lead to a build-up of oxidised NAD and a depletion of reduced NAD. This may increase oxidative stress. In *Saccharomyces cerevisiae* NAD is required for the proteins Sir2 and its homologue Hst3 to perform their histone deacetylation functions in response to genotoxic stress (Thaminy et al. 2007; Choi & Chung, 2020).

ATM has also been shown to increase the mitochondrial DNA copy number of cells when activated (Niu et al. 2012). It does this independently of human mitochondrial transcription factor A, which is believed to be one of the main regulators of mitochondrial DNA copy number (Niu et al. 2012). The increase in mitochondrial DNA copy number was also shown to likely not be due to a delay in cell cycle progression itself but due to increased reactive oxygen species levels (Niu et al. 2012). This was because the presence of antioxidants completely suppressed the increase in mitochondrial DNA copy number (Niu et al. 2012). An increased mitochondrial DNA copy number in the presence of reactive oxygen species is also seen in *Saccharomyces cerevisiae* (Hori et al. 2009). While the replication of mitochondrial DNA is initiated by the proteins Ntg1 and Mhr1 the downstream effects of Mec1 on ribonucleotide reductase are necessary for the replication of

mitochondrial DNA as it increases the amount of dNTPs available (Hori et al. 2009). Therefore, it was theorised that the lack of mitochondria may increase the amount of dNTPs available to repair nuclear DNA damage in petite strains under oxidative stress. A large-scale genetic screen has also provided evidence which suggests that defects in the DNA damage response pathway lead to an increased mitochondrial DNA copy number due to an increase in available dNTPs caused by a continually activated pathway (Puddu et al. 2019).

Another function of Mec1 outside the DNA damage response pathway is the maintenance of mitochondrial respiration in glucose deprivation in which it is recruited as part of a complex including Snf1 which phosphorylates Mec1 (Yi et al. 2017). This maintenance of mitochondrial respiration is necessary under glucose deprivation conditions for the initiation of autophagy (Yi et al. 2017). The autophagy itself is not initiated by Mec1 but by Atg1 which forms part of the complex recruited to mitochondria (Yi et al. 2017). Therefore, certain Mec1 mutations may prevent the initiation of autophagy in response to reactive oxygen species and also reduce the amount of reactive oxygen species due to less generation by mitochondrial respiration. Removal of mitochondria may also affect the initiation of autophagy as the loss of mitochondria means that there are no sites for the Snf1-Mec1-Atg1 complex to form (Yi et al. 2017).

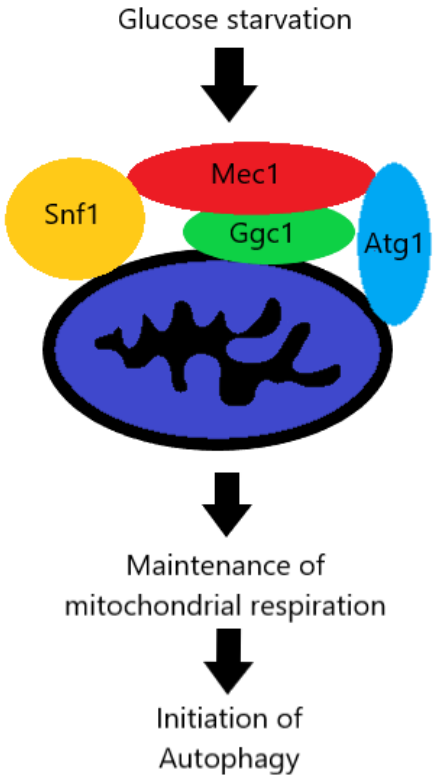

Figure 2. Depiction of how glucose starvation leads to initiation of autophagy. The Snf1-Mec1-Atg1 complex is recruited to the mitochondria at the site of the adaptor protein Ggc1. Mec1 is recruited first and is then phosphorylated by Snf1. This leads to recruitment of Atg1 and the maintenance of mitochondrial respiration leading to the initiation of autophagy as a response to glucose starvation. This image was based off of the graphical abstract of Yi et al. 2017.

1.3. Mitochondria and cellular metabolism

Mitochondria are the location of the electron transport chain and the Krebs cycle (Ernster & Schatz, 1981; King et al. 2006). Both of which are part of oxidative phosphorylation which produces the majority of the ATP in mammalian cells with ample oxygen (King et al. 2006). Mitochondria are major sources of reactive oxygen species and thus oxidative stress (Oyewole & Birch-Machin, 2015). These reactive oxygen species also act as signalling molecules which can activate transcription factors (Thannickal & Fanburg, 2000). These transcription factors may include tumour necrosis factor (Thannickal & Fanburg, 2000). High levels of endogenously generated reactive oxygen species can activate the process of mitophagy leading to

the destruction of defective mitochondria which are generating reactive oxygen species (Filomeni et al. 2015). Most reactive oxygen species from mitochondria are generated at complex I and complex III of the electron transfer chain (Finkel, 2011).

Succinate dehydrogenase and fumarate hydratase are mitochondrial proteins which are involved in the Krebs cycle (King et al. 2006). If they are mutated succinate or fumarate accumulates in the mitochondria before eventually leaking into the cytoplasm where they inhibit prolyl hydroxylase enzymes which induces a pseudo-hypoxic environment and possibly suppresses apoptosis (King et al. 2006). Loss of succinate dehydrogenase function may also increase the amount of reactive oxygen species present in the cell as it also has a role in the electron transport chain (King et al. 2006).

Apoptosis is linked to the mitochondria in humans by both proapoptotic and antiapoptotic Bcl-2 factors being recruited to the outer mitochondrial membrane which controls the release of cytochrome C from mitochondria which activates caspases and begins apoptosis (Vyas et al. 2016). Mitochondria are also involved in cellular signalling as stress sensors with metabolites generated by mitochondria affecting signalling pathways and protein activity as well as modifying histone acetylation and thus gene expression (Vyas et al. 2016).

Mitochondrial inheritance is non-Mendelian, due to the existence of a separate mitochondrial genome, and is controlled by a complex of proteins which control the attachment of the mitochondria to actin cables during cell division (Vevea et al. 2014). Mitochondria continuously fuse and divide to maintain their functionality and changes to the mechanisms which control these processes can lead to dysfunctional mitochondria and tumorigenesis (Vyas et al. 2016).

Mitochondria have their own ribosomes to translate mitochondrial RNA transcripts into proteins (Johnston & Williams, 2016). The small number of proteins it produces are primarily part of electron transfer chain complexes whose genes are retained in the mitochondrial genome (Johnston & Williams, 2016).

Mitochondria are also involved in the beta oxidation of fatty acids (Houten & Wanders, 2010). This process requires a several stage transfer of the fatty acids through the mitochondrial outer membrane as well as the conversion of acyl-CoA into acylcarnitine and then its reconversion to acyl-CoA and degradation to acetyl-

CoA (Houten & Wanders, 2010). This degradation produces electron carriers which then enter the electron transfer chain (Houten & Wanders, 2010)

1.4. Warburg effect

One of the most common characteristics of cancer cells in humans is a modified glucose metabolism termed the Warburg effect (King et al. 2006; Lu et al. 2015). It consists of an increased uptake of glucose and an increase in glycolysis even in the presence of sufficient oxygen for normal aerobic respiration involving the Krebs cycle and oxidative phosphorylation (Warburg et al. 1956; Hsu & Sabatini, 2008; Lu et al. 2015). This decrease in oxidative phosphorylation decreases the amount of reactive oxidative species produced as by-products and thus helps to inhibit apoptosis (Lu et al. 2015). Glycolysis also generates metabolites which can be used to synthesise nucleotides, amino acids, lipids, and NADPH (Lu et al. 2015). The increased concentrations of nucleotides, amino acids, and lipids speed up cell division and the NADPH which is generated by the pentose phosphate pathway is a cofactor for glutathione (Lu et al. 2015). Glutathione is a potent antioxidant and thus the increased levels of glutathione act to inhibit apoptosis and can also provide resistance to anti-cancer drugs (Balendiran et al. 2004; Lu et al. 2015). Glutathione acts by being converted from its reduced state to its oxidised state by glutathione peroxidase to convert hydrogen peroxide into water before being transported out of the cell (Baker et al. 1993; Bachhawat & Yadav, 2018). Outside the cell oxidised glutathione can be broken down by membrane bound γ-glutamyl transpeptidase (Bachhawat & Yadav, 2018). Glutathione biosynthesis is ATP dependent and requires glutamate, cysteine, and glycine (Bachhawat & Yavdav, 2018). The Warburg effect may also be linked to reduced expression of the mitochondrial pyruvate carrier genes which encode a complex composed of two proteins which mediates efficient uptake of pyruvate, the product of glycolysis required for the Krebs cycle, into mitochondria (Schell et al. 2014).

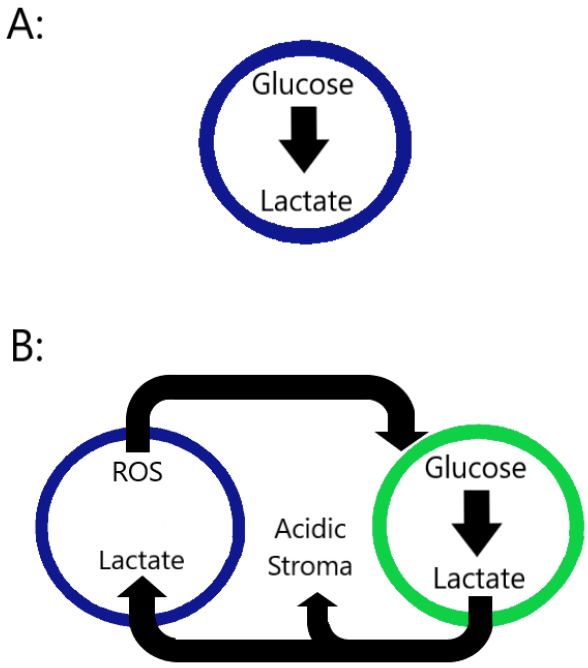

Figure 3. Comparison of the Warburg effect (A) and the reverse Warburg effect (B). In the Warburg effect the cancer cell itself switches to aerobic fermentation. In the reverse Warburg effect the switch to aerobic fermentation of glucose takes place in cancer-associated fibroblasts which have been primed to do so by reactive oxygen species from the cancer cell. The fibroblast then exports the lactate produced which is either taken up the cancer cell as an energy source or contributes to acidifying the tumour microenvironment.

Another hypothesis for how the Warburg effect occurs is the reverse Warburg effect (Martinez-Outschoorn et al. 2011; Benny et al. 2020). This hypothesis states that the Warburg effect actually happens in the cancer-associated fibroblasts instead of the cancer cells with the cancer-associated fibroblasts exporting the lactate they produce as the end product of their aerobic glycolysis after being primed by reactive oxygen species released from the cancer cells (Martinez-Outschoorn et al. 2011; Benny et al. 2020). This lactate in the stroma contributes to the generation of the tumour microenvironment as the lactate increases the acidity of the tumour microenvironment which can speed up tumour progression and proliferation (Benny et al. 2020). Some of this lactate is then imported by the cancer cells which use the lactate as their primary energy source. This necessitates the use of the Krebs cycle and therefore requires functional mitochondria (Benny et al. 2020). This model has

been demonstrated experimentally and has led to new treatment developments for cancer targeting the lactate importers and exporters, MCT-1 and MCT-4 respectively (Martinez-Outschoorn et al. 2011; Benny et al. 2020). The reverse Warburg effect model can affect the way in which the results of the experiments are interpreted due to the fact that the cancerous cells *in vivo* may have perfectly functioning mitochondria with the tumour microenvironment being generated by the associated cells whereas the DNA damage response mutants used in the *Saccharomyces cerevisiae* model to represent cancerous cells will have the modified metabolism themselves due to the destruction of their mitochondria. This will mean that they will only be able to use anaerobic respiration.

1.5. Yeast model system

Saccharomyces cerevisiae is a Crabtree positive yeast which means that even in the presence of enough oxygen *Saccharomyces cerevisiae* utilises fermentation instead of aerobic respiration when on glucose-containing media (De Deken, 1966, Pfeiffer & Morley, 2014). This has much lower levels of ATP being produced than if aerobic respiration was being utilised (Pfeiffer & Morley, 2014). Aerobic respiration requires the mitochondria, where the Krebs cycle and the electron transport chain occur, and can be utilised by *Saccharomyces cerevisiae* when non-fermentable carbon sources are present to produce energy (Diaz-Ruiz et al. 2011). This means importantly that *Saccharomyces cerevisiae* can survive without its mitochondria and combined with the fact that it is non-pathogenic and has a short generation time this makes it a very useful model (Goldring et al. 1971; Menzes et al. 2015). As mentioned above it also has homologues of the human checkpoint proteins ATM and ATR which allow the results from the testing of *Saccharomyces cerevisiae* to be used to assess how the DNA damage response in humans may function and due to the rapid growth time of *Saccharomyces cerevisiae* a large number of tests can be performed in a short period of time (Harper & Elledge, 2007; Menzes et al. 2015; Jeggo et al. 2016).

Petite strains are *Saccharomyces cerevisiae* strains which lack mitochondria (Goldring et al. 1971). Ethidium bromide treatment inhibits the incorporation of thymidine into mitochondrial DNA causing irreversible damage and removal but does not significantly damage the nuclear DNA (Naas, 1970). This allows for petite strains completely lacking mitochondria to be generated therefore allowing the Warburg

effect to be mimicked and its relation to the DNA damage response to be investigated as *Saccharomyces cerevisiae* can survive using fermentation alone (Diaz-Ruiz et al. 2011). Due to the non-Mendelian inheritance of mitochondria and their separate genome all descendants of the petite strains generated will lack mitochondria (Goldring et al. 1971; Vevea et al. 2014).

Petite strains due to their lack of mitochondria are unable to utilise non-fermentable substances such as glycerol as they can only be broken down via glycolysis the products of which require the mitochondrial processes of the Krebs cycle and oxidative phosphorylation to produce energy (Goldring et al. 1971; Jin et al. 2013). Therefore, the petite status of a strain can be confirmed by its inability to grow on media containing glycerol as the sole energy source (Goldring et al. 1971).

