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The Role of Mre11-Rad50-Nbs1 CTBP-Interacting Protein (MRN-CtIP) In Providing Resistance against Camptothecin

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SBS

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ………………………………………….. (candidate)

Date ……………………………………………..

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed …………………………………………. (candidate)

Date ………………………………………….

(Approx. 12400 words)

DEDICATION

This project research is dedicated to the almighty God for his guidance and steadfast protection during my year in this institution. It is by his abundant grace that we are able.

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LIST OF ABBREVIATION

Deoxyribonucleic acid (DNA)   ultraviolet light (UV)   ionizing radiation (IR)

Single strand breaks (SSB)   double strand breaks (DSB)

Reactive oxygen species (ROS)   DNA damage response (DDR)

Topoisomerase 1 (Top1)   Topoisomerase 2 (Top2)

Mre11-Rad50-Nbs1 (MRN)   Homologous recombination (HR)

Nijmegen breakage syndrome 1(Nbs1)   Meiotic recombination 11(Mre11)

Ataxia telangiectasia mutated complex (ATM)   Breast cancer 1 (BRCA1)

Mediator of DNA damage checkpoint 1 (MDC1)   ATM-Rad3-Related (ATR)

CTBP1-interacting protein (CtIP)   Carboxy-terminal binding protein 1 (CTBP)

ATR-interacting protein (ATRIP)   Non-homologous end joining (NHEJ)

Mis-match repairs (MMR)   Base excision repairs (BER)

Nucleotide excision repair (NER)   Global genome NER (GG-NER)

Transcription coupled repair (TCR)   Tyrosyl-DNA phosphodiesterase 1 (TDP1)

Replication protein A (RPA)   Camptothecin (CPT)

Single stranded DNA (ssDNA)   Fork head associated domain (FHA)

BRCA1 C-terminal domain (BRCT)

5-floro orotic acid (5FOA)

Recombinase mediatted cassette exchange (RMCE)

Methyl methane sulphonate (MMS)

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ABSTRACT

MRN – CtIP protein complexes in humans have been implicated in the resistance of cancer cells to camptothecin (CPT). The experiment investigated randomly mutated ctp1 gene, in *S. pombe*, which is a homolog of CtIP, to discover mutations on ctp1 DNA sequence that lead to sensitivity to CPT but not to other genotoxins. Cre - lox RMCE was performed *in vitro* to recombine cassettes carrying mctp1 into cre expression plasmid pAW8 - ccdb, and transformed into *E. coli* to generate a plasmid library. Plasmid library were transformed into *S. pombe* and cre expression on plasmid switched on to catalyze *in situ* RMCE, which recombined mctp1 into endogenous gene location in *S. pombe* base strain, in place of ura4$^+$ marker. In order to create library of mutants for screening colonies that showed sensitivity to drug agents, ura4$^-$ cells possessing mctp1 cells were selected for by the action of 5FOA and propagated to screen CPT ($4\,\mu M$ & $8\,\mu M$), MMS (0.004% & 0.008%) and phleomycin ($2.5\,\mu g/ml$ & $3.5\,\mu g/ml$). A total of eight plate-isolates sensitive to one or more genotoxins were discovered, of which one isolate showed sensitivity to CPT alone. This reiterated that defects in ctp1 results in sensitivity to genotoxins, due to inability of defective mctp1 to activate Mre11 complex and bring about DSB resection and repair. However, screening for isolates is a statistical effort that requires continuous building up of libraries to generate more data that could provide more outcomes. Thereby underlying molecular effects of unique isolate that showed phenotype to CPT could not be analyzed to draw more conclusions.

CHAPTER ONE

REVIEW

1.0)   **Summary**

DNA damage is as a result of endogenous and exogenous agents that predispose organisms to cancers and other degenerative diseases. Human body mechanisms have evolved DNA damage response and repair pathways that enable maintenance of genome integrity, in which this process is highly regulated through cell cycle checkpoint arrest. Nevertheless, DNA damage still occurs and there exists mechanistic factors that promote and defend against these damages. This results to an unending paradox that often requires treatment. Topoisomerase are key enzymes that reduce torsional stress on DNA by their ability to create nicks on the DNA during replication and transcription processes. Topoisomerase inhibitors like camptothecin are used for treatment of colorectal and ovarian cancers, and functions by locking topoisomerase 1 & 2 covalently on the DNA thereby killing fast replicating cells. Studies have shown that resistance of cells to camptothecin does arise, and are attributed to the removal of the induced covalent attachments of topoisomerase from the DNA ends to allows re-ligation of the gap, and continuation of replication and transcription processes. The MRN complex have been described as major contributors to this event alongside CtIP/Ctp1, and have been proposed as good drug targets to increase camptothecin sensitivity. The objective of this is to analyze the mechanism of Ctp1/CtIP dependent topoisomerase removal in a molecular detail, by using Ctp1, a homolog of CtIP found in *Schizosaccharomyces pombe (S. pombe)* as a cell model organism. I intend to screen for regions of its gene that are sensitive to camptothecin but are not sensitive to other DNA damaging agents. This is to be done by inserting mutated Ctp1 gene through loxP – loxM mediated Cre recombination into S.pombe base strains, and to express the gene on selected DNA damaging agents for phenotypes that shows sensitivity to them.

1.1) GENOME INSTABILITY AND DNA DAMAGE REPAIR

All biological molecules decompose spontaneously in its natural environment. Deoxyribonucleic acid (DNA), a biological molecule that encodes genetic information of living organisms, is a stable molecule that is inert outside cell environment (Lindahl, 1993). However, due to its occurrence in large amounts in the human genome of about three billion base pairs, organized into 22 pairs of chromosomes, it is often exposed to degradation and damage (Venter et al., 2001).

1.1.1) **Types Of DNA Damage**

DNA damage is simply a chemical alteration on the nucleotide base(s) of the DNA strand that if unrepaired results into mutation(s), which can lead to degenerative diseases (Cha et al., 2013). Genetic instability is brought about by DNA mutations and is fuelled by DNA replication errors and damage inflicted on DNA that goes unrepaired (Deshpande et al., 2009). Damages to DNA could be spontaneous or induced; and can be caused by exogenous or endogenous agents, which can lead to base damage, formation of clustered lesions and single strand breaks (SSB) or double strand breaks (DSB) (Hakem, 2008; McIlwraith, 2008; Iyama et al., 2013). DNA damage can also affect the sugar phosphate backbone of the strand leading to a break on the strand, or affect the nucleotide base pairing of the strand leading to formation of lesion(s) on the DNA molecule (Figure 1.1).

Exogenous induced DNA damage occurs as a result of environmental factors such as ultraviolet light (UV), genotoxic chemicals and ionizing radiation (IR), which can lead to acute DNA damage (Hoeijmakers, 2001; Cha et al., 2013). UV light remains the most pervasive agent of DNA damage creating thousands of lesions on DNA for each cell exposed (Iyama, 2013). IR from X-rays and radiation therapy can lead to SSB & DSB, while mutagenic chemical pollutants like aflatoxins & heterocyclic amines released by contaminated food materials, chemotherapeutic drugs used for cancer treatment, and alkylating agents like ethyl methane & methyl methane sulphonate (MMS) all have adverse effect on the DNA integrity by causing lesions (Jackson et al., 2009).

Furthermore, aerobic organisms face a major challenge of faithfully maintaining their genetic materials from unavoidable spontaneous attacks from hydrolysis, reactive oxygen species (ROS), and from other intracellular metabolites that modify DNA bases. These endogenous agents can be as a result of DNA replication errors that bring about mismatched base pairs (Barnes et al., 2004). DNA aberrations can be introduced during DNA replication and DNA strand break caused by abortive topoisomerase 1 (Top1) and topoisomerase 2 (Top2) (Jackson et al., 2009). Cellular metabolism can create persistent ROS generated by the mitochondria as by-products, which can cause oxidative damage to DNA (Hoeijmakers, 2001;Cha et al., 2013). ROS and reactive nitrogen are also produced by macrophages and neutrophils at sites of infection and inflammation (Jackson et al., 2009), although evolution has helped reduce the damage of this by-product through antioxidant defense mechanisms.

Spontaneous hydrolysis of DNA also causes deamination or depurination of the nitrogenous bases of DNA. In metabolic active cells, DNA occurs in fully hydrated B-forms that expose it to adduct of depurination (Lindall, 1993). Depurination leads to loss of Adenine and Guanine bases and creates apurinic sites that can cause mutation through cleavage of the glycosyl bond after the reaction (Cavalieri et al., 2012). On the other hand, deamination results from spontaneous hydrolysis of Cytosine, Adenine, Guanine and 5-methyl cytosine (Hoeijmakers, 2001). Deamination of cytosine generates an aberrant base Uracil, and it's likely to occur more rapidly in single stranded regions like replication forks and transcription bubbles, which lack complementary strands to uphold genetic integrity (Barnes et al., 2004). Also 5-methyl cytosine, which is methylated cytosine brought by epigenetic mechanisms, deaminates to thymine at a rate threefold that of un-methylated cytosine. This creates mutation and erosion of cytosine in the DNA. Adenine and guanine both deaminate to unnatural bases hypoxanthine and Xanthine respectively, which are easily recognized by certain enzymes (Barnes et al., 2004; Hoeijmakers, 2001)

1.1.2) **Consequences Of DNA Damage**

There several possible biological consequences of DNA damage, thereby cells have evolved conserved mechanisms of detecting damage to its DNA. These mechanisms ensure cell's DNA is intact before allowing replication and cell division to occur (Cha et al., 2013; Iyama et al., 2013; Kaas et al., 2010). Acute effects of DNA damage leads to cell cycle arrest at specific checkpoints G1, S, G2 & M, to allow repair of lesions before they become permanent mutation. When damage to DNA becomes too significant the affected cell goes through intricate signaling to initiate apoptosis. Failure to arrest or stop damaged DNA replication can lead to accumulation of damages contributing to oncogenesis (Deshpande et al., 2009;Hoeijmakers, 2001). It is presumed that about 10^5 DNA lesions occur in a mammalian genome each day as a result of DNA damage, which can induce mutagenesis through; base substitution, small insertion & deletion, chromosomal rearrangement & loss, of which predisposes the organism to neurological disorders, immunodeficiency, and cancer (Iyama et al., 2013; Deshpande et al., 2009; Hakeem, 2008).