1.6. Drugs used to provide different stresses for testing

Reactive oxygen species are potent oxidants which can cause irreversible damage to proteins and DNA (Davies, 1987; Imlay & Linn, 1988; Filomeni et al. 2015). There are multiple methods by which reactive oxygen species can damage DNA (Filomeni et al. 2015). Guanine is the most susceptible base and its reaction with •OH can generate 8-hydroxyguanine which is incorporated into DNA as 8-hydroxydeoxyguanosine. This is especially carcinogenic as it can pair with cytosine and thymine which does not lead to single strand breaks that would activate the DNA damage response (Filomeni et al. 2015). Other effects of reactive oxygen species such as DNA protein crosslinks are more easily detected and can stimulate autophagy and ultimately cell death (Filomeni et al. 2015). Mitochondrial DNA is also susceptible to reactive oxygen species and the results can be much more damaging as it can lead to adherent synthesis of the complexes of the electron transfer chain which leads to an even greater amount of reactive oxygen species being generated (Filomeni et al. 2015).

In terms of protein damage, the hydroxyl radical (•OH) can cause protein aggregates which impede cell functions (Davies, 1987). Detection of these aggregates ultimately leads via various pathways to the removal of the aggregates by proteolytic enzymes (Davies, 1987).

The reactive oxygen species hydrogen peroxide in cells is also essential for growth factor signalling and has been shown to have roles in a number of other pathways

including apoptosis and cell proliferation (Veal et al. 2007; Finkel, 2011). Hydrogen peroxide can cause oxidative damage by producing hydroxyl radical via the Fenton reaction in cells (Linley et al. 2012). Hydrogen peroxide media was therefore one of the stresses which was used in the tests to simulate high levels of reactive oxygen species. This allowed it to be determined whether the strains generated with the petite mutation had possibly had their signalling pathways involving reactive oxygen species disrupted. It also allowed for the potential differences in the base level of reactive oxygen species to be investigated with FACS.

Hydroxyurea is a drug first synthesised in 1869 and is a hydroxylated analogue of urea (Kettani et al. 2009; Huang et al. 2016; Singh & Xu, 2016; Vinhaes et al. 2020). It is used in the treatment of various disorders including sickle cell anaemia and malignancies such as chronic myeloid leukaemia (Huang et al. 2016; Singh & Xu, 2016). Hydroxyurea was first demonstrated to have an effect on DNA metabolism in the 1960s and further studies have determined its specific roles *in vivo* (Young & Hodas, 1964; Huang et al. 2016; Singh & Xu, 2016). Endogenous hydroxyurea has also been found in low levels in humans but its exact role has not been determined (Kettani et al. 2009; Singh & Xu, 2016).

The primary target of hydroxyurea is the RNR (Nordlund & Reichard, 2006; Davies et al. 2009; Singh & Xu, 2016). This complex is essential for DNA repair and replication as it catalyses the conversion of ribonucleotides to deoxyribonucleotides (Krakoff et al. 1968; D'Angiolella et al. 2012). There are three different class of RNRs (Singh & Xu, 2016). Both humans and the yeast *Saccharomyces cerevisiae* have class I RNRs (Singh & Xu, 2016). Class I RNRs are composed of two dimeric subunits R1 and R2 (Nyholm et al. 1993; Singh & Xu, 2016). Hydroxyurea inhibits class I RNRs by transferring an electron to a tyrosyl radical in the R2 subunit (Nyholm et al. 1993; Singh & Xu, 2016). Loss of this radical prevents the RNR from catalysing the reduction of ribonucleotides to deoxyribonucleotides (Singh & Xu, 2016). Hydroxyurea can also affect the reduction or oxidation of other elements and compounds including iron in the R2 subunit which it reduces from Fe^{3+} to Fe^{2+} (Nyholm et al. 1993). This reduces stability and causes the loss of iron from R2 (Nyholm et al. 1993). Hydroxyurea's effect on RNR leads to replication fork stalling and prevents cell cycle progression (Singh & Xu, 2016). This ability to prevent cell division and its induction of replication stress is the reason for hydroxyurea's use in

treating malignancies alongside other more targeted drugs. The presence of hydroxyurea may also lead to greater transcription of antioxidant genes due to increased levels of reactive oxygen species (DeNicola et al. 2011; Vinhaes et al. 2020).

Further evidence of the relationship between oxidative stress and hydroxyurea comes from studies in *Saccharomyces cerevisiae* strains which were modified to lack either SOD1 or LYS7 genes (Carter et al. 2005). Sod1 is copper-zinc superoxide dismutase a homodimeric enzyme which is one of three superoxide dismutases in humans (Milani et al. 2011; Sea et al. 2015). Lys7 is the copper loading chaperone of Sod1 (Carter et al. 2005). The strains lacking either of these proteins showed oxygen dependent sensitivity to hydroxyurea and other DNA damaging agents (Carter et al. 2005). This provides some evidence that the damage caused by hydroxyurea is caused by its ability to induce the formation of reactive oxygen species (Carter et al. 2005). This is supported by evidence that the antioxidant NAC supresses the effects of hydroxyurea in the same null strains (Carter et al. 2005). NAC does not, however, supress the hydroxyurea sensitivity of *mec1Δ sml1Δ* mutants (Carter et al. 2005).

Iron-Sulphur centres are metallic cofactors which have roles in maintaining the normal function of redox reactions in associated proteins (Huang et al. 2016). Hydroxyurea has been shown to decrease the levels of Iron-Sulphur centres *in vivo* but not *in vitro* which suggests that its effects on Iron-Sulphur centres are associated with a metabolic reaction (Huang et al. 2016). Overexpression of genes involved in the production Iron-Sulphur centre associated proteins increases resistance to hydroxyurea (Huang et al. 2016). Among the proteins which contain Iron-Sulphur centres are DNA polymerases such as Pol3 in *Saccharomyces cerevisiae* thus providing another way in which hydroxyurea can affect DNA replication (Netz et al. 2012; Huang et al. 2016). It also explains how hydroxyurea can affect DNA replication despite the basal pool of dNTPs not being fully depleted (Huang et al. 2016).

The effects of Iron-Sulphur centre loss can also be linked to an observation in *sod1Δ* strains of decreased NADPH levels and oxidation of the Iron-Sulphur centre of homoaconitase, which is necessary for its reactions, leads to blockage of the

pentose phosphate pathway which leads to reduced production of amino acids (Slekar et al. 1996; Carter et al. 2005).

The strains were also tested using media containing AZC. AZC is a homologue of the amino acid proline (Trasko et al. 1976; Trotter et al. 2002). This means that AZC can become incorporated into proteins in the place of proline which can cause protein misfolding (Trasko et al. 1976; Trotter et al. 2002). This can affect a wide range of different proteins and thus these misfolded proteins can result in various pathways and processes within the cell becoming dysfunctional (Trasko et al. 1976; Trotter et al. 2002).

Another chemical it was decided would be used to test the mitochondrial mutants was MMS. MMS methylates guanine and adenine (Lundin et al. 2005). This causes base mispairing and can halt replication which activates the DNA damage response (Lundin et al. 2005). Therefore, by testing mitochondrial mutants with MMS the effect of the mutations on the DNA damage response could be determined.

Chemical added to media with range of concentrations	Primary target	Mechanism of action
Hydrogen peroxide (H_2O_2) 1mM – 12.5mM	Proteins and DNA	Oxidation of the target causing either damage and/or activating the protein's effect within a signalling pathway
Hydroxyurea (HU) 2mM - 200mM	Class I RNRs	Transferring an electron to a tyrosyl radical in the R2 subunit of class I RNRs inhibits production of dNTPs and thus DNA replication and repair
L-Azetidine-2-carboxylic acid (AZC) 0.3mg/ml - 0.7mg/ml	Proteins containing proline	Incorporation into new proteins in place of proline resulting in protein misfolding
Methyl methanesulphonate (MMS) 2µl/50ml – 25µl/50ml	Guanine and adenine	Methylation of the DNA bases leads to replication fork stalling

Table 1. Summary of the chemicals used in testing and their primary mechanism of action upon their targets.

1.7. Aims of the project

The overall aim of the project was to investigate possible reasons for the Warburg effect in cancer cells. This was to be done by creating and comparing mitochondria lacking versions of *Saccharomyces cerevisiae* strains which had modifications in various proteins in the DNA damage response pathway which are homologous to proteins implicated in cancer. The first preliminary test for these strains was to perform spot tests on media with various drugs and noting any differences.

The results of the preliminary spot tests resulted in observations that the lack of mitochondria improved the survival of two strains on media containing hydroxyurea. These strains were *mec1-4* mutant and the ***dun1Δ sml1Δ*** mutant. These observations led to two hypotheses being developed and tested.

The first hypothesis was that the increased survival was due to the lack of mitochondria directly impacting the function of the DNA damage response pathway and in doing so improving the poor ability of the DNA damage response mutants to repair damage caused by exposure to hydroxyurea containing media. This was investigated in the petite and non-petite *mec1-4* mutated strains using Western blots for both Rad53 and Sml1 including some Western blots for Rad53 involving Phos-tag reagent. The phosphorylation of Rad53 and the lack of Sml1 due to breakdown being considered as markers of DNA damage response activity.

The second of the hypotheses, based on preliminary findings, was that the removal of the mitochondria reduces the total amount of reactive oxygen species in the individual *Saccharomyces cerevisiae* cells and impairs apoptosis so that when *mec1-4* mutation is present and generating reactive oxygen species the lower baseline amount of reactive oxygen species and the impaired apoptotic pathways prevent the death of the cell. That is to say that the poor survival of the mitochondria containing strains is due to the *mec1-4* or ***dun1Δ*** mutations causing a greater level of oxidative stress in the cell outside of their functions in the DNA damage response. This was tested using DCFDA, a dye which forms a fluorophore when oxidised. The fluorescence being measured using a FACS machine.

2. METHODS

2.1. Generation of petite strains

Wildtype and strains with the mutations in the DNA damage response pathway, for which it was wanted to test the effect of the mitochondria upon, were cultured overnight at 23ºC in YPD liquid (2% Peptone, 1% Yeast extract, 2% Glucose). Parts of these cultures were then diluted the following day in YPD liquid with a concentration of Ethidium bromide of 10µg/ml and incubated at 23ºC for 2 hours. 100µl of each culture was then spread onto a separate YPD media plate (2% Peptone, 1% Yeast Extract, 2% Glucose, 2% Agar). These plates were incubated 25ºC for 2 days before being replica plated onto both YPD and YPG (2% Peptone, 1% Yeast Extract, 2% Glycerol, 2% Agar) plates which were incubated at 25ºC for 3 days. The colonies on the different plates were compared with those which did not grow on the YPG plate being taken as being petite colonies from which were obtained initial populations of petite strains.

Strain name	Background	Markers
Wildtype (WT)	BY4741	his3Δ1; leu2Δ0; met15Δ0; ura3Δ0
mec1-kd	BY/SK1	MEC1::mec1-kd1 sml1::hphMX4 his3
mec1-4	SK1	ho::LYS2, lys2, ura3, leu2::hisG, ade2::LK, his4x, arg4N, mec1-4-ADE2
tel1Δ	S288C	tel1Δ::KanMX
tel1Δ mec1-4	S288C	mec1-4::NAT, tel1Δ::KanMX
rad53-K227A	BY/SK1	rad53K227A-URA3 his3
dun1Δ	BY4741	ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; dun1::kanMX4

Table 2. List of strains for which petites were created

2.2. Spot tests

Strains were grown in YPD liquid (2% Peptone, 1% Yeast extract, 2% Glucose) overnight at either 25ºC or 30ºC depending on if the spot test involved temperature

sensitive mutants. The optical densities of the cultures were then measured at 600nm and the range of cultures were normalised so that equivalent amounts of cells were plated before being diluted in a series of one tenth dilutions. The drug plates themselves were YPD based (2% Peptone, 1% Yeast Extract, 2% Glucose, 2% Agar) with the drugs being added just prior to pouring them. YPG plates (2% Peptone, 1% Yeast Extract, 2% Glycerol, 2% Agar) were also used in the spot tests to confirm whether strains were petite. The strains grown overnight were single colonies taken from replica plates of streaks for single colonies. These replica plates were YPD and YPG plates replicated from an initial streak on YPD to ensure that the strains being tested were petite and non-petite respectively. Non-petite strains were taken from the YPG replica plate and petite strains were taken from the YPD replica plate as they did not grow on the YPG replica plate confirming they were petite.

2.3. TCA Extraction

Cells were grown in YPD liquid overnight. The optical densities of the various strains were taken and (incorrectly) used to ensure equal amounts of cells were added to 30ml of YPD liquid but not properly adjusted to ensure that cells would be in the logarithmic growth phase when the hydroxyurea was added. These 30ml cultures were allowed to grow at 25^{o}C for 3 hours before hydroxyurea was added to half the culture at a concentration of 50mM. After 1 hour and 30 minutes further culturing at 25^{o}C the cells were pelleted. Protein extracts were precipitated with the use of 10% TCA solution, glass beads, and centrifugation and the pellets were resuspended in 2X Laemmli loading buffer (4% SDS, 20% glycerol, 10% beta-mercaptoethanol, 0.125 M Tris HCl and 0.004% bromophenol blue). Tris was added to these resuspensions to restore the Laemmli buffer's pH. This was done by eye as the colour changed back to a deep blue colour from the yellow colour it took on when the protein extracts were added. The samples were heated on a 70^{o}C hotplate for 5 minutes before being centrifuged with the supernatant being retained.

2.4. Western blots

Western blot analysis was performed on TCA extracts. After boiling, samples were loaded into 8% polyacrylamide gels for detection of Rad53 or 15% polyacrylamide gels for detection of Sml1. These gels were composed of two layers the 8% or 15% separation gel and a layer of 5% stacking gel with wells into which the samples and

the ladder, with visually tagged proteins of known molecular weight, were added. The separation gels were created from de-ionised water, 30% acrylamide solution, SDS-PAGE separation buffer (which contained 90 grams of Tris base and 2 grams of SDS per 500ml), 10% APS solution, and TEMED. Both the 8% and 15% gels contained 0.001% of both APS and TEMED but different volumes of de-ionised water and the 30% acrylamide solution to dilute the acrylamide to the desired concentration. The stacking gel added after the separation gel had set was created in the same manner but contained SDS-PAGE stacking buffer (which contained 30.24 grams of Tris base and 2 grams of SDS per 500ml) instead of the SDS-PAGE separation buffer. The use of different amounts of Tris base and adjustment with hydrochloric acid led to the buffers having two different pH values, 8.8 for the separation buffer and 6.8 for the stacking buffer.