SSB occur more rampantly than DSB, but DSB tends to be a more cytotoxic form of DNA damage (Kaas et al., 2010). DSB can be induced by UV light, x-rays, and mutagenic chemicals or during replication of SSB at replication forks, in which these breaks are relevant to repair machineries conserved in mammals. Cells that employ recombination activities, like B-cells and T-cells during somatic recombination and gonad cells during homologous recombination may be sensitive to DSB. When not properly regulated these lesions induce various sorts of chromosomal aberrations including aneuploidy, chromosomal deletions and translocation, which are all occurrences associated with cancers (Hoeijmakers, 2001). Therefore, it is essentially important for cells to efficiently possess a plethora of DNA damage response (DDR). If this is not done it leads to cancer development and eventually death of the organism (Hakeem, 2008)
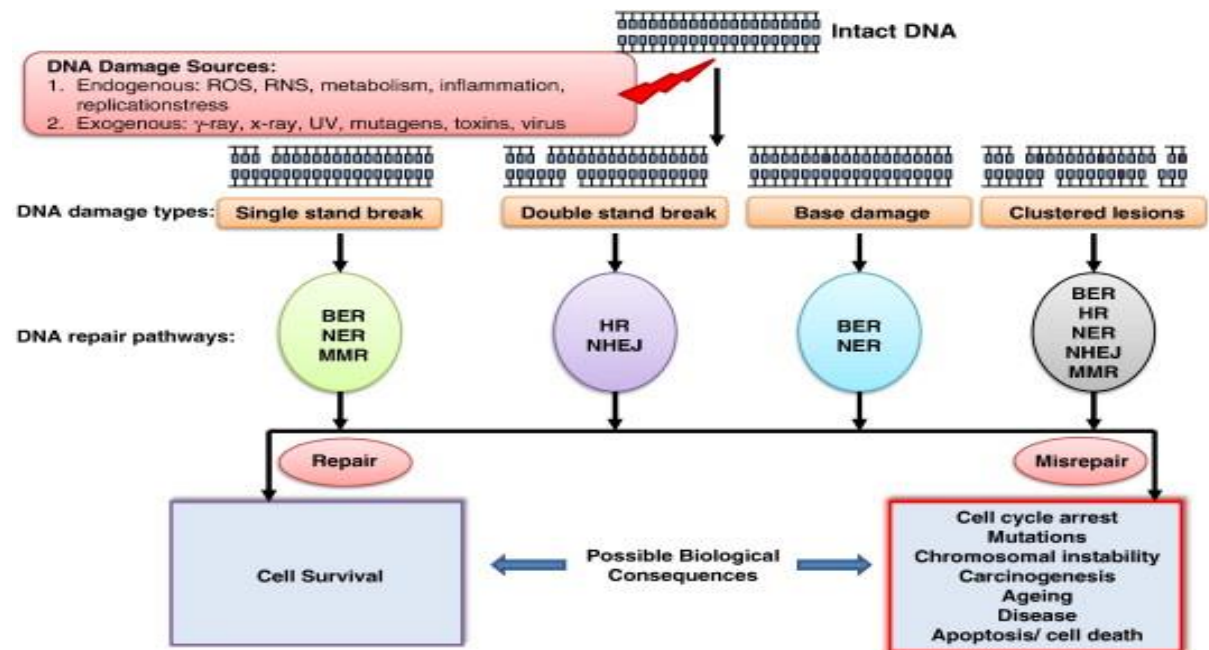
Figure 1.1: Schematic diagram illustrating the DNA Damages and DNA damage repair mechanisms [From (Aziz et al., 2012)] *BER* Base excision repair, *NER* Nucleotide Excision repair, *MMR* Mismatch repair, *HR* Homologous recombination, *NHEJ* Non-Homologous end joining
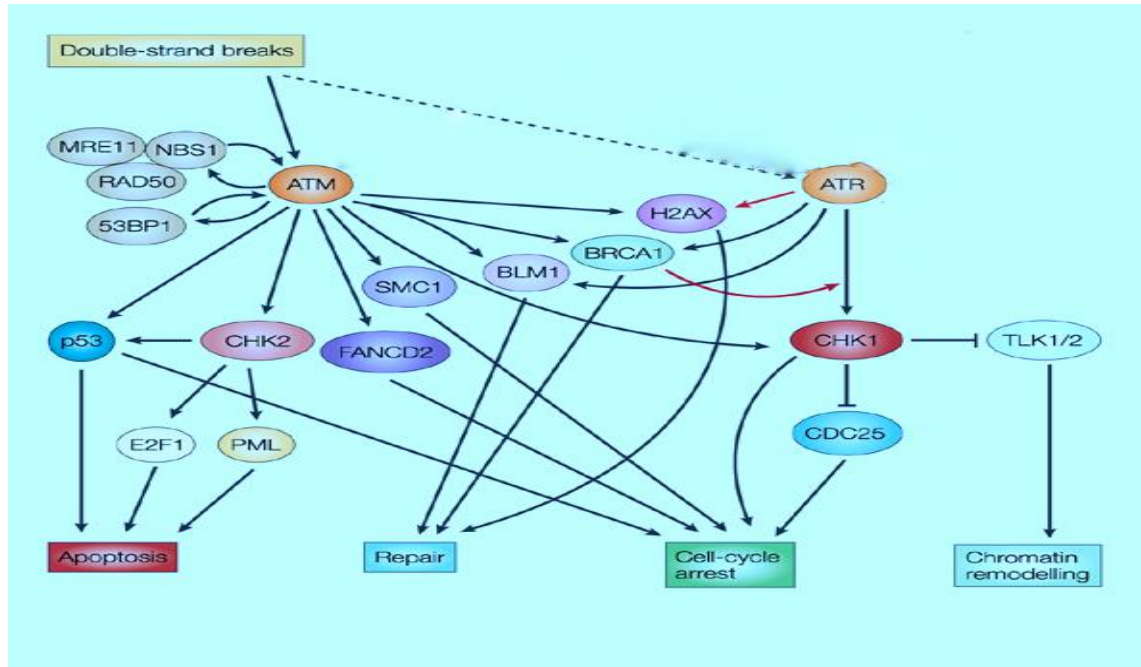
Figure 1.2: A schematic diagram illustrating how the Mre 11-Rad50-Nbs1 complex initiates DNA Damage repair by interaction with ATM, which leads to recruitment and activation of other downstream factors like that act as transducers, mediators or effectors in DNA maintenance. Their activation trigger checkpoint arrests, repair and if repair ultimately cell death. (Edited from; Zhou et al., 2004 Nature reviews)

1.2) DNA DAMAGE RESPONSE, DNA REPAIR AND CANCER.

DDR and repair is carried out through intricate sets of fine regulated signaling pathways (cell Checkpoint arrest, DNA repair and apoptosis) (Cha et al., 2013). Due to existence of several types of DNA damage events, no single repair process can be able to cope with every kind of damage. Therefore, evolution has made various DDR and repair mechanisms that cover most insult inflicted on the DNA of a cell. These repair pathways are in some instances, highly conserved in in eukaryotes suggesting that they have been in existence for a long time (Aziz et al., 2012; Hoeijmakers, 2001). To be able to repair damaged DNA, cells require checkpoint mechanisms to play crucial roles in their regulation and damage control.

1.2.1) **DNA Damage Checkpoints**

In the light of a plethora of DNA damaging agents, repair of DNA is regulated by series of signaling proteins that serve as sensors/detectors, transducers, mediators and effectors in order to result to a cellular response (Aziz et al., 2012; Cha et al., 2013). One of the outcomes of the cellular response is DNA checkpoint arrest, and this can be achieved either transiently in order to repair damage cells, or permanently so as to place cells in a senescence state (Terzoudi et al., 2005, 2011). This mechanism is best understood during its response to DSB, in which Mre11–Rad50–Nbs1-CTBP1 interacting protein (MRN-CtIP) protein complex play crucial roles. Initiation of cell cycle checkpoint arrest is dependent on transient recruitment of Mre11-Rad50-Nbs1 (MRN), a nucleus protein complex at the DSB region where it then acts as a detector of DNA damage (Hakeem, 2008; Cha et al., 2013; Iyama et al., 2013). MRN complex is highly conserved and is involved in nearly every aspect of the DNA DSB damage response, which include DNA damage sensing, signaling and repair (You et al., 2010).

Studies show homologous recombination (HR), which is a DNA repair pathway for DSB is activated when Nijmegen breakage syndrome 1(Nbs1) recruits Meiotic recombination 11(Mre11) and Rad50 into the nucleus to bind DNA DSB ends (De Lorenzo et al., 2013). When binding of the MRN protein complex to DNA DSB occur, it initiates the recruitment of ataxia telangiectasia mutated complex (ATM). However, this is done in a in a histone protein variant (H2AX) dependent manner, and it leads to the subsequent activation of the ATM complex (Hakeem, 2008). ATM is a transducer-protein kinase that induces recruitment and activation of various other key proteins by interaction with the Nbs1 subunit. On interaction with Nbs1 it

phosphorylates H2AX at its serine139 residue to form y-H2AX, leading to chromatin remodeling and more recruitment of DDR proteins, in which use y-H2AX as a loading platform for more activation (Jackson et al., 2009; Hakeem, 2008; Cha et al., 2013). ATM transduction is reported to signal recruitment of ATM substrate mediators like the mediator of DNA damage checkpoint protein 1 (MDC1), 53BP1, BLM1, Structural maintenance of chromosome SMC1, and Breast cancer 1 (BRCA1) to the site of DNA DSB, triggering checkpoint arrest and DNA damage repair through downstream kinases chk2 & chk1 and through p53 to trigger apoptosis (Hakeem, 2008; Cha et al., 2013) (Figure 1.2).

The S/G2 phase of the cell cycle is when Homologous Recombination (HR) mechanism operates, and these ATM dependent substrates attack Chk2 so as to reduce cyclin dependent kinase (CDK) activity and arrest cell cycle, under the regulation of p53 gene (Jackson et al., 2009). MRN complex is also important in the signaling of DSB repair factors critical to the HR pathway like CTBP1-interacting protein (CtIP), BRCA1 and Rad51 (Cha et al., 2013). CTBP1 (Carboxy-terminal binding protein 1) interacting protein (CtIP), play central role in cell cycle response to DSB as a tumor suppressor protein, which control the decision to repair DSB (You et al., 2010) (Figure 1.4). The CtIP proteins are conserved in eukaryotes, and they interact with N-terminus and C-terminus of Nbs1 sub-unit of MRN, localizing MRN at the site of DSB to initiate HR strand resection (Cha et al., 2013; Heyer et al., 2010). MRN complex recruits phosphorylated CtIP at DSB resection, which activate endonuclease activity of Mre11 sub unit (De Lorenzo et al., 2013). MRN-CtIP interaction initiates resection of DSB to create single strand DNA (ssDNA) overhang and recruitment of replication protein A (RPA). This brings about ATR-dependent phosphorylation by ATM-Rad3-Related (ATR), and ATR-interacting protein (ATRIP) of RAD17, BRCA1 and other repair factors and ultimately activation of Chk1 & Chk2 (Cha et al., 2013) (Figure 1.3)

However, CtIP also influences DDR choice of HR or non-homologous end joining (NHEJ) repair pathways during the S/G2 phase by its interaction with BRCA1 during DSB (De Lorenzo et al., 2013). ATM and ATR with DNA dependent kinase catalytic subunit (DNA-PK) are members of the PIKK family of serine/threonine kinases, which attack DSB by targeting Chk1 & Chk2, in order to reduce CDK activity by various interactions, some of which are mediated by action of p53 (Eyfjord et al., 2005; Cha et al., 2013). Unsuccessful DNA repair leads to p53 dependent apoptosis, or forces the cell to enter a state of senescence (Hakeem, 2008). Chk2 together with Chk1 are evolutionary conserved protein kinases that regulate cell cycle progression through

checkpoint activation and DNA repair respectively. Inhibition of CDK arrests cell cycle progression at G1-S phase, Intra–S phase and G2-M phase cell cycle checkpoints. At any phase in the cell cycle DNA DSB is repaired by NHEJ pathway, which re-ligates both ends of DNA strand while in the S & G2 phase HR is of higher priority owing to the presence of homologous sister chromatids in order to achieve high fidelity repair (Jackson et al., 2009) (Figure 1.4).
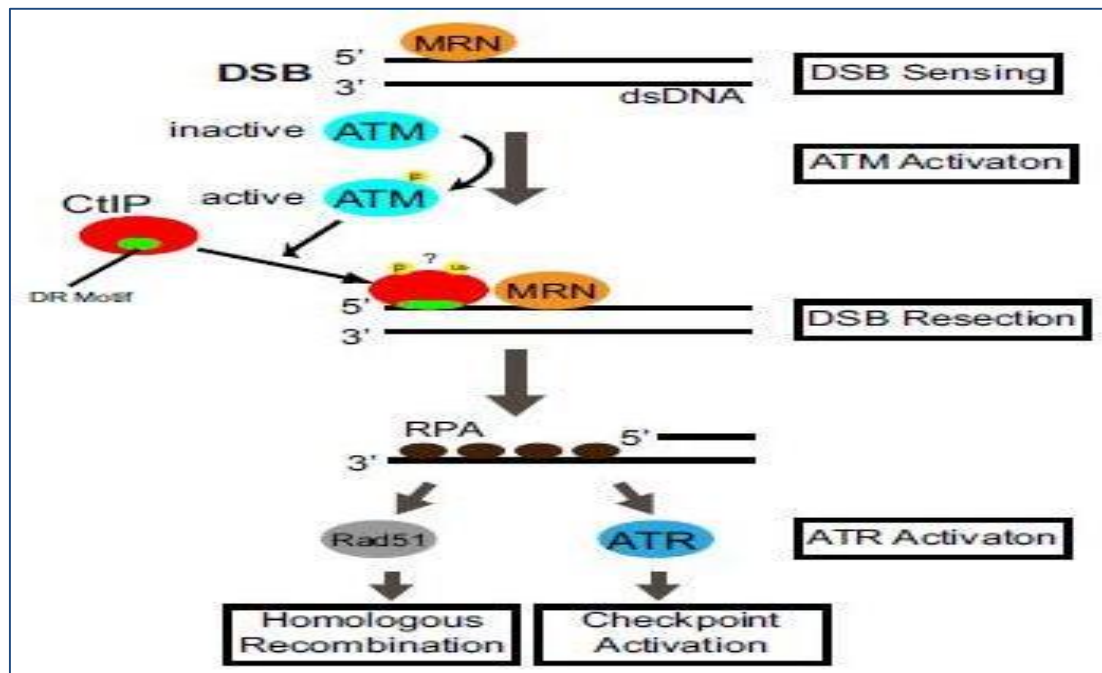
Figure 1.3: Pathway shows how MRN complex is recruited to DSB, which leads to activation of ATM and recruitment of repair factor/tumor suppressor CtIP. Phosphorylated CtIP interacts through its DNA repair (DR) motif, with Nbs1 domain of MRN complex. This leads to activation of Mre11 that initiates homologous recombination by carrying out DSB resection and recruitment of replication protein A (RPA).
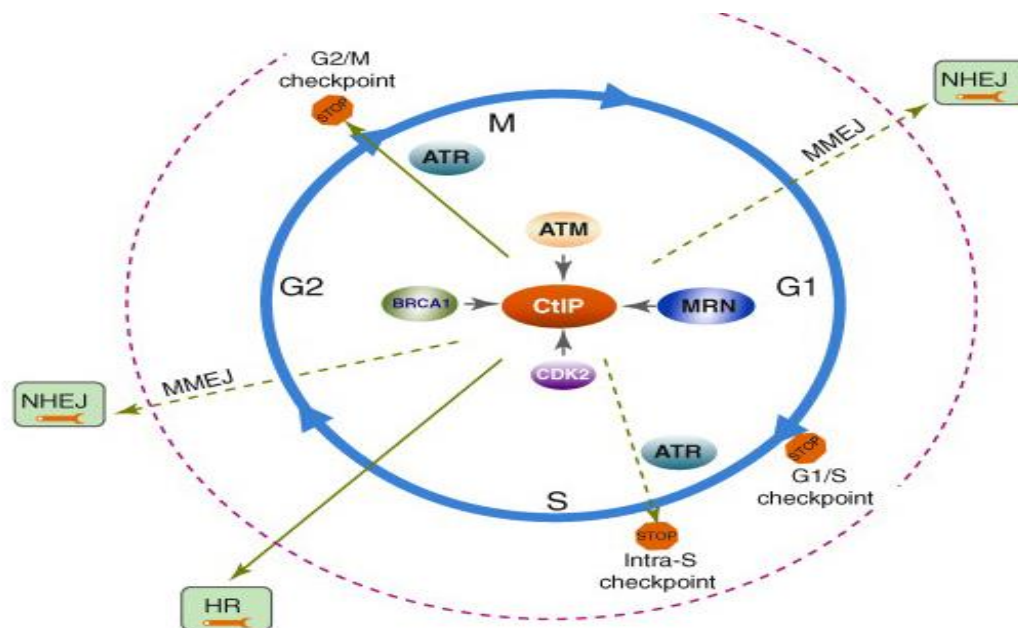
Figure 1.4: Diagram showing the role of CtIP in cell cycle response via its interaction with other DNA repair proteins, by which it controls decision to repair DNA DSB. Homologous Recombination (HR) is carried out during the S & G2 phase of the cell cycle [From (You et al., 2010)].