These gels were run until the nearest band of the ladder to the molecular weight reached near the bottom of the gel. In the case of Sml1 this was almost always accomplished by running the gel at a constant voltage of 100 volts for 10 minutes before the voltage was increased to 120 volts for a further 1 hour and 30 minutes. The amount of time taken for the Rad53 gels was much more variable in one case taking over 3 hours at 120 volts. The buffer used for the running of these gels contained 3 grams of Tris base, 18.8 grams of glycine, and 10ml of 10% SDS solution per litre.

The proteins were transferred from the gel onto nitrocellulose using tank electrophoresis at a constant current of 250 mA for 1 hour in transfer buffer that was 20% methanol with 3 grams of Tris and 14.5 grams of glycine per litre. Washing of the membrane was performed using TBST (produced by adding 0.05% Tween 20 to 1X TBS which I diluted from a stock 10X TBS solution with a pH of 7.9 which contained 24.2 grams of Tris and 175.2 grams of Sodium chloride per 2 litres). Following this the membrane was washed with water before 0.1% Ponceau S solution was added to the membrane for 5 minutes. After this the Ponceau S solution was removed and stored for further use and the membrane was washed with water approximately 3 times until the background staining was removed. Following photographing of the Ponceau stained membrane a 0.2M Sodium hydroxide solution was added to membrane to remove the Ponceau staining before the membrane was washed with water 3 more times. Blocking was then done with a 5% weight by

volume solution of milk powder dissolved in TBST. The addition of primary and secondary antibodies was done in this milk powder and TBST blocking solution. The primary antibody was incubated with the membrane overnight before being washed 3 times with TBST for 5 minutes the following day before the secondary antibody was added in fresh blocking solution and left to incubate for 1 hour. After this the membrane was washed in the same manner as after the primary antibody, the membrane was then placed between two sheets of laminating sheets and Western Lightning ECL Pro developing solution, mixed just prior, was added. The blots were then imaged using the Chemiluminescence blot setting in a BioRad Chemidoc imaging system.

The primary antibody used for detection of Rad53 was the mouse monoclonal antibody EL7E1 from abcam (ab166859). The primary antibody used for the detection of Sml1 was a rabbit polyclonal antibody from Agrisera (AS10 847). The secondary antibody for the detection of Rad53 was the horseradish peroxidase linked goat anti-mouse IgG (heavy and light chain) antibody from abcam (ab6789). The secondary antibody for the detection of Sml1 was the horseradish peroxidase linked goat anti-rabbit IgG (heavy and light chain) antibody from Cell Signalling Technology (#7074). The antibodies were added to the blocking solution at a concentration of 1µl/3ml. The total amount of blocking solution was 15ml.

Phos-tag is a molecule developed at Hiroshima University which binds in a selective manner to phosphate monoester dianions which can be conjugated to acrylamide and as such can be used in the production of gels for Western blots (Kinoshita et al. 2022). These gels create a much greater separation of proteins as the phosphorylated forms of the protein that is probed for with antibodies will not move as far through the gel during electrophoresis as the non-phosphorylated form of the protein due to the interaction between Phos-tag and the phosphate groups attached to the protein (Kinoshita et al. 2022). This has the effect of slowing the movement of the protein through the gel resulting in multiple separate bands depending on the number of phosphate groups that the protein has (Kinoshita et al. 2022).

When performing Phos-tag Western blots 7% polyacrylamide gels were used to assess Rad53 phosphorylation. These gels were different from the gels used in the other Western blots as while they used the same 30% acrylamide solution diluted

with de-ionised water and the same concentrations of APS and TEMED they used Bis-Tris buffer instead of the SDS-PAGE separation and stacking buffers that had been used in the previous Western blots. This Bis-Tris buffer was created by taking 150ml from a 5X stock solution containing 30.3 grams of Tris base and 52.3 grams of MOPS per 500ml and adding 7.5ml of a 10% SDS solution and 7.5ml of a 0.5 mol/L Sodium bisulphite solution. In addition, 40µl of the Phos-tag reagent and 80µl of zinc chloride necessary for the increased separation of phosphorylated Rad53 from unphosphorylated Rad53 were mixed into every 10ml of polyacrylamide gel produced.

These Phos-tag gels were run in parallel with a Bis-Tris containing gel which had the same samples as well as a ladder. This is due to the fact that the ladder cannot be used with the Phos-tag reagent. The same method for determining when to stop running the gels as was used for the other Western blots where the end point was determined by the position of the bands of the ladder was used for the Bis-Tris and Phos-tag gels run in parallel. The voltages used for separation of the samples in the Bis-Tris and Phos-tag gels were the same as those used in the other Western blots. Transfer of these gels onto nitrocellulose was also performed in the same way with the addition of 1% SDS to the transfer buffer and the addition of antibodies and imaging were also the same.

2.5. Image analysis of gels

Image analysis of gels was performed using ImageJ software so that differences in protein loading could be accounted for. This analysis involved comparing the ratios of the greyscale intensity of the bands on the Ponceau stain to the ratios of the intensity of the bands on the Western blot. This was done by measuring the intensity of the greyscale Ponceau stained band and inverting the value given by ImageJ (by subtracting it from the maximum possible value of 255). The intensity of the background of each lane was then also measured and inverted in the same way. The background value was then subtracted from the value derived from the Ponceau stained band to give a net intensity value for each lane. The same process was carried out on the Western blot with intensities of the band and background being measured and inverted to give a net intensity value for each lane. The net intensity

value of the band in each lane on the Western blot was then divided by the value for the corresponding lane from the Ponceau stain.

2.6. Measurement of reactive oxygen species differences

Strains were cultured overnight in 5ml of YPD at 30ºC. The optical densities of these cultures at 600nm were measured the following day to work out how much to add to 30ml of YPD so that after 2 hours these cultures would be in in the logarithmic growth phase. After these 2 hours of growth at 30ºC, the hydroxyurea or hydrogen peroxide stock solutions were added to the cultures which were then cultured for a further 1 hour and 30 minutes. DCFDA was then added before the cells were cultured for a further 1 hour and 30 minutes at 30ºC. DCFDA is a cell permeable non-fluorescent probe which when oxidised by reactive oxygen species becomes the fluorescent 2′-7′-dichlorofluorescein (Royall & Ischiropoulos, 1993). Cell numbers in these cultures were then estimated from cells counted on a hemacytometer under a microscope. 50mM sodium citrate was then added to the cultures and they were spun down in a centrifuge at 3000rpm for 3 minutes. The supernatant was then removed and the cells washed in sodium citrate before another centrifugation at 3000rpm for 3 minutes. The supernatant was again removed and the cells were resuspended in sodium citrate at a volume calculated to create a density of approximately 100000 cells per 100µl.

The resuspended cells were then passed through a FACS machine. The forward and side scatter of these cells were used to find a population of living cells. These living cells were then gated and a sample of 25000 were analysed for their fluorescence from the oxidised DCFDA.

2.7. Analysis of FACS data

Data from the machine was exported and worked on using FlowJo software. This software was used to work out gates for the analysis of reactive oxygen species levels which included living cells and excluded dead cells. The background fluorescence was also determined using FlowJo software and used to isolate the peaks of fluorescence caused by the reaction of the ROS with DCFDA. The background fluorescence was determined from the non-petite wild type without dye. This allowed for a percentage of cells showing fluorescence and thus high reactive oxygen species levels to be determined.

3. RESULTS

3.1. Petite mutation increases survival of the *mec1-4* and *dun1Δ* DNA damage response pathway mutants in spot tests

Preliminary spot tests on various different drug plates with different concentrations revealed changes in the survival of petite mutants compared to the non-petite strains they were created from on hydroxyurea plates. The petite strains were confirmed as petite by their lack of growth on YPG media as seen in Figure 4. This is due to the petite cells being unable to utilise glycerol as glycerol is non-fermentable (Goldring et al. 1971).

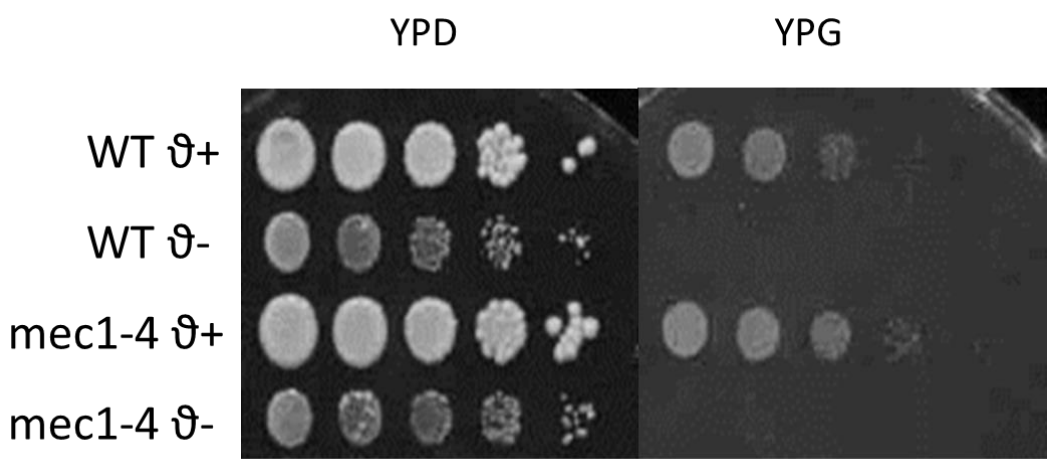

Figure 4. ϑ+ represents a non-petite strain and ϑ- represents a petite strain. The petite mutation is confirmed by lack of growth on YPG media as the energy source, glycerol, is non-fermentable and thus requires the mitochondria to break it down.

These spot tests showed that *mec1-4* mutants had increased survival on YPD plates with hydroxyurea (Figure 5 and Figure 6) with additional spot tests showing increased survival at higher temperatures as well (Figure 7) despite *mec1-4* being considered a temperature sensitive mutation. This suggests that processes involving the mitochondria are involved in the temperature sensitivity and reaction to hydroxyurea of the *mec1-4* mutant and thus suggest that there may be potential links between mitochondria and the DNA damage response. The petite *mec1-4 tel1Δ* mutant also appeared to have an increased survival on hydroxyurea media during the preliminary tests compared with its non-petite counterpart but the *mec1-kd* mutant did not appear to have any change in survival when petite. Neither of these

results are shown because the YPG plate which would have confirmed if these strains were petite was contaminated and because further experiments on these strains were not undertaken following the preliminary spot tests. The improved survival of the petite *mec1-4 tel1Δ* mutant was assumed to be due to same process as the *mec1-4* mutant as the growths of the petite and non-petite *tel1Δ* strains were equivalent to one another. The *mec1-kd* mutant was also not used in further experiments due to time constraints and because it apparently showed no difference in growth when petite. This result, however, was taken into consideration and from this observed difference the theory that the loss of mitochondria reduces the base level of reactive oxygen species present in cells and thus allows certain DNA damage response pathway mutants to survive the additional stress caused by reactive oxygen species generated by hydroxyurea was developed in contrast to the theory that the differences in survival were due to the changes in the DNA damage response pathway (Carter et al. 2005).

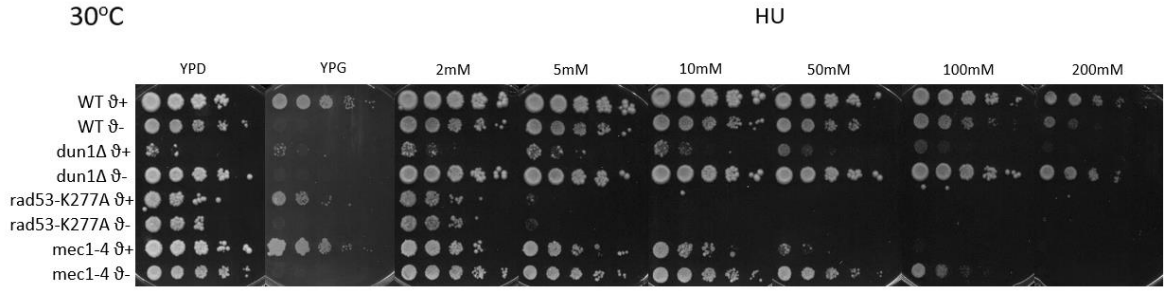

Figure 5. ϑ+ represents a non-petite strain and ϑ- represents a petite strain. The change in growth of the *mec1-4* and *dun1Δ* mutant strains on HU media when petite suggests that the lack of mitochondrial processes is beneficial to the *mec1-4* and *dun1Δ* mutants.

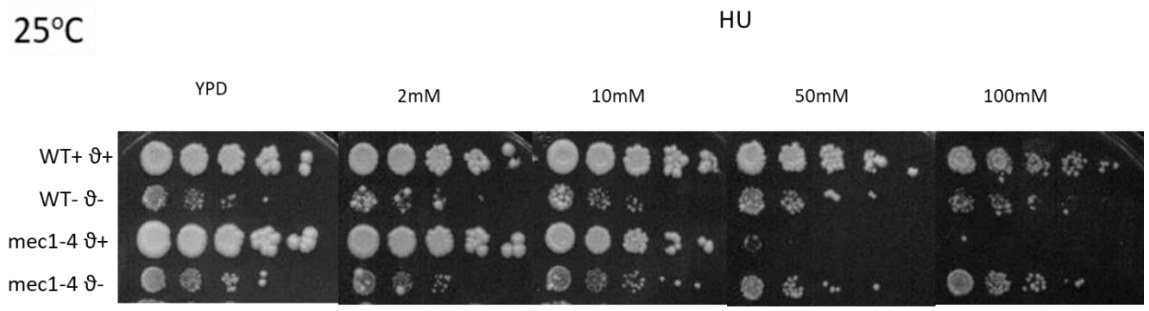

Figure 6. ϑ+ represents a non-petite strain and ϑ- represents a petite strain. The change in growth of the temperature sensitive *mec1-4* mutant strain on HU media when petite suggests that the lack of mitochondrial processes is beneficial to the *mec1-4* mutant.