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1.2.2 **Homologous Recombination**

Homologous recombination is required for accurate chromosome segregation during meiotic division necessary for sexual reproduction (Heyer et al., 2010). HR is also a high fidelity repair pathway that dominates the S/G2 phase of the cell cycle when DNA is being replicated, in which the presence of a homologous sister strand provides template for repair of DSB (Hoeijmakers, 2001; Mc Kinnon et al., 2007; Kass et al., 2010). Like the NHEJ it is critical for genome stability and it's important at stalled replication fork (RF) of SSB where it carries out resection and helps aid continuation of replication (Aziz et al., 2012).

MRN-CtIP Interactions in Homologous Recombination Damage Repair

MRN complex, which plays role in sensing DNA damage and signaling checkpoint activation, plays its most active role in DNA DSB repair (You et al., 2010). HR pathway is activated when components of Nbs1 brings its binding partners Mre11 and Rad50 to the nucleus where it binds to DNA DSB (De Lorenzo et al., 2013). This results to recruitment of DSB repair factors like phosphorylated CtIP, which activates endonuclease activity of Mre11 and lead to resection of 5' end of DNA DSB to generate a 3' overhang (You et al., 2010; De Lorenzo et al., 2013). Exonucleases elongate the resection to about several thousand base pairs, while RPA coats DNA to prevent it from annealing to form secondary structures. The RPA is then replaced with Rad51 that forms a nucleofilament to aid the ssDNA invade undamaged template in order to copy missing strand (Jackson et al., 2009; De Lorenzo et al., 2013). The entire process is linked to cell cycle checkpoint and cyclin dependent kinases (CDK2), and is thought to facilitate the phosphorylation of CtIP before it binds to MRN complex (De Lorenzo et al., 2013; Kass et al., 2010).

Cancer predisposition in organisms can be caused in HR mechanisms by defects in the ataxia telangiectasia (AT) and Nbs1 genes both linked to AT linked disorders (ATLD) and Nijimegen breakage syndromes (NBS), which also involve defective Mre11 (Jackson et al., 2009; Hakeem, 2009). Inherited defects in BRCA1 and BRCA2 have been linked to breast and ovarian cancer and further studies are evident that the components of these repair pathways could be good cancer drug targets (Hoeijmakers et al., 2001; Eyfjord et al., 2005; Cha et al., 2013). Recent studies have also inferred that mutations to human CtIP could lead to congenital microcephaly disorders, and a recessively inherited dwarfism condition known as Jawad and seckel syndrome (Makharashvili et al., 2014; Qvist et al., 2011).

1.2.3 Factors That Lead to Tumor Growth and the Role of CtIP

Genomic instability is one of the major drivers of tumor growth (Cha et al., 2013). Ultimate goal of cell division in non-cancerous cells is to multiply its genetic information into two daughter cells, which means any aberrant cell division would result in various alterations in daughter cells' genetic makeup (Shen, 2011). Therefore, to prevent tumorigenesis cell division is a tightly regulated process and employs various mechanisms to maintain genome integrity as stated above. Tumors are complex tissues composed of multiple distinct cell types, with the ability to generate stemness and participate in heterotypic interactions with one another (Hanahan et al., 2011). The lack of regulation in tumor cells is an indication that a collapse in regulation of proliferating cells can lead to its uncontrolled growth. This lack of regulation can be as a direct effect of somatic mutation of activated growth factor receptor like the B-raf protein, constituting to prolonged signaling through the Raf mitogen activated proteins (MAP), or disruption of negative feedback signaling that regulates proliferative cell signaling (Hanahan et al., 2011). This enables the cell to resist apoptosis while proliferating continuously and inducing metastasis through angiogenesis.

CtIP is a transducer/effector nuclease protein that is a tumor suppressor, which controls decision to repair DSB by either HR or NHEJ (You et al., 2010; De Lorenzo et al., 2013). CtIP is believed to be essential for cellular proliferation as it forms a complex with MRN in budding yeast as well as mammalian cells. According to Nakamura et al. (2010) studies have also shown that CtIP interacts with BRCA1 to repair trapped Topoisomerase I (TopI) from DNA strand break ends, by degradation of the Top1 compound. The CtIP through its catalytic property also plays important role in endonuclease dependent removal of damaged nucleotides from DSB (Nakamura et al., 2010). CtIP has an indirect role in checkpoint activation and it promotes ATR signaling and DSB resection by interaction with MRN in mammals (You et al., 2010; Sartori et al., 2007) (Figure 1.4). However, CtIP catalytic activity has a clear preference for 5' strands ends at RF that are often generated by Topoisomerase II (TopII) and Spo11 (Makharashvili et al., 2014; Wang et al., 2014). Studies by You et al. (2010) have shown that knockout of one CtIP allele predisposes mice to multiple types of cancers, while mutations in it have been found in human tumor cell lines. Mammalian CDK is believed to control HR through modulation by cell cycle dependent phosphorylation, as well as DNA damage – induced phosphorylation of CtIP proteins by ATM & ATR kinases of which CtIP promotes DNA end resection activity of other nucleases (Wang et al., 2013; Makharashvili et al., 2014).

1.3   MRE11 RAD50 NBS1 – CTB1 INTERACTING PROTEINS.

Studies have shown that MRN complex is implicated in telomere maintenance, meiotic recombination and DNA damage repair where it facilitates Homologous Recombination (Zioikowska-Suchanek et al., 2013; Reczek et al., 2013). The MRN complex has been implicated in its role in removal of covalently bound human TopI & TopII, from DNA strand break ends in cell-lines treated with Camptothecin (CPT). Human topoisomerases has also been identified as an important drug target for the treatment of cancer (Lee et al., 2012).

1.3.1   **Meiotic Recombination 11**

Mre11 is a highly conserved protein nuclease that possesses ssDNA endonuclease activity and 3'-5 exonuclease activity at DNA DSB dead ends (Stracker et al., 2011; Thompson, 2012). Genetic studies on *S. cerevisiea* used in providing useful starting roles in humans have also implicated this protein in removal of covalently bound 5'-TopII from DNA strands both *in vivo* and *in vitro* (Lee et al., 2012). Importantly cells deficient in Mre11 exhibit a major repair defect as nucleolytic processing by Mre11 is of fundamental importance to DNA repair, which is distinct from MRN-mediated control of ATM signaling (Quennet et al., 2011). According to Zioikowska-Suchanek et al. 2013, molecular variant of Mre11 gene have been identified in breast and ovarian cancers, but is not implicated in laryngeal cancer. Mre11 associates with the Walker A&B and coiled-coil domains of Rad50 through its central globular domain and its DNA binding site easily accommodates ssDNA (Stracker et al., 2011). Studies by Stracker et al. 2011 also indicated that its 3'- 5' exonuclease activity and ssDNA endonuclease activity do not depend on Rad50 and Nbs1. It has been proposed that Mre11 nuclease activity is involved in removal of topoisomerase-like spo11 protein and also affects the removal of covalently bound DNA – Top I & II complex (Hartsuiker et al., 2009; Stracker et al., 2011).
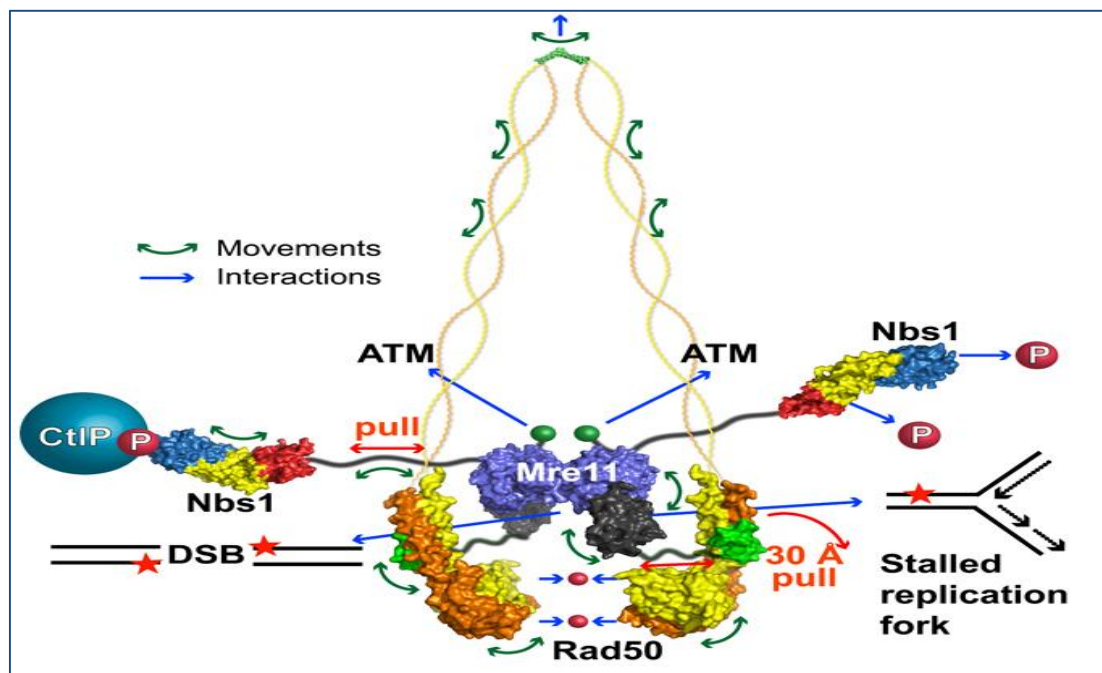
Figure 1.5: shows a diagram of the MRN complex interaction through the FHA domain of Nbs1 with phosphorylated CtIP at site of DNA double strand break. Nbs1 a substrate of ATM recruits binding partners Mre11 & Rad50 unto double strand break, and with the influence of CtIP activates Mre11 to initiate damage repair. [From (William et al., 2011)]

TABLE 1.1: Summary of proteins involved in double strand break end resection

PROTEIN (MAMMALS)	KEY PATHWAY	ORTHOLOGS S. pombe/ S. cereviscae	FUNCTIONS	Camptothecin Sensitivity in mammalian cells
MRE11	HR	Mre11	Single strand endonuclease & 3'-5' exonuclease activity	Deficient cells are sensitive to CPT
RAD50	HR	Rad50	Stimulates Mre11 3'-5' exonuclease activity	_
Nbs1	HR	NBS1/Xrs2	Brings in binding partners into the nucleus & has an N-terminal which contains FHA domain that interacts CtIP/Ctp1	Deficient cells sensitive to CPT
CtIP	HR	Ctp1/Sae2	Tumor suppressor which interaction with MRN activates Mre11 activity and facilitates end resection	Deficient cells Hypersensitive to CPT
Exo1	HR	Exo1	Elongates resection of DSB	-
BLM	Replication	Rqh1/Sgs	RecQ family of DNA helicases	Deficient cells sensitive to CPT

1.3.2 __RAD50__

Rad50 is said to show similarities with the structural maintenance of chromosome (SMC) proteins and it has a Walker A & C terminal and a Walker B motif separated along coiled-coil regions (Raji et al., 2006). Raji et al. 2006 stated that Rad50 folds back allowing Walker A & B to form a globular ABC ATPase domain, with the coil-coiled region that joins them forming an apex containing Cys-X-X-Cys domain, in which two of such domains can form a hook allowing Rad50 to form intermolecular dimers. Rad50 interaction with Mre11 stimulates the exonuclease and endonuclease activity of the later (Raji et al., 2006), and the hook domain of Rad50 has been implicated in telomere maintenance by Mre11 protein (Stracker et al., 2011).

1.3.3 __Nijmegen Break Syndrome 1__

Nbs1 is a substrate of the ATM and is the least conserved member of the MRN complex, which is important for regulation and nuclease activity of the Mre11 while influencing its binding to DNA (Stracker et al., 2011). The N-terminal region of NBS1 contains two phosphopeptide binding modules; which is a fork head associated domain (FHA) and a tandem BRCA1 C-terminal domain (BRCT domain). Analyses of these domains in *S. Pombe* Nbs1 homolog have revealed novel modular architecture that allows phosphorylation dependent protein interactions with its binding partner Ctp1 (CtIP in humans) (Stracker et al., 2011; Wang et al., 2013; YasuhiroTsutsui et al., 2011). MDC1 is also thought to bind to Nbs1 through its FHA domain and this phosphor-binding-induced influence has regulatory influence on Mre11. Its C-terminal region is conserved and contains binding sites that lead to Mre11-Rad50 translocation into the nucleus, ATM auto phosphorylation and its activation (Yasuhiro Tsutsui et al., 2011; Thompson, 2012).

1.3.4 __CtBP Interacting Proteins (CtIP)__

CtIP initially characterized for its role as a transcription cofactor repressor for CtBP and binding partner to Retinoblastoma protein and BRCA1 in regulation of cell cycle, have been shown to play important role in cell cycle checkpoint arrest (You et al., 2010). It is thought to physically and functionally interact with MRN complex in DDR and repair and its depletion by (si)RNA caused hypersensitivity of cells towards Topoisomerase poison CPT (Sartori et al., 2007). CtIP tends to activate nuclease activity of Mre11, which leads to BRCA1-CtIP-MRN complex promoting DNA end resection (Thompson, 2012). CtIP levels in human cells are very low in G1 phase but expressed in S/G2 phase of cell cycle in relatively higher amounts, of which it is regulated through post transcriptional mechanisms (Yu and Baer, 2000). Its role in

cellular response to DSB is further enhanced by its promotion of ATR in S/G2 phase and controlling cell choice of DSB repair pathways.

Results have suggested that CtIP phosphorylation at Ser327 and interaction with BRCA1 ensures that end resection and HR repair occurs (Thompson, 2012), by processing DSB ends with secondary structures that promote HR. It is apparent that CtIP exhibits specifity for the 5' ssDNA ends and does not process 3' ssDNA tail ends (Wang et al., 2014). Sartori et al. 2007 states that CtIP C-terminus is highly conserved in its ortholog, in other eukaryotic species. From studies, CtIP has two CDK dependent and two ATM dependent phosphorylation sites, of which one of the CDK dependent site have been located at the Thr847 of the conserved C-terminal domain. Mutations of CtIP Thr-847 influences genome instability and this alteration in CtIP have been reported in cancers (Huertas, 2009). The ctp1 a homolog of CtIP in *S. pombe* has been shown to carry out resection of DNA DSB ends, via its endonuclease activity in the absence of Mre11 of up to several hundred base pairs *in situ*. CtIP is required to work more efficiently with Mre11 to create a clean ssDNA to initiate strand invasion into its sister chromatid and DNA repair. Recent studies have further shown that the not conserved N-terminal of CtIP in humans is important for its endonuclease activity as it has been also shown that mutating this residues in human cells result to sensitivity to CPT (Makharashvili et al., 2014).

1.4   CAMPTOTHECIN AND TOPOISOMERASE POISONING

Camptothecin was first identified from Chinese tree *Camptotheca acuminata,* in which three water soluble derivatives were approved for clinical use: topotecan, ironotecan, and belotecan (Pommier et al., 2010). CPT are clinically important drugs used in the treatment of cancers, which act on proliferating cells by locking topoisomerase covalently to DNA, thereby interfering with replication and transcription and ultimately resulting in the programed cell death (Hartsuikeret al., 2011). Their molecular target has been firmly established to be human Top I where it forms reversible Top I – CPT – DNA complexes in treatment of colorectal and ovarian cancers (Liu et al., 1996; Leppard and Champoux, 2005). This class of drug trap Top1 by docking at the enzyme DNA interface between the DNA base pair and tyrosine 723 site of Topo1 in a rapid manner, which can lead to stalling RF and cell death. However, cancer cells have been reported to resist treatment of these drugs, which have been attributed to Top I nucleolytic removal at RF by components of the MRN-CtIP complex during DSB repair.

Topoisomerase on the other hand are nuclear enzymes that modify topological state of the DNA by relaxing torsional stress, which they do by making single strand or double strand nicks. They participate in fundamental metabolic processes such as replication, transcription, DNA repair, recombination, and chromosome segregation (Jacob et al., 2001). The human genome encodes six topoisomerases which are classified into Type I (SSB) and Type II (DSB) (Pommier et al., 2010; Hande., 1998). They are:

 (1) Nuclear top I,   (2) mitochondrial top I,

(3) (4) top II α and β,   (5) (6) topIII α and β.

Germ cell restricted Spo11 is often classified as a topoisomerase due to its topoisomerase properties in meiotic recombination (Tomicic et al., 2013).

1.4.1 **Removal of Covalently Bound Topoisomerase I&II From DNA**

Nuclear proteins like Tdp1 have been implicated in the removal of Top I as a result of stalled transcription machinery; by hydrolyzing phosphodiester bond between the tyrosine residue of Top I and the 3' phosphate of the DNA. Knockout of Tdp1 in both *S. cerevisiea* and *S. pombe* confer mild CPT sensitivity (Hartsuiker et al., 2009). MRN-CtIP also cleaves ssDNA at stalled RF at the 5' termini blocked by covalently attached topoisomerase caused by CPT (You et al., 2010; Hartsuiker et al., 2009). However, not enough studies have been carried out on MRN-CtIP complex to ascertain the region of the protein complex involved in removal of covalently bound topoisomerase.

1.5   HYPOTHESIS

We propose that CtIP, an effector protein through its interaction with Nbs1 FHA/BRCT domain is required for activation of Mre11 endonuclease activity, and the assembly of Rad51 on resected DSB to facilitate HR (Haber et al., 2010; Nakamura et al., 2010; Wang et al., 2013). Mutations to CtIP and its homolog Ctp1 in *S. pombe* have shown hypersensitivity to CPT (Yasuhiro Tsutsui et al., 2011), when compared with other proteins that effect DSB resection. Recently CtIP has also been shown to maintain stability of DNA fragile sites by end resection independent of endonuclease activity, and are very important in resistance of cells to CPT (Wang et al., 2014; Sartori et al., 2007; Reczek et al., 2013). I intend to analyze the mechanism of Ctp1 using *S. pombe* as a model organism, of which are very adaptable to genetic manipulation and functional analysis of genes (Forsburg, 2001). Ctp1 shows homology with CtIP at its C-terminal although, unlike CtIP its CDK phosphorylation sites have not been identified (You et al., 2010; Yasuhiro Tsutsui et al., 2011). By screening mutagenized-Ctp1 genes selectively inserted into *S. pombe* base strain on DNA damaging drug agents MMS, phleomycin and CPT, I intend to study most especially colonies that show phenotypes to CPT but do not to the other DNA damaging agents.

The relevance of this project research is that CtIP/Ctp1 are critical for the initiation of resection that removes Topo-CPT-DNA from DSB, which results to HR but the underlying molecular defects in its expression are largely unknown. The aim would be to find part of the gene of Ctp1 in *S. pombe* that show phenotypic properties when expressed on CPT but does not on other DNA agents used in the screen. Further studies could be carried out, like site directed mutagenesis to re-create mutant phenotypes that have shown interesting phenotypes on the drug agents, by which further analysis could be carried out. This could in turn be good drug target that could help increase the effects of cancer drugs to damaged sites on DNA while mitigating damages to other molecular processes involved in DNA repair.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MUTAGENIC PCR OF pAW8-ctp1 AND PURIFICATION OF PAW8-ccdb PLASMID

Prior to the start of my experiment, pAW8-ctp1 plasmids were prepared as a template for error prone PCR by restricting the plasmid with *BgI*I restriction enzyme and isolating the fragment of plasmid of about 1kb band from the gel. Standard gel purification was then performed on the fragment DNA.

2.1.1 Mutagenic PCR of pAW8-ctp1 plasmid

The aim of this procedure is to randomly mutate the ctp1 gene in the plasmid, for insertion into an *S. pombe* base strain. Amplification of ctp1 from pAW8-ctp1 plasmid using error prone PCR, was performed with primers pAW8loxextA - F 5' GTTGTGTGGAATTGTGAGCG 3' and pAW8loxextA - R 5' GCGATTAAGTTGGGTAACGC 3' (Table 3.1). In order to create enough products, four reactions were run alongside a non-mutagenic control. The total volume for each tube was 100µl and the mutagenic PCR tube contained 62 µl of dH_2O, 1 µl of pAW8-ctp1 plasmid (5 ng), 1 µl of 10 pmol pAW8loxextA - F & pAW8loxextA - R forward and reverse primers, 10 µl of 10x PCR buffer (no $Mgcl_2$), 12 µl of 25 mM $Mgcl_2$, 2 µl of 25 mM $Mncl_2$ 10 µl of 10x mutagenic dNTP and 1µl of 5 units/µl of NEB *Taq* polymerase enzyme. The non-mutagenic PCR tube contained the same mix with the exception of 1µl 10 mM dNTP used in place of mutagenic dNTP, 6 µl of 25 mM $Mgcl_2$, $Mncl_2$ was omitted and the mix was complemented with dH_2O. Tubes were placed in the thermo-cycler and PCR reaction programmed for 95℃ for *30s*, then 15 cycles of (95℃ for *30s*, 60℃ for *30s*, 72℃ for *three minutes*) and a final extension of 72°c for *five minutes.*

2.1.2 **Purification of pAW8-CCDB plasmid**

A single colony of stored pAW8 *E. coli* cells containing ccdb plasmids was streaked onto freshly prepared LB agar plates, containing 0.1% ampicillin and incubated *overnight*. After incubation single colonies were then inoculated, each into two tubes containing 10ml of LB media and 0.1% ampicillin, and incubated in a shaking incubator *overnight*. The culture was then harvested by centrifugation in preparation for a mini-prep plasmid purification procedure. The mini prep plasmid purification was performed using Nucleobond xtra mini – plus kit following Machery-nagel July 2010/rev.07 user manual for plasmid DNA purification, using its protocol for isolation of low-copy plasmids construct or cosmids. The ccdb plasmid product after isolation was suspended 50 μl of dH$_2$O, and an aliquot of the ccdb plasmid mix then run on 0.8% agarose gel.

2.1.3 **Restriction digest of pAW8 - ccdb plasmid with *HindIII* and *SpeI***

To verify the identity of the pAW8 - ccdb plasmid, a *HindIII* restriction enzyme digest was carried out on 1 μl of purified pAW8 - ccdb plasmid with 1 μl *HindIII* enzyme (by Promega), 1 μl of buffer E, 1 μl of BSA (Bovine Serum Albumin) and 6 μl of dH$_2$O. Also, *SpeI* restriction digest was performed using 1 μl of the plasmid with1 μl of 10x buffer mix, 1 μl of *SpeI* enzyme and 7 μl of dH$_2$O. A control tube containing 1 μl of the plasmid and 9 μl of dH2O was prepared, and all tubes were then incubated at 37℃ for two hours and at 70℃ for *10 minutes*. The digest mixes were run on 0.8% agarose gel, in order to ascertain if the resultant bands obtained were of the predicted molecular weight. *SpeI* restriction enzyme digest was then performed on 20 μl of the isolated pAW8 - ccdb plasmid to linearize the plasmid constructs using relative ratio of the initial *SpeI* digest. The *SpeI* enzyme cuts close to one of the lox sites, which greatly promotes the *in vitro* cre recombination reaction.