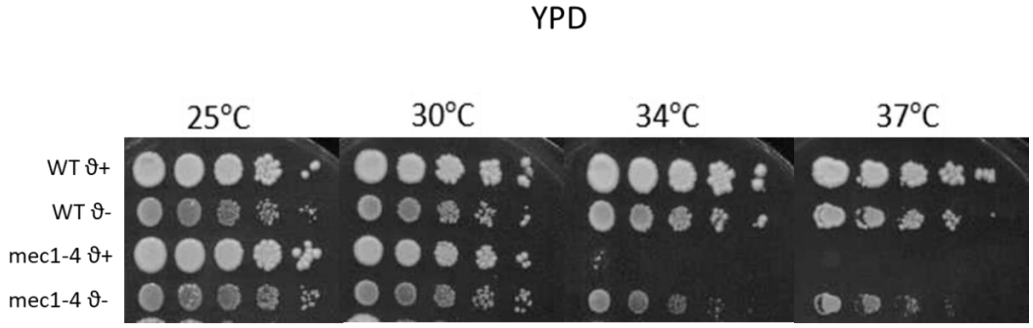

Figure 7. ϑ+ represents a non-petite strain and ϑ- represents a petite strain. The temperature sensitivity of the *mec1-4* mutant appears to be drastically reduced by the loss of mitochondria

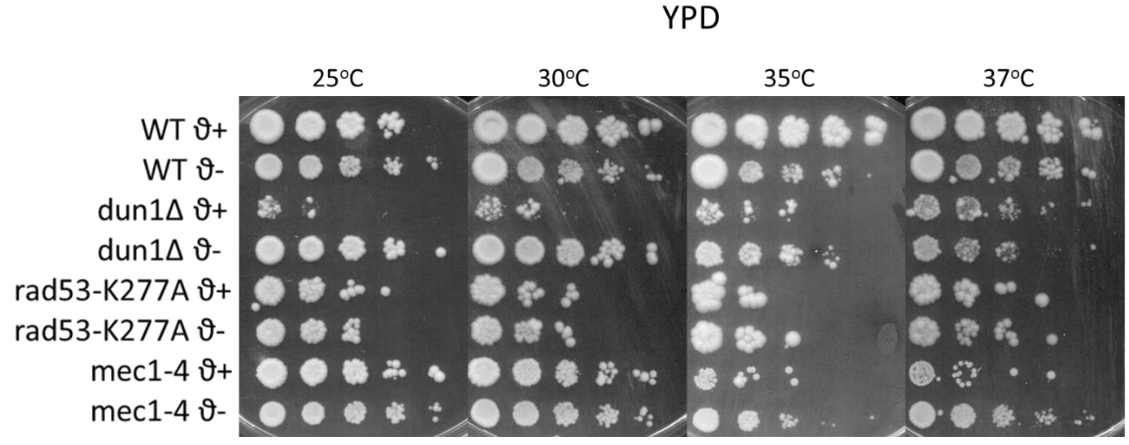

Figure 8. ϑ+ represents a non-petite strain and ϑ- represents a petite strain. *dun1Δ* appears to have cold sensitivity that is reduced by being petite which suggests that the lack of mitochondrial processes is beneficial to the *dun1Δ* mutant.

In Figure 8 the *dun1Δ* mutant tested shows cold sensitivity with reduced growth on media cultured below 37^{o}C while the *dun1Δ* mutant with the addition of the petite mutation does not show this cold sensitivity. This difference in growth therefore suggests that the loss of mitochondria is responsible for the improvement providing an interesting starting point for future investigations linking mitochondrial processes and the DNA damage response.

The petite *dun1Δ* mutant also shows increased survival on hydroxyurea media when compared to the non-petite *dun1Δ* mutant (Figure 8) providing evidence that the ability of DNA damage response mutants to survive hydroxyurea treatment is linked to the presence or absence of mitochondria. This fits with the theory that removing mitochondria reduces the levels of reactive oxygen species in the cell. However, the fact that the *rad53-K227A* strain does not have any change in growth on hydroxyurea media when the petite mutation is present (Figure 8) complicates the situation in regards to the theory proposed above as Rad53 lies between Mec1 and Dun1 in the phosphorylation pathway, being phosphorylated by Mec1 and in turn phosphorylating Dun1 (Figure 1).

3.2. Differences in Rad53 activation in response to hydroxyurea

The increased survival of the *mec1-4* mutant on hydroxyurea containing media when petite (Figures 5 and 6) suggests that the petite phenotype may affect the phosphorylation of Rad53 by Mec1. To test if this was the case Western blots were run with the petite and non-petite wildtype and *mec1-4* mutants using an anti-Rad53 antibody and a luminescent anti-anti-Rad53 antibody as this would allow the phosphorylation of Rad53 to be visualised. Later these Western blots were performed using Phos-tag to increase the visible difference between phosphorylated and unphosphorylated Rad53.

The observable differences in the migration of the various treated and untreated strains of individual Western blots are often contradictory. However, the first response was that overall Western blots for Rad53 (Figure 9 as an example) seem to show that the petite mutation does not affect the phosphorylation of Rad53 in response to hydroxyurea. However, several Western blots (including that shown in Figure 9) appeared to show that the petite mutation in the wildtype increased the base level phosphorylation of Rad53 bringing it in line with that of the *mec1-4*

mutants while untreated. The appearance of the non-petite wildtype proteins on the Ponceau stain suggest there may be a different reason for this apparent result as the migration of the extracted proteins of untreated non-petite wildtype strain appears darker and more smudge-like. This was not seen on every Western blot. Figure 10 for example does not seem to show a great change in the migration in the Rad53 of the untreated non-petite wildtype strain when compared to the untreated petite wildtype strain. The migration of the hydroxyurea treated wildtypes on Figure 10 appear, however, to be slightly affected by the petite mutation with the petite wildtype showing what could be interpreted as a slight reduction in phosphorylation when compared with the hydroxyurea treated non-petite. Another blot (Figure 11) also shows this apparent reduction in phosphorylation in the hydroxyurea treated wildtype when petite. This blot (Figure 11) also shows the aforementioned difference in the migration of the protein extracts of the petite and non-petite wildtypes when untreated. The appearance of the untreated non-petite wildtype strain's proteins on the Ponceau stains as a whole suggest that the apparent increase in phosphorylation of the untreated wildtype when petite should instead be interpreted as a repeated unexplained change in the migration of the protein extract of the untreated non-petite wildtype likely due to an experimental error. This impacts the usefulness of the data as it is meant to be the control.

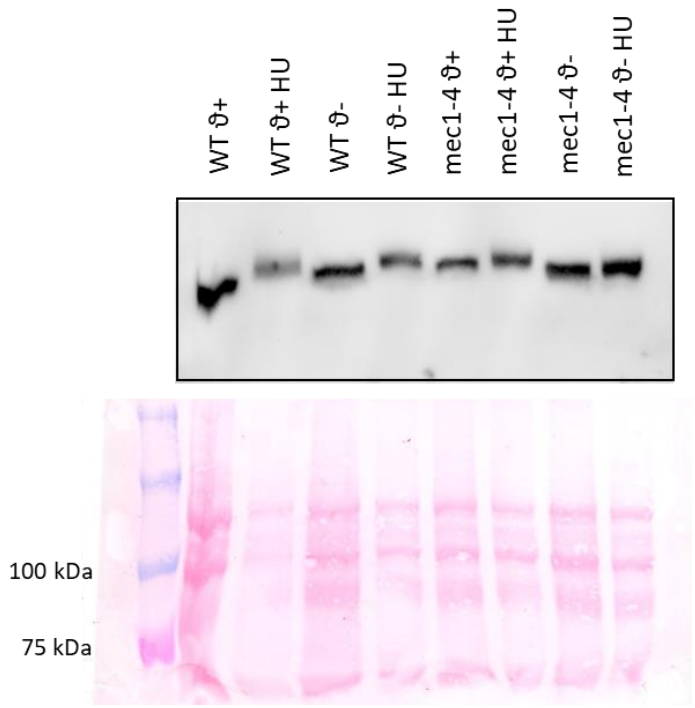

Figure 9. Western blot for Rad53 with Ponceau stain. The result of this blot appears to show that the petite mutation doesn't affect the phosphorylation of Rad53 in response to hydroxyurea but does slightly increase the phosphorylation of wildtype under non-stress conditions. However, this is likely due to an experimental error.

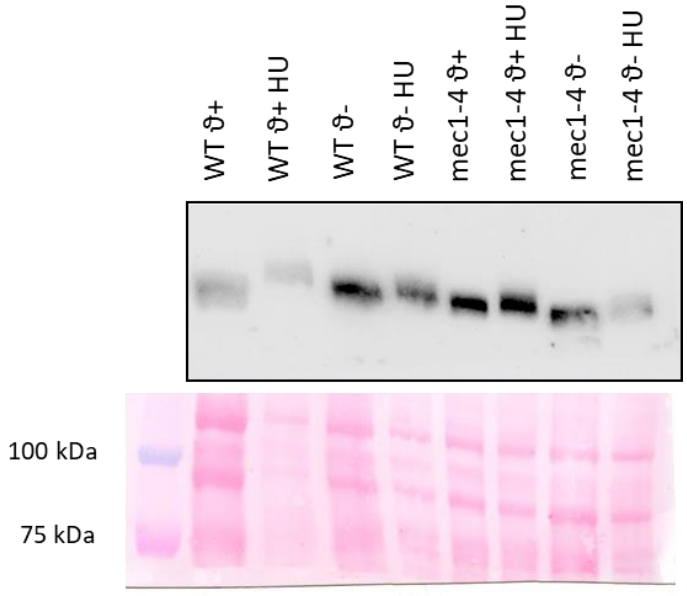

Figure 10. Western blot for Rad53 with Ponceau stain. The result of this blot appears to show that the petite mutation slightly affects the phosphorylation of wildtype in response to hydroxyurea.

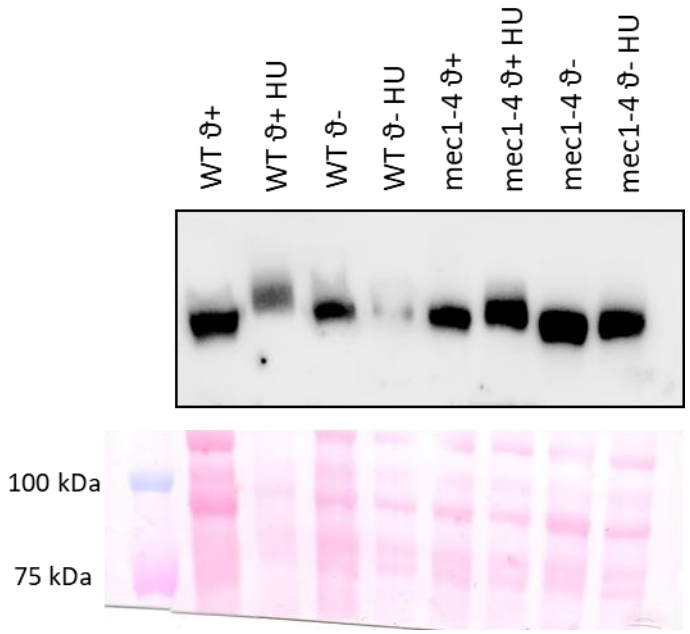

Figure 11. Western blot for Rad53 with Ponceau stain. The result of this blot appears to show that the petite mutation slightly increases wildtype phosphorylation under non-stress conditions and decreases it under the stress of hydroxyurea.

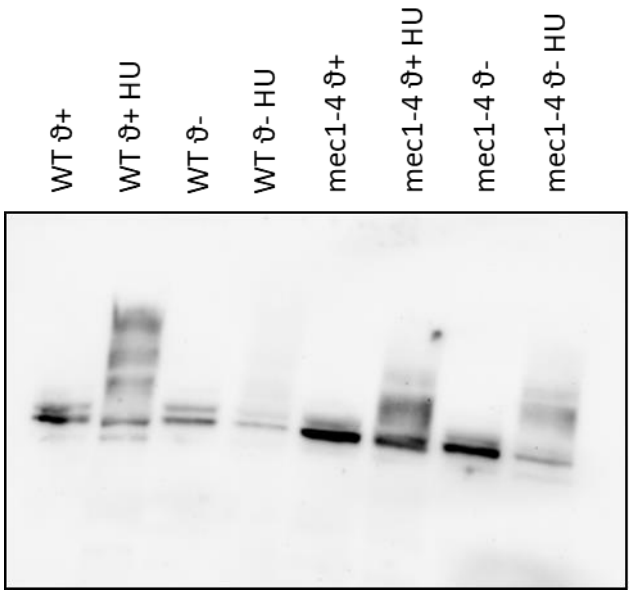

Figure 12. Western blot using Phostag. The petite mutation does not seem to affect the phosphorylation of Rad53 in response to hydroxyurea in the *mec1-4* mutant but potentially does affect its phosphorylation in the wildtype

Phos-tag Western blots for Rad53 (an example of which is shown in Figure 12) at first appear to show that there is no difference in phosphorylation between the petite

strains and the non-petite strains they are derived from when treated with 50mM hydroxyurea for 1 hour and 30 minutes. However, an argument could be made that the lighter appearance of the hydroxyurea treated petite wildtype does in fact show that the petite mutation reduces the phosphorylation of Rad53 in response to hydroxyurea in the wildtype corroborating the results seen in figures 7 and 8.

The Phos-tag Western blots also show no major difference in the phosphorylation of the untreated non-petite wildtype and the untreated petite wildtype. This, alongside the appearance of the non-petite wildtype on the Ponceau stain of Western blots, means therefore that another explanation for the dramatic difference in migration of the non-petite wildtype demonstrated on some Western blots when compared with the other strains must be found.