Phenol-chloroform purification was carried out separately on both pAW8ctp1 insert and pAW8 - ccdb plasmid vector by; adding 0.1M Nacl and equal volumes of Phenol chloroform relatively, with the volumes of ccdb plasmid (200 μl) and mctp1 PCR product (400 μl) and mixed thoroughly with a vortex. Afterwards, the mixes were centrifuged at top speed for 15mins and the aqueous layer precipitated into a fresh tube. 100% ethanol of the same volume of each mix was then added to the aqueous solution containing the DNA, and the tubes were put at -80℃ for one hour, after which they were centrifuged at max speed and supernatant discarded. The pellets were then washed in 70% ethanol and the liquid was immediately discarded. The DNA pellets were air-dried and the individual products suspended in Tris-EDTA (TE) buffer *overnight*. The migration of DNA fragments of the individual products were run in 0.8% agarose gel and viewed using a UV trans-illuminator and DNA concentration obtained.

2.2   THE CRE-LOX RECOMBINASE REACTION AND RMCE IN *S.POMBE*

The *in vitro* cre - recombinase reaction was carried out with commercially available NEW ENGLAND BioLabs cre-recombinase. Reactions for mutated and wild type pAW8 - ctp1 DNA insert was done alongside a plox2 positive control reaction and a negative control reaction without the ctp1 insert. A total volume of 10μl was attained for all reaction (See appendix I). The plox2 reaction contained 1 μl of linearized plox2, 1 μl of 10x Cre buffer, 1 μl of 25% polyethylene glycol (PEG) solution, 0.5 μl of 5 units/μl cre recombinase and supplemented with dH$_2$O to 10 μl. The reactions for mutated and wild type pAW8 - ctp1 DNA insert contained 2 μl 25% PEG solution, 1 μl 10x cre buffer, 0.5 μl of 5u/μl cre recombinase, 2.3 μl dH$_2$O, and 1.9 μl linearized pAW8-ccdb plasmid vector, 1.8 μl mutated ctp1 (mctp1) & wild type ctp1 respectively. Negative control contained all agents used in the actual cre reaction with the exception of the ctp1 inserts and was supplemented with dH$_2$O. All reaction tubes were placed in a thermo-cycler at 37℃ for *30 minutes* and 70℃ for *10 minutes.*

2.2.1 <u>**Transformation of recombined mutated Ctp1 into competent *E. coli* cells**</u>

Products of the cre – reactions were then transformed into electro-competent and chemically competent DH5 alpha *E. coli* cells, both commercially made by NEB and made locally in the laboratory using the following protocols:

<u>**Transformation into NEB 5-alpha electro-competent *E. coli* cells**</u>;   cuvettes, 1.5 ml micro-centrifuge tubes, NEB 5-alpha electro-competent *E. coli* cells and products of the cre-reaction were all placed on ice for about *10 minutes*. SOC outgrowth medium and selective plates containing LB agar and ampicillin were warmed at 37℃. For each cre-reaction product, 25 μl of the cells was added to 1 μl of each DNA solution in micro-centrifuge tubes, carefully transferred into chilled cuvettes and pulsed on an electro-pulse apparatus at EC2 settings. 975 μl of SOC medium was then added to each cuvette, mixed and transferred to a culture tube and placed in a shaking incubator at 180 rpm for *60 minutes* at 37℃.

<u>**Transformation of NEB 5-alpha chemically competent *E. coli* cells**</u>;   SOC outgrowth medium and selective plates containing LB agar and ampicillin were warmed at 37℃. Then 1.5 ml micro-centrifuge tubes (transformation tube), NEB 5-alpha competent cells and products of cre-reaction reaction were all placed on ice for about *10 minutes*. Then 50 μl of competent cells were gently mixed with 2 μl DNA solution from linearized plox2 positive control, negative control reaction & each product of cre reaction into individual micro-centrifuge tubes. All Tubes were placed on ice for *30 minutes*, heat shocked at exactly 42℃, and placed on ice for *five minutes*. 975 μl SOC media was added to each tube to bring total volume to 1 ml and placed in a shaking incubator at 180 rpm for *60 minutes* at 37℃.

<u>**Preparation of DH5-alpha electro-competent *E. coli* cells for electroporation**</u>:

The *E. coli* DH5-alpha cells were grown in 1 L LB at 37℃, to an Abs_{600} 0.5-1.0. They were harvested and twice washed with 100 ml chilled, distilled water at 4000 rpm at 4℃. The cells were then resuspended in 20 ml of 10% glycerol and centrifuged at 4000 rpm at 4℃ for *15 minutes.* The pellets were resuspended in 2 ml 10% glycerol, distributed into Eppendorf tubes and stored at -80℃. Cells were to be used within *six months*.

Transformation of laboratory made DH5-alpha electro-competent *E. coli* cells;
cuvettes, micro-centrifuge tubes, DH5-alpha electro-competent cells and products of
the cre-reaction were placed on ice for *10 minutes*. 25 μl of cells was added to 1 μl of
DNA solution in micro-centrifuge tubes and carefully transferred to respective chilled
cuvettes and pulsed at EC2 settings. Then 975 μl of SOC was added to the cell mix,
transferred to individual micro-centrifuge tubes, and incubated at 30℃ for *60 minutes*
in a shaking incubator at 180 rpm.

**Preparation of DH5-alpha chemically competent *E. coli* cells for chemical
transformation;**

A 200 ml culture of *E. coli* DH5-alpha cells were grown to Abs_{550} 0.5, chilled for *five
minutes*, then harvested at 4000 rpm for *five minutes*. It was then resuspended in 80
ml TfbI solution [30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15%
(v/v) glycerol, adjusted to PH 5.8 with 0.2 M HOAc], and left on ice for *five minutes*.
Cells were harvested again and resuspended into 8 ml TfbII solution [10 mM MOPS,
75 mM $CaCl_2$, 10 mM RbCl and 15% (v/v) glycerol, adjusted to PH 6.5 with 1 M
KOH], and incubated on ice for *15 minutes*. Cells were distributed into 1.5 ml tubes,
rapidly frozen in liquid nitrogen and stored at -80℃.

**Transformation of laboratory made DH5-alpha chemically competent *E. coli*
cells;** chemically competent DH5-alpha cells were thawed at room temperature and
then chilled on ice with micro-centrifuge tubes and DNA solutions for about *10
minutes*. 50 μl of competent cells was mixed with each DNA solution and placed on
ice for *45 minutes*, before being heat shocked at 42℃ for *90s*, and returned on ice for
about *2 minutes*. Afterwards, 450 μl of sterile LB solution was added to the mix and
placed in a shaking incubator at 180 rpm for *60 minutes* at 37℃.

After the incubation period, 10, 100 and 1000 fold serial dilutions of all the cell mixes
were made, before 100 μl was plated on each LB agar selective plates containing
0.1% ampicillin and incubated. For both of the NEB cell mixes for electro-competent
cells and chemically competent cells, incubation was carried out at 37℃ *overnight*.
For both laboratory made cell mixes for electro-competent and chemically competent
cells, they were incubated at 30℃ for *two days*.

2.2.2 **Checking the Mutation Rate of mctp1 PCR product**

Single colonies of *E. coli* transformed cells were picked and streaked on fresh LB agar selective plates and incubated *overnight*. Then colony PCR preparation was performed placing a very small amount of each streaked cells into 50 μl of 2x HF buffer from Phusion polymerase kit and heated for *5 minutes* at 99℃. This was to lyse cell walls and release DNA from nucleus of streaked cells. 1 μl of each buffer mix containing DNA was added to a PCR mix that contained 25 μl of 2x Bioline MyTaq mix, 22 μl of sterile water and 1 μl of pAW8 loxextA - F and pAW8 loxextA – R primers. All reactions were prepared on ice. PCR mixes were then put in a thermo-cycler and programmed as follows; 95℃ for *1 minute*, then 25 cycles of 95℃ for 30sec, 55℃ for 30sec, 72℃ for *1 minute*; and then a final extension of 72℃ for *10 minutes*. PCR mix was then run on 0.8% agarose gel electrophoresis to ascertain the molecular weight of DNA fragments.

In order to prepare samples to be sent of for sequencing, all PCR mixes that showed band of the desired molecular weight were assembled and 20 μl of resultant PCR reaction mixes were treated with 2 μl of Shrimp alkaline phosphatase (SAP) and Exo1 enzymes, incubated at 37℃ for *15 minutes* and deactivated at 85℃ for *15 minutes*. Then 10 μl of the treated DNA mixtures were mixed with 1.5 μl of ctp1 int2-F 5' AAGTTCCGACGGTGAAATGC 3' forward primers, and 3.5 μl of sterile water in individual sequencing tubes and sent for sequencing.

2.3   TRANSFORMATION OF pAW8 – mctp1 PLASMID CONSTRUCT INTO *S.POMBE* & SWITCHING ON *IN SITU* CRE-EXPRESION GENE

The *Schizosaccharomyces pombe* base strain used for the procedure was EH1053 *h⁻smt0 ade6-704 ctp1::loxP-ura4-loxM leu1-32 ura4-D18*, which had its leucine gene knocked out . 350 ng/μl of mctp1 plasmid construct was used to transform *S. pombe* base strain using Lithium acetate transformation as described by Okazaki et al. (1990). 100 μl of transformed *S. pombe* cells were plated onto minimal media plates containing YNBA + ade ura, to select for *S. pombe* cells that carry the mctp1 plasmid products as episomes. This is possible, as cells would require the leucine marker on the plasmid to synthesize the leucine. Plates were incubated at 30℃ for *5 days*.

2.3.1   Switching On Cre- Expression pAW8 – mctp1 to influence *in situ* RMCE of mctp1 gene Into Endogenous Gene Location of *S. pombe*.

Individual colonies from the transformation reaction were picked and transferred into 96 well plates containing 200 μl of YE liquid, and incubated at 30℃ for a day. This is to allow viable propagation of cells without the need to have a plasmid. After incubation, with the aid of a 96-prong replicator, propagated colonies in the 96 well plates were replicated into YE agar plates containing 5-fluoro orotic acid (5FOA) and incubated for *2 days* at 30℃. Selective pressure of 5FOA enables only cells that have lost plasmids and incorporated mctp1 to propagate. The *S. pombe* cells colonies that had propagated were replicated into 100 μl YE liquid in 96 well plates and incubated at 30℃ for *two days*. In order to store the *S. pombe* cells carrying mctp1 in endogenous location, the cells in 96 well plates of YE liquid were suspended in 50% glycerol in 100 μl of YE liquid and stored at 80℃.

2.4   SCREENING OF LIBRARY FOR MUTANT PHENOTYPES

The agents for screening; Camptothecin (CPT), Methyl Methane Sulphonate (MMS) and Phleomycin were prepared in their respective concentration of 4 μM & 8 μM, 0.004% & 0.008%, and 2.5 μg/ml & 3.5 μg/ml from stock solutions in 50 ml of sterile YE agar media. Plates were then labelled and stored for *24 hours* at room temperature, one for each 96 well plate to be screened on each DNA damaging agent, and a control plates that had no drug agent.   Sterilized replicators were used to transfer mutant cells growing in YE liquid transfer plate onto respective YEA + drug agents target plates, control plate and then were incubated at 30℃ for *five days*.

2.5   SEQUENCING ISOLATES OF INTERESTS

Colonies of *S. pombe* on control plate was used to reference colonies on individual YEA plates containing DNA damaging drug agents CPT, MMS and Phleomycin. *S. pombe* colonies that showed sensitivity on individual drug plates were selected on control plates, inoculated into 5 ml of YE liquid and incubated *overnight* at 30℃.

2.5.1   Yeast lysis and DNA extraction from S.pombe cells

The 5 ml tubes containing *S. pombe* cells were centrifuged at 3000 rpm for *5 minutes*, supernatants were discarded, and cell pellets were suspended in 1 ml of water and then transferred into 2 ml screw cap tubes. The cells were centrifuged at 4000 rpm for *5 minutes* and the supernatant were removed. Mechanical lysis preparation was carried out using glass bead and 200 μl of lysis buffer made with; 1 ml of 100x triton, 5 ml 10% SDS, 0.5 ml of 1 M Tris, 5 ml of 1M Nacl and 100 μl of 0.5 M EDTA mixed with 50 ml of sterile water and re-suspended with the aid of a pippete. Then 200 μl of phenol chloroform was added and the mixture tubes placed in a ribolyzer at max speed for *2 minutes*. Cell mixes were then centrifuged at 13000 rpm for *12 minutes*, and the topmost aqueous layer containing the DNA was carefully transferred into individual 1.5 ml micro-centrifuge tubes, after which 100% ethanol of about 3x the volume of DNA liquid was added to each tube and incubated at -80℃ for *one hour*. DNA mixes were centrifuged at top speed for *12 minutes*, the supernatant was discarded and the pellets washed in 70% ethanol, which was quickly poured out, and the tubes left to air-dry for 15 minutes. Cells were then re-suspended in 50 μl of TE overnight ahead of PCR.

PCR preparations were made by adding 5 μl of 10 mg/μl RNaseA into each of the DNA mixes. 1 μl DNA mix was extracted and a 10x dilution with sterile water performed to be used as DNA template for PCR. The PCR reaction mix was prepared with 1 μl DNA template, 25 μl 2x my taq ready mix, 1 μl Ctp1 ext-F 5' CGTATTAGGATGGTAGTCGC 3' forward primers, 1 μl Ctp1 ext-R 5' GGTAAGATGCTACTTCGTCG 3' and 22 μl of sterile water. PCR program was 95℃ for *three minutes* then 30 cycles; 95℃ for *15s*, 55℃ for *30s*, 72℃ for *45s*, then an extension for 72℃ for *10 minutes*. DNA mixes were run on 0.8% agarose gel to detect band of about 1.8kb molecular weight. DNA molecules showing desired molecular weight were then treated and prepared for sequencing same using protocols as *E. coli* plasmid DNA. The primers for sequencing were ctp1int – F and ctp1int2 – R used for separate batches of sequence mixes.

CHAPTER THREE

RESULTS

3.0   RESULT OVERVIEW

The system was designed to mutagenize ctp1 isolated from pAW8 - ctp1 plasmid through PCR, and then recombine the PCR product with pAW8 - ccdb plasmid via RMCE. The ccdb gene that had been incorporated into the multiple cloning site (MCS) of the pAW8 plasmid is cytotoxic and brings about post-translational killing of cells after the plasmid loss, which allows selection for cells that have incorporated the recombined mutagenic ctp1 (mctp1) plasmid after transformation into *E. coli* cells. Lithium acetate chemical transformation was used to transform mctp1 into *S. pombe* base strain, with mctp1, the donor cassette flanked by lox - P and lox – M3 sites in the plasmid that also expresses cre recombinase enzyme (Figure 3.1).

The pAW8 plasmid is based on *S. pombe pAL19* plasmid that carries protein coding *ars1*$^+$ marker, and a *LEU2*$^+$ gene that compliments the knocked out leucine gene on the *S. pombe* base strain. Selectable ampicillin resistance gene (*AmpR)* ensures that only cells that have taken up the plasmid are selected for. The *S. pombe* base strain EH1053 *h$^-$smt0 ade6-704 ctp1::loxP-ura4-loxM leu1-32 ura4-D18* also has lox – P and lox - M3 sites that flank a selectable *ura4*$^+$ marker, which enables cre - recombinase site specific cassette exchange *in situ* between the *ura4* gene and mctp1 insert. The cre - recombinase synthesized by its gene on the cre - expression plasmid (pAW8) sufficiently carries out the recombination in *S. pombe* (Figure 3.1), and due to the hetero-specific nature of the lox – P and lox – M3 sites, stable products can be formed (Watson et al., 2007). The pAW8 plasmid can exist as episomes in the cytoplasm of *S. pombe*, however, the palindromic make-up of the cre - reaction results to two outcomes when *cre* is expressed; one of which the *ura4*$^+$ marker is still expressed on its base strain and the other being mctp1 successfully incorporated into the base strain in exchange for *ura4* (Figure 3.1).

The workflow was carried out *in vitro* to create mutagenized ctp1, and with the aid of the cre - lox reaction, to recombine mctp1 with pAW8-ccdb plasmid & transform recombinants into competent *E. coli* cells. Successful PCR products from several transformation reactions were then pooled to form a plasmid library, and transformed into *S. pombe* as described by Okazaaki (Okazaaki et al., 1990). *LEU2* is important for leucine biosynthesis and the *leu2$^-$* marker on *S. pombe* base strain, ensures that

only cells that have taken up the plasmid can synthesize the amino acid. The expression of cre-recombinase *in situ,* leads to RMCE between mctp1 and *ura4* cassettes that are flanked by lox sites. The cell colonies were allowed to propagate without any selective pressure in fresh YE liquid media, which allowed free growth of transformants without any need to have the pAW8 plasmid. This resulted in a luxuriant growth of colonies that had *ura4*+ markers either incorporated in pAW8 plasmid or in the genomic DNA of *S. pombe* base strain, or *ura4*- colonies that had mctp1 incorporated into the base strain of *S. pombe*. The reagent 5FOA was then used to select for *ura4*- colonies that have lost the plasmid. 5FOA is a precursor of 5-ura4 that is toxic to *ura4*+ cells and lead to selection for *ura4*- cells in *S. pombe* cells. This subset of cells were then used to generate a library of *S. pombe* mutants carrying mctp1, which were used to carry out screens on CPT, alkylating agent MMS and phleomycin (Figure 3.2). The aim of the screen is to discover colonies that show phenotypes to the DNA damaging agents, and most especially colonies that show phenotypes to CPT but do not show phenotype to the other DNA damaging agents.

TABLE 3.1: Primers used in PCR and sequencing of mctp1 gene construct.

Primers	Primer Sequence (5' – 3')
pAW8 loxext A - F	GTTGTGTGGAATTGTGAGCG
pAW8 loxext A - R	GCGATTAAGTTGGGTAACGC
Ctp1 int - F	TGGAAGTGATACAGTGGACG
Ctp1 int - R	CAATCCGGACATTCGTAAGC
Ctp1 int2 - F	AAGTCTTCAGGTGACCTTGC
Ctp1 int2 - R	ATGTCTTCAGGTGACCTTGC
Ctp1 ext - F	CGTATTAGGATGGTAGTCGC
Ctp1 ext - R	GGTAAGATGCTACTTCGTCG

Figure 3.1: Schematic diagram of the *in situ* switch-on of RMCE in *S. pombe*
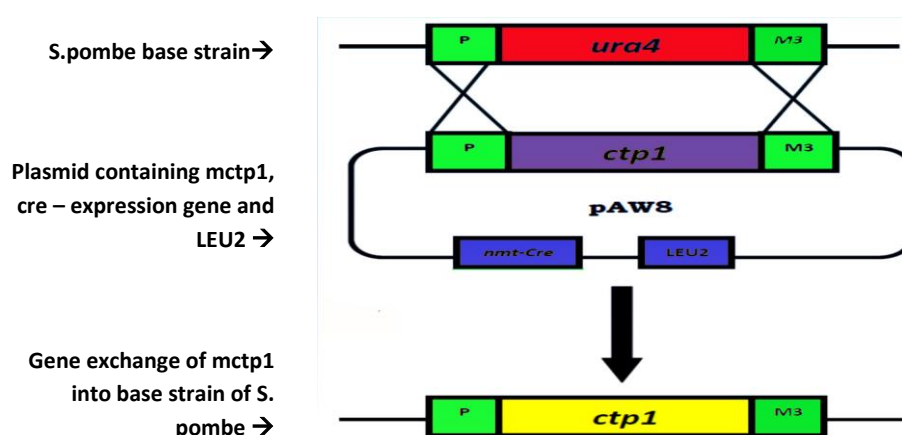
Figure 3.1 shows RMCE in *S. pombe* during site-specific recombination used to insert mctp1 into *S.pombe* base strain. Cre recombinase synthesized by its gene on the plasmid catalyzes the reaction sufficiently. [Image from Jaendling, A. (2014)]

Figure 3.2: A schematic diagram of the workflow to switch on cre – expression.
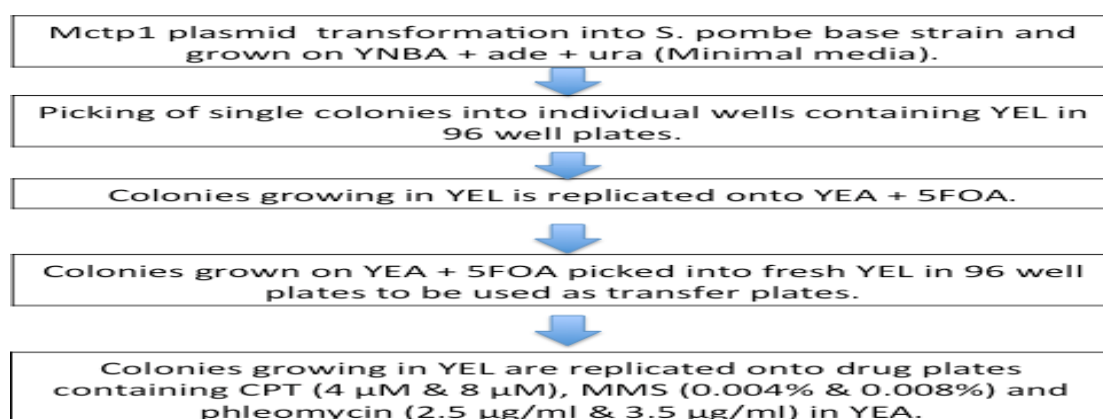
Figure 3.2 shows the workflow involved in transformation of mctp1 plasmid into *S. pombe* base strain and how cre – expression is switched on, to bring about *in situ* recombination and propagation of cells in YEL. Colonies grown on YEL are then replicated onto YEA + 5FOA to select for colonies that have incorporated the mctp1 episomes into the base strain of *S.pombe.*

3.1 RESTRICTION OF PAW8 - CCDB PLASMID WITH *SPEI* AND *HINDIII* ENZYMES

In order to prepare the pAW8 - ccdb plasmid for cre - lox recombinase mediated cassette exchange (RMCE), the plasmid was isolated using the mini-prep low copy plasmid purification protocol. The pAW8 - ccdb plasmid is 8867 bp with 43% G C content and 57% A T content, which is influenced by three open reading frames (ORFs) being *LEU2, AmpR and Cre* (Figure 3.4). An aliquot of pAW8 - ccdb plasmid was digested for verification with *HindIII*, in which two staggered cuts were created at 3855 bp/3859 bp and 6652 bp/6656 bp (Figure 3.4). The cuts produced two bands of molecular weight 2797 bp for the smaller fragment and 6070 bp for the larger fragment (Figure 3.5A). The gel image analysis of the two bands confirmed the identity of pAW8 - ccdb plasmid that had been purified from pAW8 – ccdb *E. coli* cells. The *SpeI* restriction digest of the plasmid was also carried out for verification of plasmid identity, with a single band of molecular weight 8867 bp present on the gel image. Both restriction digest were performed alongside an undigested pAW8 - ccdb plasmid that served as control (Figure 3.5).

The *SpeI* is a stable restriction enzyme that produced a single cut close to the Lox - M3 site at 4549 bp/4553 bp (Figure 3.4), resulting in a linearized plasmid. This greatly promotes *in vitro* cre recombinase reaction by increasing frequency of the cre –lox recombination event between the lox sites of the plasmid vector and the mctp1 insert. Also, linearization of plasmid vector prevents cre recombinase from producing secondary structures caused by excision and cyclization of the DNA between lox sites. To this effect, 20 μl of the purified ccdb plasmid was digested with 4 μl of *SpeI* restriction enzyme, 156 μl of purified water and 20 μl of 10x buffer to result in a total of 200 μl. This was stored at -20℃ to be used as the vector for cre-lox reaction RMCE.

On the other hand, pAW8 - ctp1 plasmid (Figure 3.3) to be used as template for mutagenic PCR had been restricted with *Bgl*I in preparation for mutagenizing ctp1 GOI via PCR, in order to prevent mis-priming of the DNA sequence during PCR. The PCR was performed in four folds to boost product of reaction, alongside a non – mutagenized control using pAWloxext A – F + R primers. By decreasing the fidelity of PCR through action of Mgcl$_2$ and Mncl$_2$, in which Mg^{2+} stabilizes non-complementary strand and Mn^{2+} decreases fidelity of Taq polymerase, ctp1 was amplified creating random mutation at various loci of the gene. The PCR products of mctp1 and Non-mutagenized ctp1, along with *spe*I restricted pAW8 - ccdb plasmid vector were purified using phenol-chloroform purification of DNA, in order to remove impurities and achieve better nucleic acid concentration. Agarose gel image of mutated ctp1 and non-mutated ctp1 showed the expected bandwidth of 1160 bp after the phenol purification protocol (Figure 3.5B). The final concentration of *Spe*I cut plasmid vector was determined using a nanodrop device at 91.4 ng/ µl, while that of the mctp1 insert was determined using the DNA hyper ladder and estimated at 50 ng/ µl (Table 3.2).