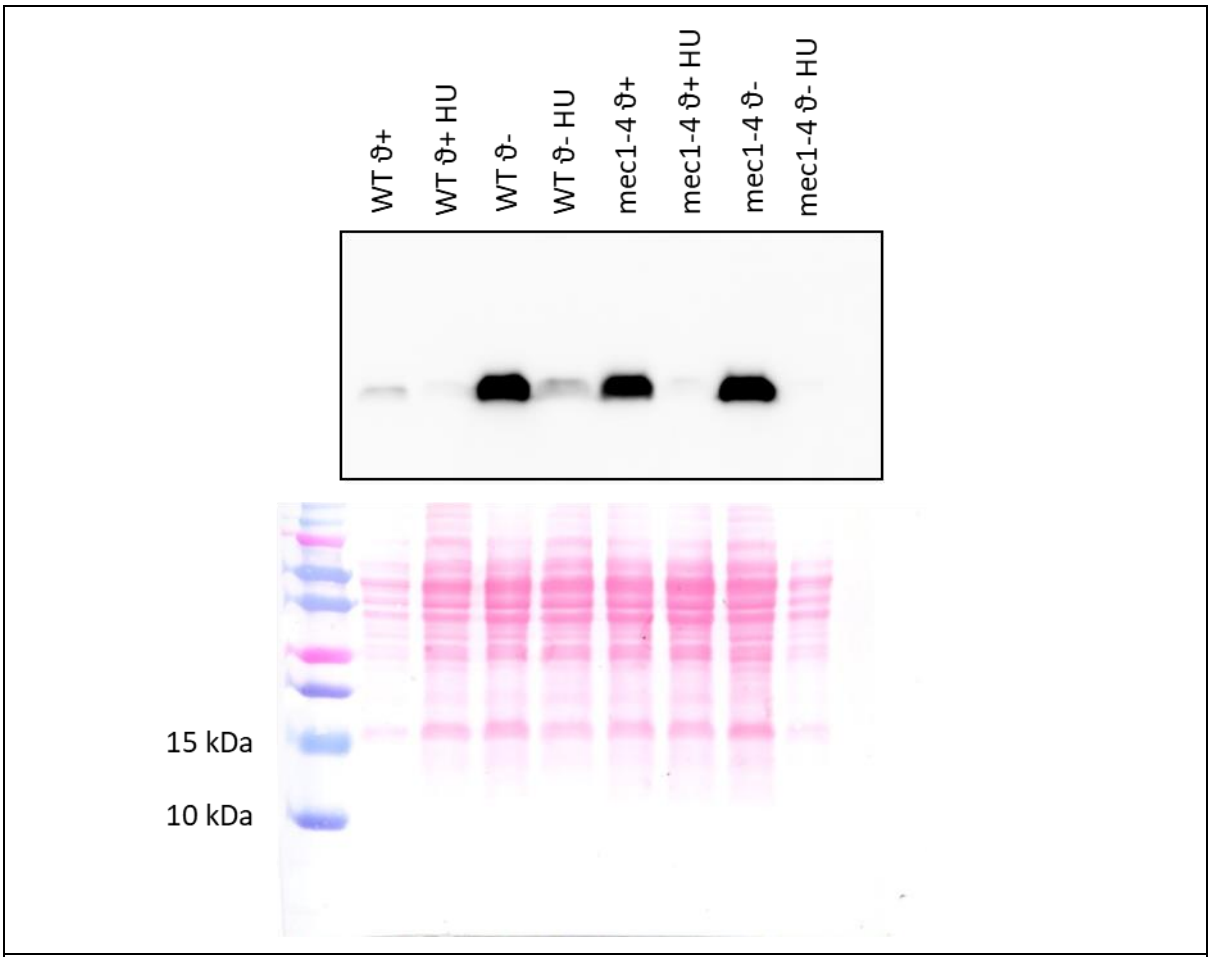

Figure 13. Western blot for Sml1 with ladder from Ponceau stain. The petite mutation does not appear to significantly affect Sml1 breakdown in the presence of hydroxyurea.

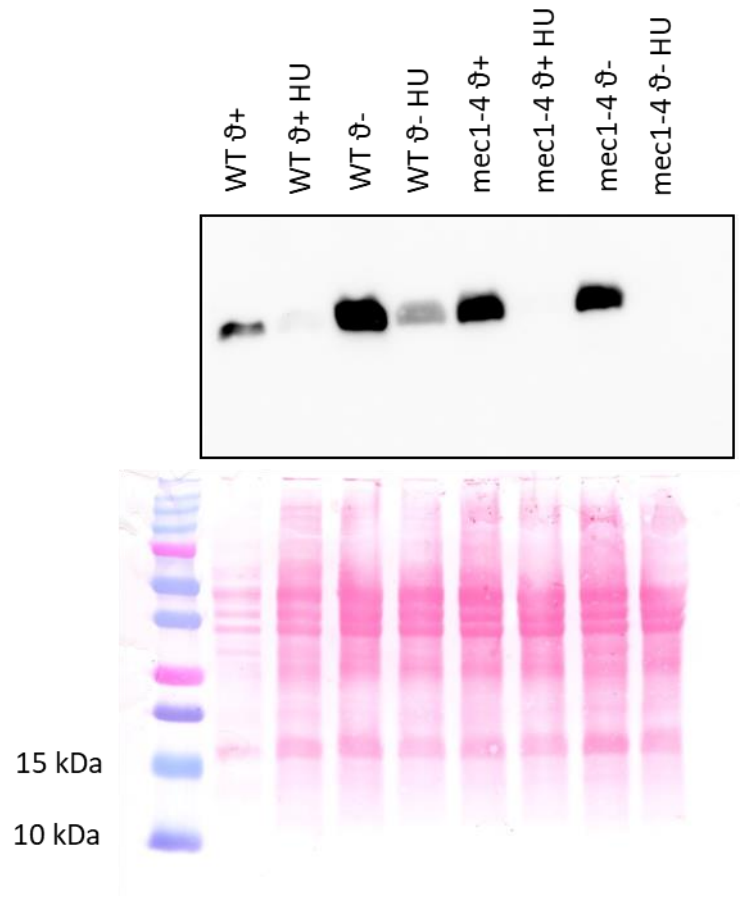

Figure 14. Western blot for Sml1 with ladder from Ponceau stain. The petite mutation does not appear to significantly affect Sml1 breakdown in the presence of hydroxyurea.

3.3. Differences in Sml1 removal in response to hydroxyurea

Western blots for Sml1 were performed in the same manner as those for Rad53 with the only change being the use of an anti-Sml1 antibody and corresponding anti-anti-Sml1 antibody rather than an anti-Rad53 antibody and an anti-anti-Rad53 antibody. The reason for performing these was, like the Rad53 Western blots, to see whether the petite phenotype affecting the Mec1-mediated DNA damage response.

The results from the Western blots for Sml1 (Figure 13 and Figure 14) appeared to show that the petite mutation does not affect the breakdown of Sml1 when the strains are exposed to hydroxyurea. Interestingly the petite strains appear, on some blots, to have an increased expression of Sml1 under normal conditions. However, the Ponceau stain shows that there is less total protein in the non-petite wild type lane. Because of this ImageJ analysis was used to account for these differences in total protein loaded and after accounting for this difference in protein loading Figure

15, the graph for the difference between the total protein and the Western blot in Figure 13, showed a noticeable difference in the intensity of the Sml1 band for the wildtype when petite and non-petite. This could be interpreted as an increase in the presence of Sml1 when petite or alternatively a decrease in the presence of Sml1 when non-petite. The non-petite *mec1-4* mutant itself on this blot also shows an increase in the intensity of the Sml1 bands. None of this is true of the blot in Figure 14, however, as can be seen in Figure 16. Both Figure 15 and Figure 16 show, however, an increase in intensity in the presence of hydroxyurea and thus what can be interpreted as a decrease in the breakdown of Sml1 in the petite wildtype when treated with hydroxyurea.

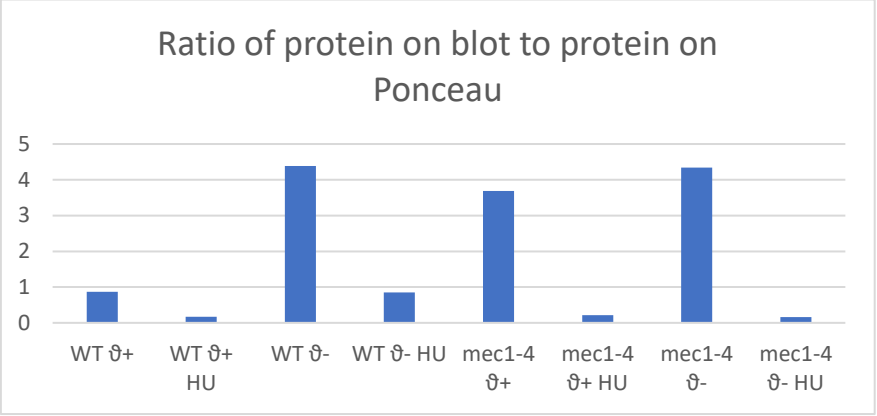

Figure 15. Bar graph demonstrating the difference in the intensity data from ImageJ for the bands of the Ponceau stain and the chemiluminescence image of Figure 13. This provides data which can be used as a comparison of Sml1 abundance.

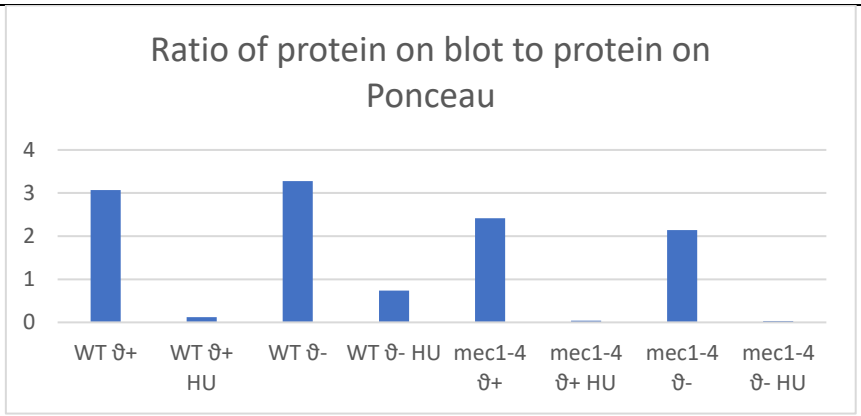

Figure 16. Bar graph demonstrating the difference in the intensity data from ImageJ for the bands of the Ponceau stain and the chemiluminescence image of Figure 14. This provides data which can be used as a comparison of Sml1 abundance.

3.4. No increase in reactive oxygen species seen in response to hydroxyurea

A second hypothesis unrelated to the DNA damage response mediated pathway was also developed. This hypothesis was that removal of the mitochondria reduces the total amount of reactive oxygen species in the individual Saccharomyces cerevisiae cells and impairs apoptosis so that when mec1-4 mutation is present and generating reactive oxygen species the lower baseline amount of reactive oxygen species and the impaired apoptotic pathways prevent the death of the cell. This was tested using DCFDA, a dye which forms a fluorophore when oxidised. The fluorescence was measured using a FACS machine.

The results from fluorescence analysis using a fluorescence-activated cell sorting machine are not what was expected. It was believed that the cells treated with hydroxyurea would show an increased fluorescence when treated with DCFDA indicating an increased presence of reactive oxygen species (Royall & Ischiropoulos, 1993). However, the results gained from DCFDA treatment and analysis with FACS (Figure 17) show that every strain treated with hydroxyurea has a similar fluorescence profile to its untreated counterpart and to the untreated non-petite wildtype which acts as the negative control (seen compared to its undyed counterpart in Figure 18). The fluorescence peaks above the background demonstrated in the cells which were treated with hydrogen peroxide, the positive control, show that the experiment as a whole was performed correctly and thus

shows that the results seen with hydroxyurea are likely true to the reality in the cells. This being that following a 90-minute treatment in a 50mM hydroxyurea solution the levels of reactive oxygen species in the treated cells are not increased.

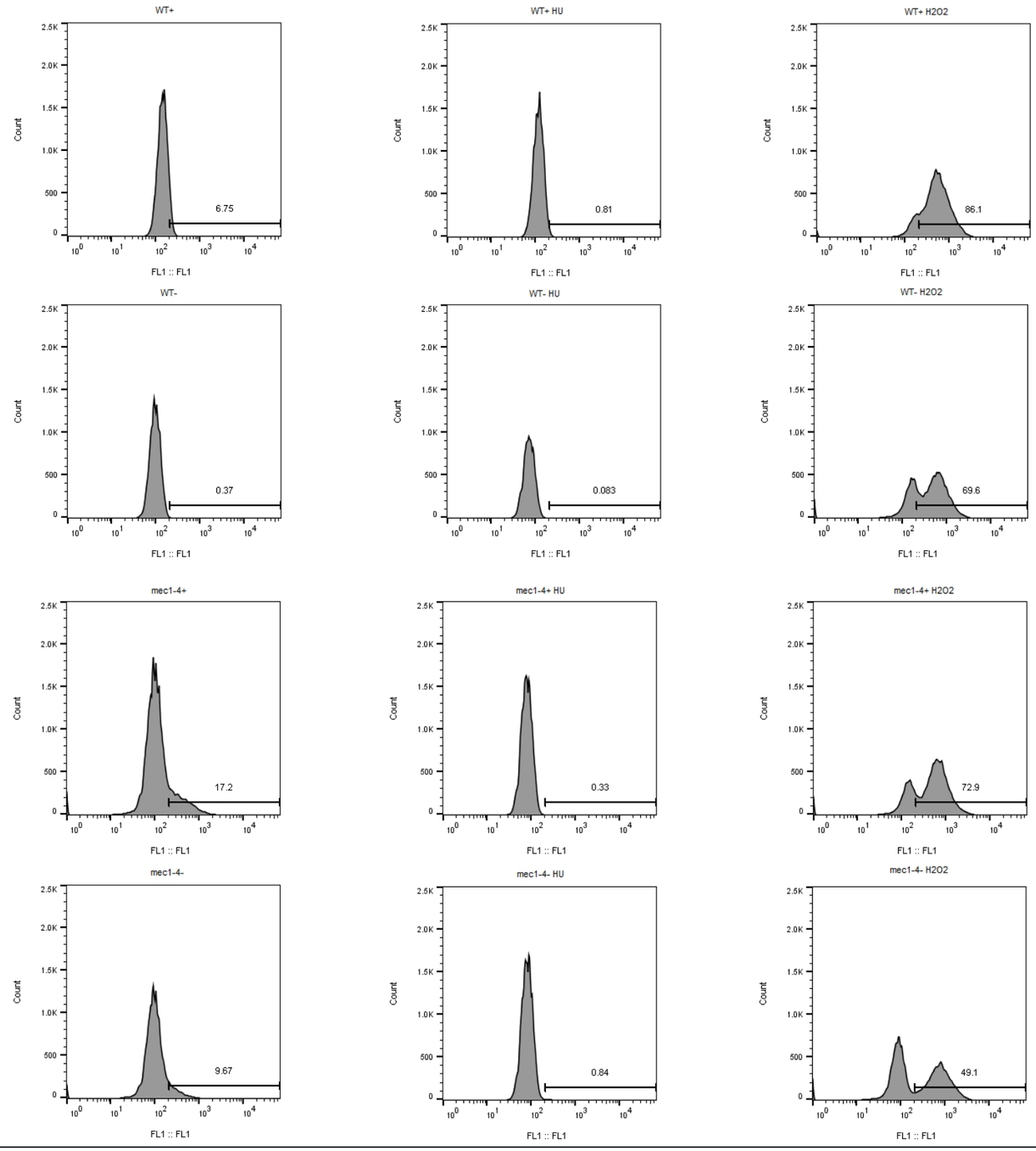

Figure 17. FlowJo data showing the percentage of cells from each sample with fluorescence above background. Shifts to right indicate a greater number of cells in which the DCFDA dye has reacted with reactive oxygen species.