FIGURE 3.3: Plasmid map image of pAW8-ctp1.
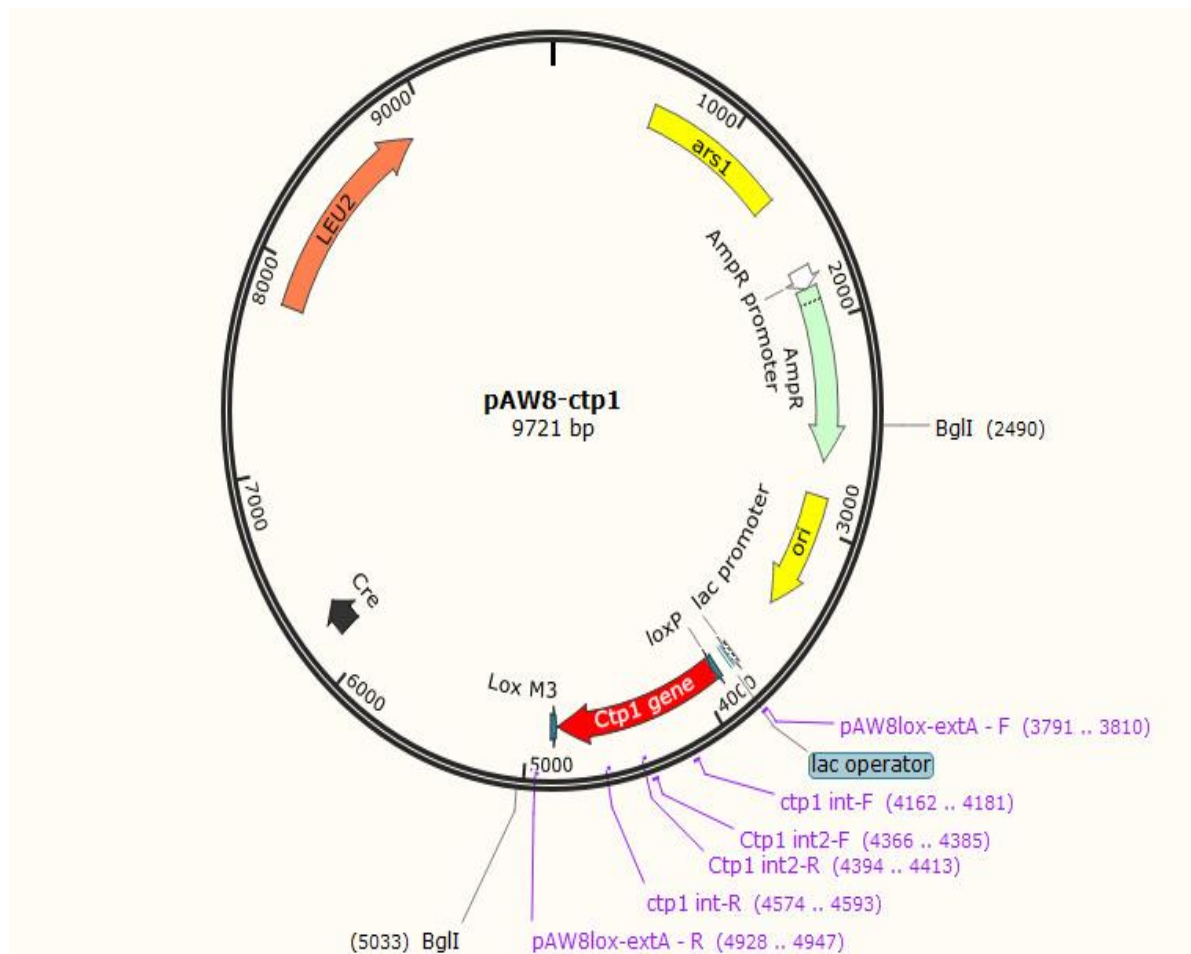
FIG 3.3: This shows pAW8 - ctp1 plasmid containing a LEU2 gene allowing selection in *S. pombe* transformation, an autonomous replicating sequence (ars1) marker expressed for *S. pombe* replication, Ampicillin resistance gene (AmpR), Origin of replication (Ori) expressed for *E. coli* replication and importantly, the ctp1 gene & cre recombinase expression marker. The GOI, ctp1 is flanked by lox P and Lox M3 sites, which aid Cre enzyme mediated recombination between the lox sites.

The figure also shows the site of the *Bgl*I restriction enzyme and the position of different primers used for PCR and sequencing of ctp1 gene.

Figure 3.4: plasmid map Image of pAW8-ccdb vector
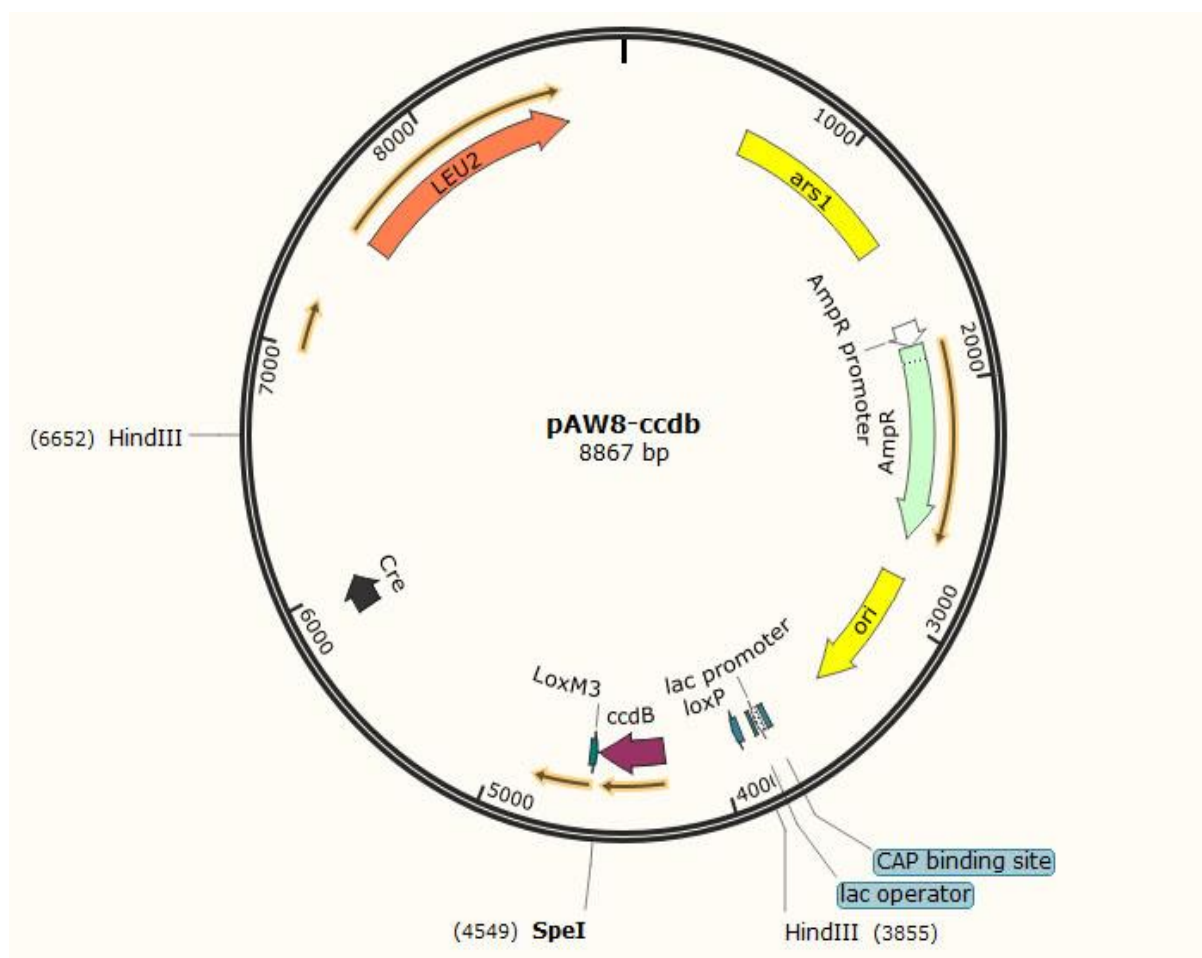
FIG 3.4: pAW8 - ccdb plasmid containing a LEU2 gene, an autonomous replicating sequence (ars1) marker, Ampicillin resistant gene (AmpR), Origin of replication (Ori), and the ccdb gene. The ccdb is flanked by lox P and Lox M3 sites, which aid Cre enzyme mediated recombination between the lox sites that leads to RMCE between the vector and insert.

The figure also shows *HindIII* and *SpeI* restriction sites implicated in restriction digest of plasmid.

FIGURE 3.5: Agarose gel image showing (A) pAW8 - ccdb plasmid cut with restriction enzymes, *HindIII* and *Spel*. (B) Plasmid preparations to be used for Cre-recombinase reaction.

A   B
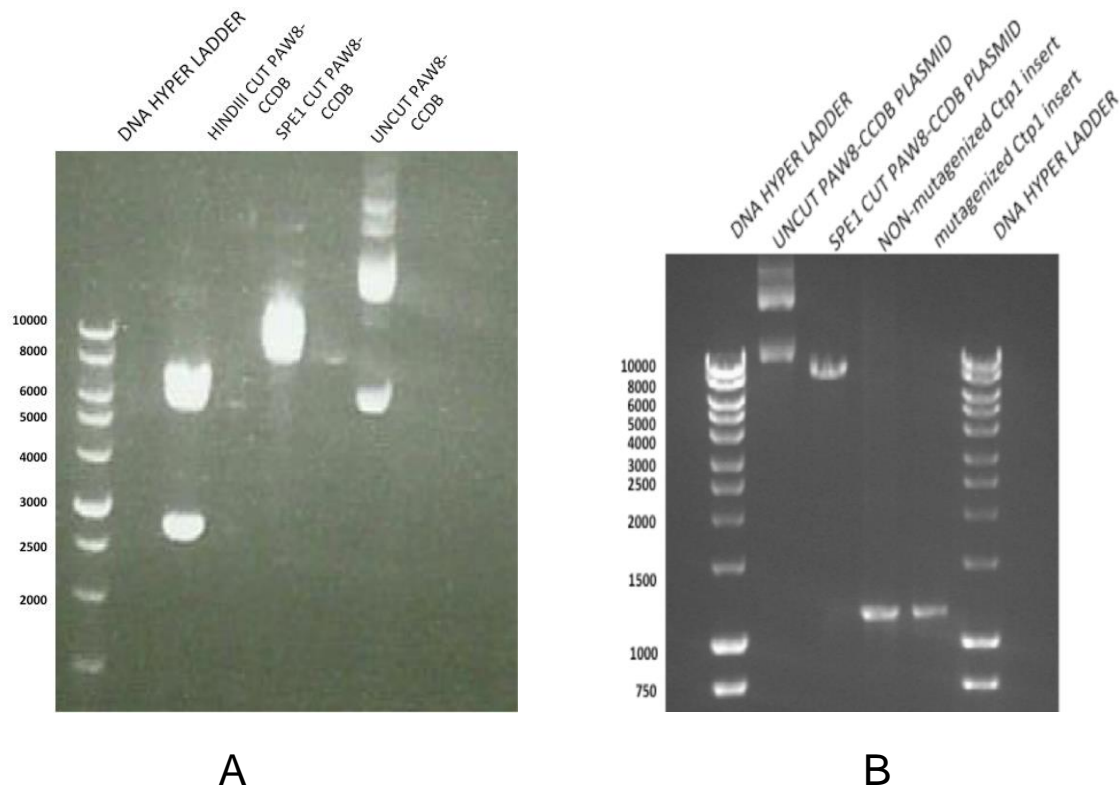
FIG 3.5 shows agarose gel image for (A) pAW8 - ccdb plasmid restricted with *HindIII* and *Spe1* restriction enzymes, which generated two cuts and a single cut respectively, alongside a super coiled uncut pAW8 - ccdb plasmid in the last lane. (B). Uncut pAW8 - ccdb plasmid (8867 bp), *Spel* cut pAW8 - ccdb plasmid vector used for Cre recombinase reaction with a molecular weight of 8867bp, non-mutagenized ctp1 PCR product and the mutagenized ctp1 insert both of the same size of 1160 bp.

TABLE 3.2: DNA concentration of *Spel* cut ccdb plasmid vector & ctp1 PCR products.

	Final DNA Conc. (ng/μl)	A260/A280	Molecular Weight (bp)
***Spel* cut pAW8 ccdb plasmid**	91.4	1.88	8867
Non - Mutagenized Ctp1 insert	50	-	1160
Mutagenized Ctp1 insert (GOI)	50	-	1160

Table 3.2 shows different concentration of DNA used in cre lox recombination reaction. *Spel* cut DNA plasmid vector was measured using a nanodrop spectrophotometer, while that of ctp1 (mutagenic & non-mutagenic) was estimated by comparing it with hyper ladder 1kb molecular weight marker.

3.2   CRE-LOX   RECOMBINASE   MEDIATTED   CASSETTE   EXCHANGE REACTION (RMCE)

In order to incorporate the mutagenized ctp1 PCR product (mctp1) into the linearized pAW8 - ccdb plasmid vector, nucleic acid concentrations were measured (TABLE3.2). The amount of vector/insert needed to achieve a 4:1 molar ratio was calculated for *in vitro* RMCE (Appendix 1). This calculation showed that 1.9 µl of ccdb plasmid was needed to react with 1.8 µl of mctp1 insert. To achieve a total volume of 10 µl, 2 µl of 25% PEG, 1 µl of 10x Cre –buffer and 1 µl of cre recombinase were augmented with 2.3 µl of sterile water in the cre reaction mix. Negative control was made without mctp1 insert, while the plox2 positive control contained 1 µl of linearized plox2, 2 µl of 25% PEG, 1 µl of 10x cre buffer, 0.5% cre recombinase and 5.5 µl sterile water. All reactions were placed in a thermo-cycler at 37℃ for *30 minutes* and 70℃ for *10 minutes* on preparation for transformation into competent *E. coli* cells.