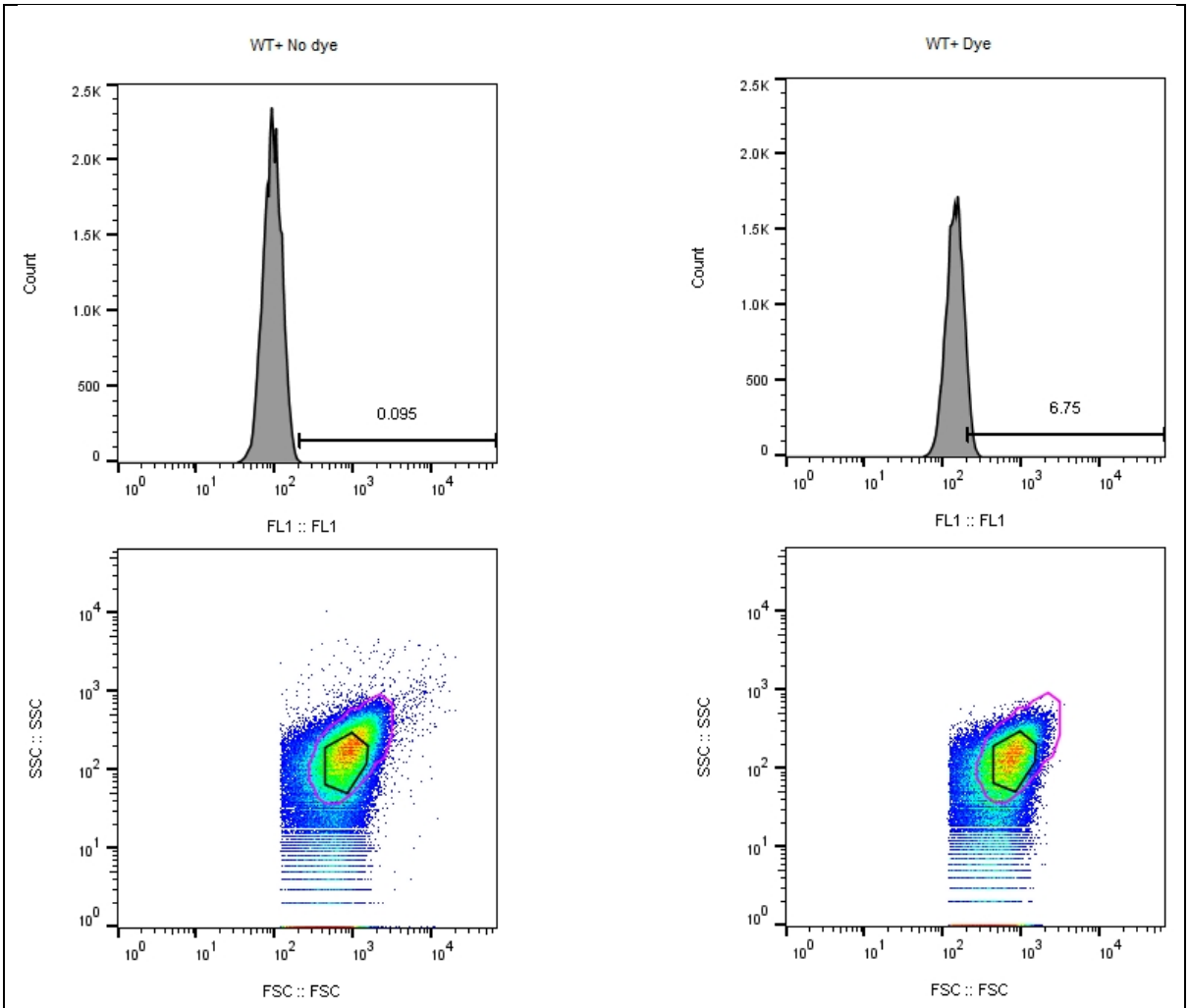

Figure 18. FlowJo gating used to select a population to investigate to ensure the cells being looked at were living and to determine the background fluorescence of normal cells without treatment with the ROS sensitive dye DCFDA.

4. DISCUSSION

4.1. The petite mutation has an effect on survival in *mec1-4* and *dun1Δ* mutants

At the beginning of the project, petite versions of a number of different strains were generated each with a mutation of a gene where the protein encoded for was a component of the DNA damage response pathway. These petite strains were grown along with the strains they were generated from on YPD plates containing different concentrations of various drugs. These drugs were hydroxyurea, hydrogen peroxide, methyl methanesulphonate, and carbonyl cyanide m-chlorophenyl hydrazone. The result seen amongst the drug plates during these spot tests was the increased survival seen on hydroxyurea plates of increasing concentrations of the petite versions of three strains. These strains were the *mec1-4* and *mec1-4 tel1Δ* strains and the *dun1Δ* strain. This result was not the only result seen, as changes in temperature sensitivity of both the *mec1-4* and *dun1Δ* strains were also seen, but was the result for which it was decided that further experiments would be performed to elucidate the reasons for the apparent phenotype seen in relation to hydroxyurea. This was due to time restrictions which also led to only the petite and non-petite *mec1-4* mutant strains being used in the further experiments. Another reason for the petite and non-petite *dun1Δ* mutants not being used in the further experiments was that no cells of the exact petite used for the spot tests were collected and frozen at -80^{o}C.

When the spot tests were being performed, as well as the drug plates and the YPG plates for the confirmation of petite status, there were YPD plates which were incubated at various different temperatures. The petite strains of the *mec1-4* and *dun1Δ* deletion mutants tested on these plates seem to be less temperature sensitive than their non-petite counterparts. In the case of the *mec1-4* mutant this is seen with an increased survival at higher temperatures compared with the non-petite *mec1-4* strain. Conversely the petite *dun1Δ* strain shows improved growth at lower temperatures compared with the non-petite *dun1Δ* strain. These results combined with the observed increase in survival on the hydroxyurea containing YPD media plates suggests that the loss of mitochondria in some way reduces severity of the *mec1-4* and *dun1Δ* mutations. However, the fact that on both the hydroxyurea plates

and the YPD plates at different temperatures there is no real perceivable difference between the petite and non-petite *rad53-K277A* strains complicates the picture. This is because Rad53 falls in between Mec1 and Dun1 in the DNA damage response signalling pathway (Zhou & Rothstein, 2002; Corcoles-Saez et al. 2018). Why therefore does there appear to be no increase in survival for the petite *rad53-K277A* strain? Does it suggest that the increase in survival has nothing to do with Mec1's or Dun1's roles in the DNA damage response and instead indicates secondary roles of Mec1 and Dun1? Perhaps. This is why Western blots looking at the phosphorylation of Rad53 and Sml1 (the target of Dun1 in the DNA damage response pathway) were performed.

Another possible reason behind the improved survival of the *mec1-4* strain and the *dun1Δ* strain was also theorised that being that the loss of mitochondria was reducing the base line level of reactive oxygen species in the cells of the *Saccharomyces cerevisiae* strains and that the effect of the hydroxyurea on the non-petite strains was linked to its generation of reactive oxygen species within cells and not its effect on the RNR complex (Nordlund & Reichard, 2006; Davies et al. 2009; Singh & Xu, 2016). This would mean that not only would DNA damage from reactive oxygen species be reduced but that autophagy pathways based on the presence of reactive oxygen species or the damage they cause to cellular components would not be activated.

This theory was developed based on the previous knowledge that mitochondria are a major source of reactive oxygen species in normal healthy cells due to electrons from the electron transport chain leaking into the cytoplasm (Finkel, 2011; Oyewole & Birch-Machin, 2015). Hydroxyurea has also been shown to influence the levels of reactive oxygen species in cells due to a demonstration of oxygen dependent sensitivity in *Saccharomyces cerevisiae* cells lacking the reactive oxygen species detoxifying protein Sod1 (Carter et al. 2005). This effect was shown to be suppressed by the presence of the antioxidant NAC further supporting the idea that hydroxyurea damages cells via reactive oxygen species (Carter et al. 2005). However, the same study which demonstrated this effect also showed that the addition of NAC did not improve the survival of *mec1Δ sml1Δ* mutants suggesting that hydroxyurea's effects on DNA damage response pathway mutants is not based upon its generation of reactive oxygen species (Carter et al. 2005). This proves a

problem for the theory as if the effect of hydroxyurea on the *mec1Δ sml1Δ* mutants in this previous study were as demonstrated not due to hydroxyurea's reactive oxygen species generation then it is possible that the results seen with the DNA damage response mutants, *mec1-4* and *dun1Δ*, are also not due to the presence or absence of reactive oxygen species. Another piece of evidence suggesting against the likelihood of this theory is that of the DNA damage response mutated strains tested only the *mec1-4* strains and the *dun1Δ* strain show differences in their growth when petite. The *rad53-K227A* petite strain does not show these differences. This therefore suggests that the presence or absence of reactive oxygen species does not directly affect the DNA damage response pathway and thus the results seen with the petite *mec1-4* and *dun1Δ* strains are potentially due to interactions that Mec1 and Dun1 have that are independent of the DNA damage response.

Due to the potential interactions that DNA damage response pathway proteins have there is a possibility that the results seen with the loss of mitochondria are due to complex interactions that the DNA damage response pathway proteins have with apoptotic factors which bind to the outer membrane of the mitochondria (Vyas et al. 2016). However, the removal of mitochondria removes the source of cytochrome C for the activation of caspases and the initiation of apoptosis which theoretically should affect all petite strains in the same way (Vyas et al. 2016). The removal of the mitochondria could also affect gene expression due to histone acetylation which could have a combined effect with the *mec1-4* and *dun1Δ* mutations resulting in the observed results (Vyas et al. 2016).

4.2. Subtle differences seen between petite and non-petite strains in phosphorylation of Rad53 and Sml1 breakdown

As mentioned above the Western blots performed only used the *mec1-4* petite and non-petite strains and the petite and non-petite wildtype strains for comparison and not the *dun1Δ* strains due to the loss of the specific petite strain seen on the spot tests as well as time restrictions. These time restrictions came about due to the number of times the original Western blots looking at Rad53 had to be repeated due to the author's lack of experience with this technique and the number of repeats that were done to confirm the observation that there was nothing different in the abundance or phosphorylation of the Rad53. This is because this observation was

not universal amongst the Western blots performed. Some blots appeared to show that the petite mutation didn't affect the phosphorylation of Rad53 in response to hydroxyurea but that it did slightly increase the phosphorylation of Rad53 in the wildtype under non-stress conditions. It was determined, however that this was likely due to an experimental error during the extraction of proteins. The exact nature of this error will be discussed later in this document. Another blot appeared to show that the petite mutation slightly reduced the phosphorylation of Rad53 in response to hydroxyurea. Another blot yet appeared to show that the petite mutation slightly increased phosphorylation of the wildtype under non-stress conditions and decreased phosphorylation when the wildtype was treated with hydroxyurea.

It is because of these somewhat conflicting results that the decision was made to perform Western blots using Phos-tag. This would allow for a much clearer picture to be obtained of the phosphorylation of Rad53 in the various strains, petite and non-petite, as the Phos-tag increases the separation of proteins based upon their phosphorylation (Kinoshita et al. 2022). The results of these Western blots using Phos-tag were initially interpreted as showing that there was no difference in phosphorylation between the petite strains and the non-petite strains from which they were derived when the strains had been treated in the same way as the other Western blots. However, a slight difference in the appearance of the migration of Rad53 from the petite wildtype treated with hydroxyurea on the Phos-tag Western blots and some of the Western blots performed without Phos-tag could suggest that the petite mutation does have the effect of reducing phosphorylation of Rad53 but only in the wildtype. This is because a corresponding change in the migration of hydroxyurea treated petite *mec1-4* strain compared to the non-petite *mec1-4* strain is not seen on either the Phos-tag Western blots or the original Western blots. It seems odd that this would be the case but this observation may be key to understanding the tolerance of hydroxyurea seen in the *mec1-4* and *dun1Δ* strains when they are petite. Looking at the growth of the petite and non-petite wildtype strains from spot tests on hydroxyurea containing plates does not show that the petite wildtype strain has an increased survival when exposed to hydroxyurea. In fact, the opposite appears to be true with the petite having reduced growth when grown on plates with higher concentrations of hydroxyurea. This difference in phosphorylation could therefore provide a partial explanation for the difference in survival between the

petite wildtype and the petite *mec1-4* strains. To see if this theory has any credence the phosphorylation of the petite and non-petite *dun1Δ* strains would have to be looked into using Phos-tag Western blots with the result being that the phosphorylation of the Rad53 of the hydroxyurea treated petite *dun1Δ* strain is shown to be similar to that of the petite *mec1-4* strain and the phosphorylation of the petite wildtype to be shown to be consistently decreased in comparison to the non-petite wildtype. The theory based upon these observations, however, has some unanswered questions. For example, why would a decrease in phosphorylation occur in only the wildtype and not the *mec1-4* mutant and, consequently, what about the *mec1-4* mutation could affect the way in which the lack of mitochondria impacts the response to hydroxyurea?

If the first interpretation of the results is correct, these results from the Western blots using Phos-tag provide evidence that the reason for the improved survival of the *mec1-4* mutant strain when petite has nothing to do with the lack of mitochondria modifying Mec1's role in phosphorylating Rad53 in the DNA damage response pathway. However, the second interpretation which suggests that the *mec1-4* mutant's survival is in part due to it modifying the effect of the petite mutation on Rad53 should be kept in mind when considering the results of the Western blots looking at Sml1 phosphorylation.

The Western blots performed for Sml1 showed similar results in regards to phosphorylation. The phosphorylation of Sml1 is easier to determine as the result of phosphorylation of the full quantity of Sml1 in the strain's cells would present as a fully blank band as phosphorylation of Sml1 causes it to be broken down (Zhou & Rothstein, 2002). The results showed that the phosphorylation and breakdown of Sml1 in *mec1-4* strains is not affected by the petite mutation when the strains are exposed to hydroxyurea. The petite mutants, however, did appear to have an increased expression of Sml1 when not exposed to hydroxyurea based purely on the appearance of the bands on the blot. When the Ponceau stain was looked at a more mundane explanation for this apparent result was found. This was that the appearance of the bands was potentially due to differences in the amount of protein loaded in the wells of the gel. To confirm if this was the case ImageJ software was used on the Ponceau stain and the Western blot to normalise the data from the Western blot. After this was done, the results of the two best blots on which this

normalisation was performed were that one blot (Figure 14) showed no differences in expression between the unstressed petites and non-petites and the other (Figure 13) showed an increase in the intensity of the Sml1 band for the petite wildtype strain compared to the non-petite wildtype when unstressed. The non-petite *mec1-4* mutant on this blot also showed an increased intensity in comparison to the non-petite wildtype. These results from the Western blots looking at the status of Sml1 in the petite wildtype and *mec1-4* strains in comparison to the non-petite strains they were generated from could be interpreted as showing that the lack of mitochondria in the petite strains has no effect on the phosphorylation of Sml1 as part of the DNA damage response pathway in the *mec1-4* mutant and when combined the interpretation of the results from the Western blots looking at Rad53 both with and without Phos-tag that Rad53 phosphorylation is not affected by the petite mutation makes the idea that the petite mutation does not affect the DNA damage response of the *mec1-4* mutant seem plausible. However, another look at the ImageJ data showed a result which suggests that the second interpretation of the Rad53 Western blots provides the more accurate picture of the petite mutation's interactions with the *mec1-4* mutation.

The other result made apparent by the ImageJ analysis of the Sml1 blots (Figures 12 and 13) is the increased intensity of the band for the hydroxyurea treated petite wildtype on both of the Western blots when compared to the non-petite wildtype treated with hydroxyurea. This suggests that in the wildtype background the petite mutation causes the breakdown of Sml1 to be impaired. Since this is not seen with the hydroxyurea treated petite and non-petite *mec1-4* strains the results of the spot tests were re-examined to determine the possible effect this difference in the breakdown of Sml1 has upon on the survival of the different *Saccharomyces cerevisiae* strains. Looking again at the spot tests upon hydroxyurea containing plates in figures 2 and 3 it can be seen that the petite wildtype has decreased survival compared with the non-petite wildtype this is the opposite effect to that observed in *mec1-4* which, as mentioned above, has an increased survival when petite. The results of these spot tests (Figures 2 and 3) and the data from the ImageJ analysis of the Western blots looking at the status of Sml1 (Figures 12 and 13) when viewed together therefore suggest that the reason for the increased survival of the *mec1-4* strain when petite may in part be due to the *mec1-4* mutation influencing the

way the in which the petite mutation affects the breakdown of Sml1 with one of the effects of the loss of mitochondria being a decreased breakdown of Sml1 in response to the stress of hydroxyurea and the mutation in Mec1 nullifying this. This data when combined with the findings from the Phos-tag Western blots for Rad53 as the Phos-tag Western blot (Figure 12) shows a decreased phosphorylation for the petite wildtype exposed to hydroxyurea compared to the non-petite. Therefore, providing evidence that the mutation in Mec1 was affecting the phosphorylation and breakdown of Sml1 through the traditional phosphorylation route of the DNA damage response by preventing the changes to Rad53 phosphorylation and thus via Dun1 reducing Sml1 phosphorylation. This therefore lends more credence to the idea discussed above that the *mec1-4* mutation changes the impact of the loss of mitochondria on the DNA damage response. How this happens and why this improves survival are questions which must be answered and this would have to be accomplished through further experimentation.

Another possibility that should still be considered is that, although seen in the data from the two blots for which the data from ImageJ analysis has been provided, the perceived change in Sml1 breakdown in the petite wildtype strain is an artefact caused by the handling of the samples. The same result seems to occur on the other Western blots for which analysis using Image J was performed although this data should be considered poorer due to the blots it was taken from and in addition to this the same protein extracts as the two Sml1 blots presented were used in these blots. Due to this it would be useful for the blots for Sml1 to be repeated with a new set of protein extracts.

Overall, these results from the Western blots performed do not allow for one to be confident in saying that the improved survival of the *mec1-4* mutant strain on hydroxyurea containing YPD media is not due to any change in the operation of the DNA damage response pathway and show that these experiments need to be repeated and further experiments performed to determine if the tantalising observation of the modified response to the petite mutation is real and to determine the method by which this observed change is affected.

Obviously, however, the data from these Western blots deals solely with the response of the petite and non-petite *mec1-4* strains to 50mM hydroxyurea in culture

and so one must be careful in the conclusions drawn from these results. The improved survival of the *dun1Δ* strain may not be due to changes in the DNA damage response pathway and instead due to some as yet unknown change in the cell caused by the petite mutation which may also be what is affecting the survival of *mec1-4* mutant. The changes seen in growth at different temperatures for both strains may or may not also be due to changes in the phosphorylation of proteins in the DNA damage response. Further experiments are therefore required. Despite this the results already obtained suggest that the petite mutation's effect on survival in the DNA damage response mutants of *mec1-4* and *dun1Δ* is not solely due to the roles of Mec1 and Dun1 in the DNA damage response pathway or the modification of the way in which the loss of mitochondria affects this pathway.

4.3. No differences in reactive oxygen species demonstrated in petite strains

To test the theory that the increased survival of *mec1-4* mutants when petite was due to a reduction in the base level of reactive oxygen species instead of Mec1's role in the DNA damage response pathway the dye DCFDA was used to determine the presence of reactive oxygen species. The theory was based on the knowledge that as well as targeting the RNR and causing replication stalling hydroxyurea also causes damage in an oxygen dependant manner and the so reason for the development of this theory was to explain why the *mec1-4* and *dun1Δ* mutants showed increased survival when petite but the *rad53-K227A* mutant did not show this same increase in survival when petite (Carter et al. 2005; Nordlund & Reichard, 2006; Davies et al. 2009; Singh & Xu, 2016). The thinking was that the difference between the *mec1-4* and *dun1Δ* strains and the *rad53-K227A* strains was to do with the amount of reactive oxygen species damage each mutated cell could tolerate and the amount of reactive oxygen species present in each cell due to the different mutations in the DNA damage response pathways. Under this theory the assumption was that all three mutants had a greater level of reactive oxygen species than the wildtype even when unstressed and that the addition of the hydroxyurea was adding to the amount of reactive oxygen species in the *Saccharomyces cerevisiae* cells. The common interpretation for why the *mec1-4*, *dun1Δ*, and *rad53-K227A* mutants had lower tolerance for the hydroxyurea treatment would be that they are DNA

damage response pathway mutants and that this would make them more susceptible to hydroxyurea's effect on the RNR the ultimate target of the DNA damage response pathway resulting in a greater number of cells suffering from replication fork stalling, the halting of the cell cycle, and ultimately cell death (Nyholm et al. 1993; Singh & Xu, 2016). However, another possible interpretation for why these mutant strains had reduced survival was that they had a higher level of reactive oxygen species and that the addition of hydroxyurea was therefore simply increasing the level of reactive oxygen species in these mutants to an intolerable level leading to cell death. With this interpretation the removal of the mitochondria a major source of endogenous reactive oxygen species was increasing the survival of the *mec1-4* and *dun1Δ* mutants by decreasing the base level of reactive oxygen species and so increasing the amount of hydroxyurea needed to generate enough reactive oxygen species to reach this intolerable level. The explanation for the *rad53-K227A* mutant not being affected by the petite mutation being that the *rad53-K227A* mutation either produced a much greater level of reactive oxygen species itself or that the *rad53-K227A* mutant was more susceptible to the damage of reactive oxygen species than the *mec1-4* and *dun1Δ* mutants.

The results of the experiments performed which looked at the nature of the levels of reactive oxygen species in petite and non-petite strains of *mec1-4* exposed to hydroxyurea unfortunately do not corroborate this theory that the petite mutants had dramatically lower base levels of reactive oxygen species (Figure 17). The results suggest in addition that the treatment of the cells with 50mM hydroxyurea doesn't increase the levels of reactive oxygen species in the cells. This is because no shift in fluorescence is seen in the data gathered from the fluorescence-activated cell sorting machine (Figure 17). The positive control where the cells were treated with hydrogen peroxide does, however, show a significant shift in cells demonstrating fluorescence (Figure 17). This suggests that the experiment was performed correctly and as such that the entire theory as discussed above is incorrect. There is, however, the possibility that the results seen are due to DCFDA not being sensitive enough to reactive oxygen species other than hydrogen peroxide rather than these reactive oxygen species not being present. There is also a potential flaw in the method which could have led to the results for hydroxyurea being incorrect which will be discussed later.

Assuming that the results are correct, the most likely scenario, what they show is that the explanation for the survival of the *mec1-4* and *dun1Δ* mutant strains when petite does not lie in changes to the functioning of the DNA damage response or in changes to the levels of reactive oxygen species. This therefore means that other potential explanations for this observed survival must be developed and tested. As mentioned previously there is the potential that the survival of the petite versions of the strains is due to the removal of interactions between the DNA damage response pathway proteins and mitochondrial proteins which could affect apoptosis or that the removal of the mitochondria would affect gene expression (Vyas et al. 2016).

4.4. Potential sources of errors

One of the possible reasons for the confusing picture painted by the results may be that the results themselves do not accurately reflect the real situation. This could be due to one of several experimental errors or a combination of them all.

4.4.1. Calculations from optical densities

The main experimental error which is known was made during the course of the project was to do with not ensuring that cells, from which proteins were extracted from for Western blots, were growing exponentially in YPD solution when they were exposed to hydroxyurea. The optical densities of the various strains in culture were measured as should have been done. However, what was done next was to form a ratio with these optical densities which was then used to work out the volume of each culture to add to 30ml of YPD solution in such a way as would create cultures with equivalent concentrations of cells. This has no real purpose as differences in the concentrations of cells are almost eliminated when Laemmli loading buffer is added to the protein extracts. What should have been done, which is what was done for the cultures used for reactive oxygen species measurement later, is to use the optical densities to calculate how much of each culture to add into 30ml of YPD solution so that after the 3 hours of growth at 25^{o}C they were going to receive they would have an optical density at 600nm of between 0.3 and 0.4 which would indicate that they were in the logarithmic growth phase. Differences in cell sizes may also affect optical densities and thus calculations of cell numbers.

The optical densities of the cultures could have been measured after the 3-hour incubation to check if this was the case as well before adding the hydroxyurea treatment.

The reason why this error could have affected the results is that if the strain cultures had reached lag phase, and thus the cells within the culture were not actively dividing and therefore replicating their DNA, the treatment with hydroxyurea would not show its full effects. In the worst-case scenario where some cultures had reached lag phase while others were still in the logarithmic growth phase this could lead to the effect of hydroxyurea appearing less pronounced in some strains despite this not being the case. This could lead to incorrect conclusions on the effects of mitochondria on the DNA damage response and tolerance of hydroxyurea being made

The results of the Western blots do not on the surface appear to have been affected by this mistake but the effect of the removal of mitochondria on the response to hydroxyurea appearing to affect the phosphorylation of Rad53 and Sml1 could be due to this mistake. This is because as mentioned above it could be that the effect is seen because all of the strains except for the petite wildtype were in lag phase when the hydroxyurea treatment took place and so any subtleties in the effect of the mitochondria on the DNA damage response to hydroxyurea are not seen as the cells were not actively dividing. This is unlikely though as a sizeable number of Western blots were performed and all showed very similar results despite the fact that these Western blots used protein extracts from multiple repeat cultures and extractions. The likelihood that every time the cultures were performed they always ended up in lag phase is low but it is not impossible that all of the results seen from the Western blots for both Sml1 and Rad53 do not accurately represent the true effects of the removal of mitochondria.

4.4.2. Drug concentrations

Another experimental error which occurred during the project was the addition of incorrect volumes of hydroxyurea stock solution to YPD plates resulting in the plates with hydroxyurea having concentrations one tenth of what they should have been. Luckily this error was spotted the following day when reviewing the calculations made to work out the amount of hydroxyurea stock solution to add to the YPD agar

and these results were discarded. This discovery led to the calculations for other drug plate concentrations being re-examined. This search did not reveal any other instances of incorrect concentrations of drugs being added to plates.

4.4.3. TCA protein extraction procedure

The appearance of the non-petite wildtype column on the Ponceau stain of some of the Western blots for Rad53, such as in Figure 9, of a darker and more smudge-like migration of the protein may suggest that there was something that was done differently during the protein extraction from the non-petite wildtype strain. This is backed up by the fact that the non-petite wildtype column does not always appear as dark and smudge-like suggesting that the appearance of the column on these particular Ponceau stains is due to an error in the handling of the non-petite wildtype strain whose extracted proteins were used in these particular Western blots. One reason that could possibly explain this appearance is that during the TCA protein extraction part of a pellet may have been left in the supernatant containing the proteins which means that when it came to separating the proteins their movement was influenced by the presence of other cellular components. This is important as the blots for Rad53 where the non-petite wildtype column has this appearance show a band for Rad53 lower down the nitrocellulose membrane than the other strains and so the likelihood that this is an artefact created via a mistake and not a genuine phenotype must be considered.

4.4.4. Western blot antibody issues

When performing the primary antibody incubation for Western blots sometimes the blocking solution containing the primary antibody would be reused to reduce costs. This can be done as long as Sodium azide is added to the solution and the solution is frozen at -20^{o}C to prevent the degradation of the primary antibody. The addition of Sodium azide was not always done, however, leading to degradation of the antibody which likely resulted in the increased levels of background seen in the images of blots and more importantly this could have reduced the ability of the secondary antibody to bind to the primary antibody. This would result in less intense bands and may affect the way in which ImageJ analysis of the intensity of bands is interpreted. For this reason, the only ImageJ band intensity analysis results presented are those of blots performed using fresh primary antibody containing blocking solutions.

4.4.5. Different levels of oxygen

Another possible but unlikely reason for the differences in results seen could be due to the effect of hydroxyurea being oxygen dependent (Carter et al. 2005). Therefore, if the amount of oxygen the cells were exposed to during the spot test, while culturing for TCA protein extraction, and while culturing for reactive oxygen species measurement with DCFDA was different then the effect of the hydroxyurea would be altered.

4.5. Further experiments

Plainly the results of the experiments leave a large number of questions unanswered. The main question being what is the reason for the improved survival of *mec1-4* mutant on hydroxyurea containing media as well as at increased temperatures when it lacks mitochondria. Two possible reasons for the survival on hydroxyurea containing media have been tested and seemingly disproved.

The first being the lack of the mitochondria affecting the phosphorylation of proteins downstream of Mec1 in the DNA damage response pathway in response to hydroxyurea. This was tested by the use Western blots with antibodies for Rad53 and Sml1. These provided no full explanation for why the *mec1-4* mutation had increased survival when petite but did provide an interesting result which suggested that the *mec1-4* mutation reversed the decreased survival caused by the loss of mitochondria.

The second possible reason tested was that hydroxyurea caused an increase in the levels of reactive oxygen species inside the cells leading to cell death and that the removal of mitochondria would reduce the base levels of reactive oxygen species. The results from the use of the dye DCFDA, which is converted into a fluorescent form by reaction with reactive oxygen species, do not show differences substantial enough to show that the increased survival of the strains on hydroxyurea media was due to reduced levels of reactive oxygen species.

However, investigations into both of these tested possibilities could be continued because the data gathered for both the Western blots and the DCFDA assay comes only from strains cultured for 90 minutes in a 50mM hydroxyurea YPD solution. Therefore, the experiments could be repeated but with higher concentrations of hydroxyurea or a longer incubation time as the effect of the mitochondria on the response to hydroxyurea may be more discernible in more extreme conditions as when the strains were grown on media for the spot tests they were exposed to hydroxyurea for a much greater period of time than the strains incubated in YPD liquid for these experiments were. The experiments should also be performed without hydroxyurea with incubation at various temperatures so that the increased survival of the petite *mec1-4* strain at higher temperatures may be investigated. The experiments with DCFDA could also be redesigned to use other fluorescent probes which may be more sensitive to different reactive oxygen species which the DCFDA is not very sensitive to.

The experiments that were performed on the wildtype and *mec1-4* strains should also be performed on the petite and non-petite *dun1Δ* strains as well as at various temperatures like the *mec1-4* strains to investigate both the survival on hydroxyurea media and the apparent effect on the *dun1Δ* mutant's cold sensitivity. Performing Western blots for Sml1 may be useful in determining the reason for the survival of the *dun1Δ* mutant when petite. This is because in theory the results for Western blots for Sml1 in both petite and non-petite *dun1Δ* strains should show no breakdown of Sml1 due to there being no phosphorylation by Dun1. Any differences would therefore be very significant.

If the results of the Phos-tag Western blots for Rad53 for the hydroxyurea treated *dun1Δ* strains and both strains at their normal restrictive temperatures also showed the same results for phosphorylation whereby the wildtype petite shows reduced phosphorylation and the mutant shows no difference in phosphorylation it would provide further evidence that the increased survival of the mutants seen on the spot tests was due in part to the negative effects on survival of both the petite mutation and the *mec1-4* and *dun1Δ* mutations somehow counteracting one another. This would inform further investigations as potential links between the downstream effects of both mutations would need to be investigated. If the results do not show the same results in regard to the mutant strains having no change in phosphorylation while

petite but the wildtype still shows decreased phosphorylation of Rad53 and Sml1 in response to hydroxyurea then it would show that the improved survival of the petite *mec1-4* and *dun1Δ* strains compared to their non-petite counterparts is not due to the interaction between the petite mutation and the DNA damage response pathway as a whole, at least not in all cases. If the results showed that the petite wildtype did not consistently display the phenotype seen whereby it has reduced phosphorylation of DNA damage response pathway components then the results and the interpretation of them would be overturned and new explanations would need to be found for the improved survival in response to hydroxyurea of the *mec1-4* mutant when petite. In all of the cases above there is a set of experiments which would be useful in determining the reason for the effects seen due to the loss of the mitochondria.

This set of experiments which could be performed and had been planned but were not performed would be to generate strains which as well as having the *mec1-4* mutation or the *dun1Δ* mutation would be deletion mutants for various different mitochondria associated proteins such as the well conserved autophagy-related protein 8 which is involved in mitophagy (Antón et al. 2016). The growth of these strains on the same media and under the same conditions as petite and non-petite wildtype, *mec1-4*, and *dun1Δ* strains as well as petite and non-petite versions of the strains modified for testing would allow any novel interactions between these other proteins associated with the mitochondria and the DNA damage response pathway to be determined based on any differences in growth or survival. If novel interactions between these mitochondrial proteins and the DNA damage response were discovered it would provide a possible explanation for the results seen in petite strains but would then require further research into the connection between the two proteins.

Another possible experiment that could be undertaken is to generate multiple petites of the same strains and to grow all of these on the same media and under the same conditions as the already generated petites to ensure that the effect seen in the petites generated is due to the lack of mitochondria after ethidium bromide treatment and not the result of a spontaneous mutation or random mutation caused by the ethidium bromide treatment. If this turned out to be the case then all of the results presented would likely be irrelevant and quite useless as the random mutation that

caused the phenotypes seen would be unknown unless the genomes of the mutants were fully sequenced and compared to the genotype of the strains they were derived from. However, if these other petites generated showed the same results as the petites in this experiment it would provide a much more robust case for the results seen being the effect of the loss of mitochondria.

When generating these petites, strains with the same *mec1-4* or *dun1Δ* mutations but with different strain backgrounds could be used to determine if there is any difference in the effect of the petite mutation based on the subtle differences in the genotypes of the different backgrounds. This should be performed before other potential experiments as if the differences in these backgrounds are shown to have an effect on the phenotype presentation of the petite mutation it may influence the way in which the other experiments suggested would be performed. This is because it would mean that the *mec1-4* or *dun1Δ* strains selected for ethidium bromide treatment to generate petite versions of the strains would have to have the same background as each other and every other strain being tested whether they are wildtype or as suggested strains with modifications in the expression of different mitochondria associated proteins.

5. CONCLUSIONS

Overall, the results should be viewed as a starting point for further experiments and research. This is because the data gathered does not fit with either of the two theories developed after the preliminary investigation. The first of these theories was that the improved survival of the *mec1-4* and *dun1Δ* mutants when petite in response to hydroxyurea and differences in temperature was due to a modification in the operation of the DNA damage response pathway, to which both Mec1 and Dun1 belong, caused by the lack of mitochondria. The second of these theories was that the petite versions of the strains had a lower baseline level of reactive oxygen species due to the removal of the mitochondria which when carrying out oxidative phosphorylation to generate ATP also generate reactive oxygen species due to incomplete reactions. This lower baseline level of reactive oxygen species was theorised to improve the survival of the *mec1-4* and *dun1Δ* mutants because it meant that when there was additional reactive oxygen species generated by the hydroxyurea or as a consequence of the *mec1-4* and *dun1Δ* mutations at the different temperatures the level of reactive oxygen species in the individual *Saccharomyces cerevisiae* would not reach the point at which they would trigger cell death through either triggering the apoptotic pathways or due to the effects of the reactive oxygen species upon the cellular components.

Both of these theories in retrospect had issues from the start. In the case of the first theory the main issue, which was noticed when first developing the theory, was that the *rad53-K227A* mutant was unaffected by the lack of mitochondria in the petite strain. This creates an issue in the theory because Rad53 lies downstream of Mec1 and upstream of Dun1 in the DNA damage response pathway. Therefore, if the effect of the petite mutation was to modify the operation of the DNA damage response as a whole why would the *rad53-K227A* mutant not be improved in the same manner as the *mec1-4* and *dun1Δ* deletion mutants? It may be possible that the reason for this apparent issue with the theory was that the effect of the petite mutation which was causing improved survival required unaltered Rad53. This explanation, however, still has issues due to the *mec1-4* mutation and the *dun1Δ* mutation being in opposite positions in the DNA damage response pathway. Another issue with the first theory is that the petite wildtype strain has worse survival than its non-petite counterpart. If the lack of mitochondria in the petite *mec1-4* and *dun1Δ*

strains is improving their survival by improving the functioning of the DNA damage response in the DNA damage response pathway mutants then why is it reducing the survival of the wildtype which has a healthy and normally functioning DNA damage response? This reduced survival of the petite wildtype strain when compared to the non-petite wildtype strain is also an issue for the second theory. This is for the same reason as the first theory that if the improved survival of the *mec1-4* and *dun1Δ* mutants is due to there being a reduced baseline level of reactive oxygen species then the survival of the wildtype when petite should at a minimum be the same as the survival of the non-petite wildtype, if not greater. Since this does not appear to be the case from the spot tests this throws the entire theory into question based solely upon this result.

To test these two theories two sets of experiments were devised. The set of experiments for the first theory were a series of Western blots using antibodies for Rad53 and Sml1 both of which are part of the DNA damage response with the blots for Rad53 later being performed using Phos-tag reagent which increase the separation of the phosphorylated Rad53 from the unphosphorylated Rad53 allowing for a more accurate picture of the phosphorylation status to be determined. This set of experiments was only performed on protein extracts from the petite and non-petite wildtype and petite and non-petite *mec1-4* mutant under two conditions these being under no stress and after a 1-hour 30-minute treatment in a 50mM hydroxyurea solution. The reason for this was time constraints caused by a number of failed or low-quality Western blots due to the author's inexperience with this method.

The findings from the Western blots were initially quite confusing and seemingly contradictory for the Western blots for Rad53 which led to Phos-tag reagent being used in the Western blots. This produced a result which initially appeared to be that there was no difference in the phosphorylation of Rad53 between the petite and non-petite strains in response to hydroxyurea. However, comparing the results of the Phos-tag Western blots with the initial Western blots for Rad53 it became apparent that the phosphorylation of the wildtype in response to hydroxyurea may have been reduced when petite. This appeared to be quite an odd result but, when looking back at the spot tests involving hydroxyurea, it was found that the wildtype petite had a reduced survival on hydroxyurea media. This suggested that the petite mutation was determinantal to the survival of the wildtype which might fit with the apparent result

from the Western blots that the wildtype had a reduced rate of phosphorylation of Rad53 in response to hydroxyurea when petite. Comparing the patterns seen in the petite wildtype to those of the petite *mec1-4* strain brings up a number of questions as the petite *mec1-4* strain shows no difference in the phosphorylation of Rad53 in response to hydroxyurea compared to the non-petite *mec1-4* strain. It was theorised that this apparent difference may provide a partial explanation for why the petite *mec1-4* strain has improved survival on the hydroxyurea containing media when compared to the non-petite *mec1-4* strain. This theory is that while both the *mec1-4* mutation and the loss of mitochondria are detrimental to survival the ways in which these changes reduce survival run counter to one another and so when both are present these detrimental effects are nullified, but this is completely untested and so further experiments must be performed. The first experiment amongst these should be to perform another set of Western blots using the same strains but at different temperatures and concentrations of hydroxyurea whilst also ensuring that the *Saccharomyces cerevisiae* cells are not in lag phase and are actively replicating at the point that the cultures are exposed to the stress which is being tested. The Western blots for Rad53 would also need to be performed for the petite and non-petite *dun1Δ* strains as alongside the other experiment the results can be used to determine whether the results described above actually have any link to the improved survival of the *mec1-4* and *dun1Δ*. However, even if the results of these further experiments did not confirm the theory it would still leave an interesting oddity that may be worthy of further experimentation. Therefore, another experiment that could be performed would be to compare different petite strains which have been generated using both spot tests and Western blots to determine whether the results seen are consistent between the different petite strains.

Amongst the data gathered from the Western blots for Sml1 the most important result came from the ImageJ analysis of two of the blots which showed that there was an increase in the presence of Sml1 in the petite wildtype in the presence of hydroxyurea. This fits with the data from the Western blots for Rad53 as the downstream effect of a decreased phosphorylation of Rad53 is a decreased phosphorylation of Sml1 as shown in Figure 1 and thus a reduction in the breakdown of Sml1 meaning that there is a greater presence of Sml1 when measured. While this is useful in confirming the observations of the Western blots for Rad53 it also

leads to further questions about how the *dun1Δ* mutant fits into the proposed theory which must be determined by the further experimentation already described. Another experiment which would be especially useful for determining the nature of the petite mutation's impact on survival would be to generate strains which as well as having the *mec1-4* or *dun1Δ* mutations would also have deletion mutations of the genes for a number of different mitochondria associated proteins. These mutants could then be tested in the same manner as the petite and non-petite strains have been in the experiments that have been performed. This would possibly allow for the exact proteins which are impacting survival to be determined providing a starting point for further research into the possible reasons for the Warburg effect being observed in cancerous cells.

The experiment to test the second theory was to add DCFDA dye to cultures of the petite and non-petite wildtype and *mec1-4* strains with and without hydroxyurea as well as hydrogen peroxide which is used to provide a positive control. The DCFDA dye is taken into cells and reacts with reactive oxygen species to become a fluorophore. By using a FACS machine and comparing the fluorescence of a fixed number of cells of the strains exposed to hydroxyurea to the positive control it was determined that when unstressed the petites had similar levels of fluorescence suggesting that the removal of mitochondria did not reduce the baseline level of reactive oxygen species during this experiment. When exposed to hydroxyurea the wildtype and *mec1-4* strains both petite and non-petite showed the same level of fluorescence. When this is compared to the cells of the same strains exposed to hydrogen peroxide, the positive control, and the unstressed negative control it shows that there is no change in the fluorescence in the strains exposed to hydroxyurea suggesting that there is no change in the levels of reactive oxygen species when *Saccharomyces cerevisiae* cells are treated with 50mM hydroxyurea for 1 hour and 30 minutes. Overall, these results, due to the change in the fluorescence of strains exposed to the positive control, suggest that the theory that the removal of the mitochondria is improving survival due to a reduction in reactive oxygen species within the cells is completely inaccurate. Despite this, further repeats of this experiment should be performed using not only the same concentration of hydroxyurea for the same time period but also larger concentrations of hydroxyurea or longer incubation periods to fully dismiss this theory. Another reason for this is

that there may have been problems with the experiment involving an inadequate supply of oxygen to the cells during incubation with hydroxyurea which would affect the impact of hydroxyurea on cells.

To conclude, the results of the project are limited and the impact of these results and their causes will require further experimentation to determine the implications of what has been observed.

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