Transformation was carried using a variety of competent cells and transformation protocols to try and optimize transformation conditions, and investigate which cell strain and methodology produced the most desired outcome (TABLE 3.2). High efficiency commercially available NEB 5-alpha chemically competent and electro-competent *E. coli* cells were used together with laboratory made DH5-alpha chemically competent and electro-competent *E. coli* cells. They were placed in the incubator at 37℃ for NEB commercial cells and 30℃ for laboratory made cells (Materials and methods). Incubation temperature and period varied with NEB competent cells incubated for a day and laboratory made competent cells incubated for two days as stated in their respective protocols.

Three transformations were carried out on each sub-set cells as shown in Table 3.2. Each set of transformation reactions were carried out simultaneously to reduce experimental variation and eliminate all bias. All reactions were carried alongside a plox2 positive control and a negative RMCE control that did not contain a mutagenized PCR product. A total of 1000 µl of transformation mix was attained for each group of competent cells and serial dilution was made up to a hundred folds (Materials and methods). Then 100 µl of each mix and its serial dilutions were plated on prepared selective LB agar plates and incubated. After incubation the colonies on the plates were counted (Table 3.2) and some randomly selected, and streaked on fresh selective LB agar in preparation for colony PCR.

Result presented in Table 3.3 shows NEB electro competent cells produced a total of 370 colonies from three reactions, and laboratory made electro – competent cells produced a total of 543 colonies from two reactions. This is due to contamination in transformation three which was excluded. The average colonies of all groups were calculated over three transformation reactions, which resulted in laboratory made electro – competent cells still producing the highest overall average of 181 colonies (Table 3.3). Its transformation 2 which produced its best result of the three had a transformation efficiency of 61.5 transformants/ng of DNA. Transformation 3 of the NEB electro-competent cells had a transformation efficiency of 60.7 transformants/ng of DNA, while that of NEB chemically competent cells had a transformation efficiency of 20.4 transformants/ng of DNA (Table 3.4). Recombination efficiency of the transformation reaction showed that laboratory made electro-competent DH5-alpha cells resulted in higher transformation frequency compared to the other three. It produced a total of 543 colonies with a recombination efficiency of 594.1 transformants/μg, with NEB electro-competent cells having a recombination efficiency of 404.8 transformants/μg (Table 3.4). This could indicate that electroporation transformation reaction produces more efficient outcomes.

A small amount of the streaked single colonies on LB agar were each suspended in 50μl of HF phusion buffer from phusion polymerase kit and heated at 100℃. 1 μl of the reactants were then used as templates for PCR. PCR was carried out to amplify ctp1, which was then run on agarose gel and the bands checked under UV. 24 PCR products out of 30 produced bands at 1366bp mark, which showed that mctp1, had correctly been incorporated into the plasmid (Figure 3.6). The successful PCR products were then prepared for sequencing by treating them with *SAP* and *ExoI,* and sent for sequencing using ctp1int2 – F and ctp1int - F primers.

Sequence data was returned and analysis made using Snapgene, Mega5, and finch TV software packages to investigate mutations in the DNA sequences and analyze them. Analysis showed the total mctp1 nucleotides sequenced was 9335 bases; a total of 37 mutations were noted within the CDS of the mctp1 gene sequenced (Table 3.5), of which three frame shifts were recorded, 13 were silent, 15 missense, and two were nonsense as they either coded for a stop codon or changed a protein codon into a stop codon. It is worthy of note that only about 75% of total ctp1 gene was sequenced due to choice of primers. The Mutation rate was ascertained at 3.9 x 10^{-3} mutations per Kb, within the ORF and intron region of ctp1 gene sequenced. This indicates the cre-reaction was successful and plasmid library can be created from the leftover cre-reaction. A total of roughly 150 individual transformed colonies was expected to be generated per plate, to be able to pool roughly 2000 colonies for

extraction of their low copy plasmid and purification. However, I was not able to generate this plasmid yield to create my own library. Figure 3.7 shows a linearized map of pAW8 – ctp1 plasmid that features primer annealing sites, lox P and M3 sites and the CDS of ctp1. The ctp1 gene is a 929 bp element that has two reading frames separated by a 44 bp intron region. The ctp1 internal primers were used for carrying out sequencing because external primers produce inconsistent result due to mis-priming of the lox sites of the external region of the gene.

Table 3.3: result of transformation reaction performed to ascertain transformation efficiency of different competent *E. coli* strains and dilutions

Dilution factor	Transformation 1			Transformation 2			Transformation 3			Total (Average)
	0	10	100	0	10	100	0	10	100	
NEB Electro competent cells	10	-	-	9	3	-	316	31	1	370 (123.3)
NEB chemically competent cells	86	5	-	19	-	-	106	11	1	228 (76)
Laboratory Made Electro competent cells	160	31	-	320	30	2	C	C	C	543 (181)
Laboratory Made chemically competent cells	5	-	-	4	1	-	32	2	-	44 (14.6)

Table 3.3 shows different transformation performed with NEB 5-alpha chemical & electro-competent *E. coli* cells and laboratory made DH5-alpha chemical & electro-competent cells. Numerical figures show amount of colonies in each dilution, - signifies zero growth, while C signifies a contamination.

Table 3.4: The recombination efficiency of resultant transformants

	Transformation efficiency (transformants/ng of DNA) (Colonies)	Total number of transformants	Recombination efficiency (transformants/µg)
NEB Electro-competent 5-alpha cells	60.7 (316)	370	404.8
NEB chemically competent 5-alpha cells	20.4 (106)	228	249.5
Laboratory made electro-competent DH5-alpha cells	61.5 (320)	543	594.1
Laboratory made chemically competent DH5-alpha cells	6.2 (32)	44	48.1

Table 3.4 shows the transformation efficiency of the most successful transformation outcome (in bracket) for each group of *E. coli* cells, the total number of transformation resultant for each group of *E. coli* cells and their respective recombination efficiencies. Laboratory made elecro-competent DH5-alpha cells produced the best outcome of all sub set cells having a recombination efficiency of 594.1 over three transformation reactions.

FIGURE 3.6: colony PCR image of mctp1 from transformed competent E.coli

(1)

(2)
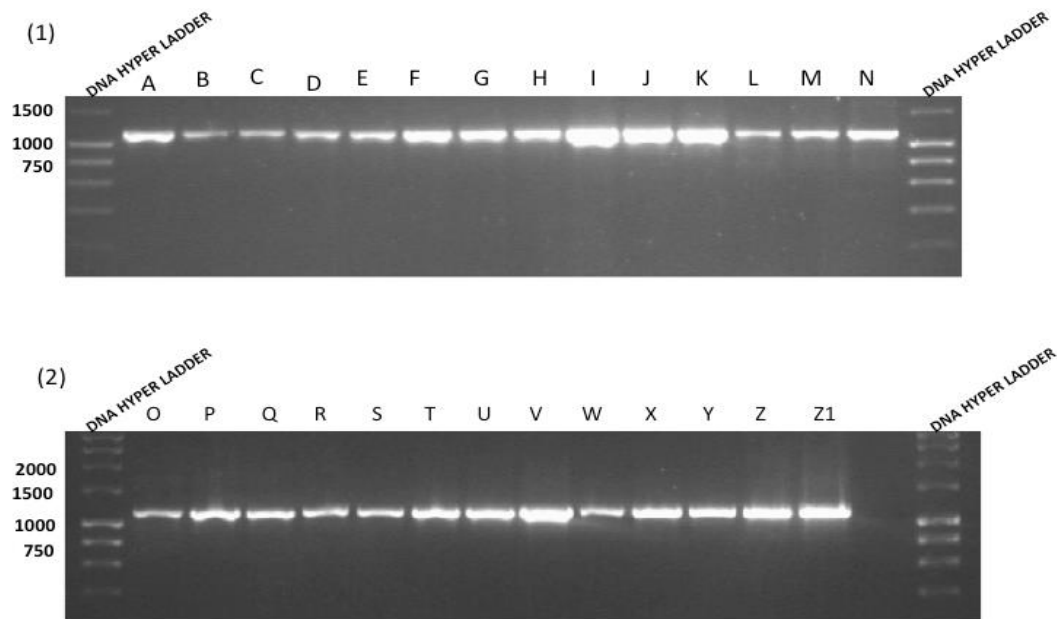
Figure 3.6 shows PCR products of cre-mediated reaction and the subsequent transformation. Single bands appear at 1366 bp that implies that mctp1 was inserted in all products prepared for sequencing.

Table 3.5: The mismatches accrued by mctp1 gene sequenced from transformed *E. coli* plasmid containing mutagenic ctp1 (mctp1)

Number of bases sequenced	Changes into A	Changes into C	Changes into G	Changes into T	Deletions
9335 bases	11	10	11	2	3

Table shows mismatch within the ORF + intron of mctp1 gene amplified by PCR and sequenced. Records show a total of 34 mismatches and three deletions were achieved form a total of 9335 bases of mctp1 sequenced. A mutation rate of 0.00396 was achieved or 4 point mutation per 1000 nucleotide.

Figure 3.7: linearized map of pAW8 - ctp1 and highlighting primer sites CDS and intron
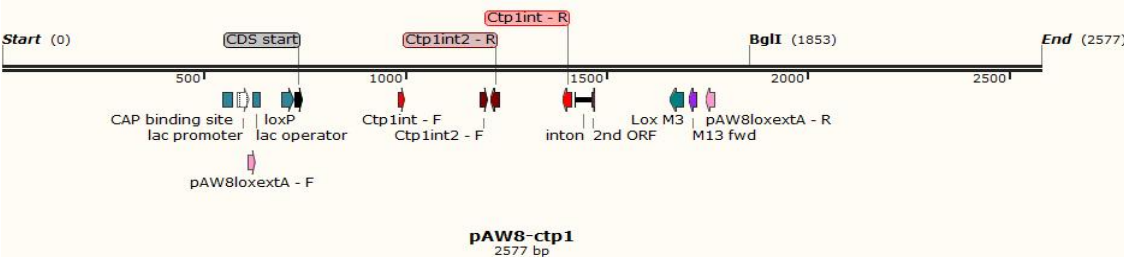
Figure shows region of linearized pAW8 - ctp1 that contains mctp1 and highlights the primer annealing sites of the gene. ctp1int – F and ctp1int2 – F were used for sequencing.

3.3 SCREENING OF THE LIBRARY OF MUTANTS ON SELECTIVE DRUG PLATES CONTAINING CAMPTOTHECIN, MMS & PHLEOMYCIN.

In preparation for transformation of mctp1 plasmid library into *S. pombe*, and subsequent *in situ* recombination event of mctp1 into endogenous base strain of the *S. pombe*; 1×10^7 cells/ml culture was prepared and lithium acetate transformation as described by okazaaki et al. was used to transform 1 μl of 350 ng/μl mctp1 plasmid library, into harvested cells suspended in 100 μl of lithium acetate. A ten-fold serial dilution was carried out and 100 μl of the cells were plated on five sets of YNBA + ade + ura plates and incubated for *five days* at 30℃. *S. pombe* base strain had its leucine gene knocked out to allow for selection of colonies that had taken up the plasmid, which would be able to synthesize leucine due to presence of leucine marker on the plasmid. The ten-fold dilution of the *S. pombe* transformants produced the best outcome on the plates, with an average of 88.2 colonies per plates. In order to switch on cre recombination of mctp1 into its endogenous location in *S. pombe*, single colonies were picked into individual wells in 96 well plates containing 100 μl of YE liquid. The cell colonies were incubated at 30℃ for three days. The colonies were replicated onto YEA + 5FOA plates, which selects for *S. pombe* cells that had incorporated mctp1 into its endogenous location and had lost plasmid carrying ura4 selectable marker. Colonies on the 5FOA media plates were replicated into YE liquid, and grown to serve as transfer plates for the drug target plates (Figure 3.2).

The screening of the library of mutants on drug agents was done by replicating colonies growing on transfer plates onto YEA plates, containing 50 μl of CPT (4 μM & 8 μM), MMS (0.004% & 0.008%) and phleomycin (2.5 μg/ml & 3.5 μg/ml). A control plate without a drug agent was also prepared as a reference plate for the procedure. They were all incubated at 30℃ for *five days* and examined for the presence of colonies showing sensitivity to the drug agents (Table 3.6). Result of the screening procedure yielded few interesting results, with 14 colonies showing some level of sensitivity to the one or more drug agents. However none of the colonies produced a totally sensitive to CPT, MMS and phleomycin. The colonies showing phenotypes to the agents were picked from their respective isolates on reference plates into separate 5 ml YE liquid media, and incubated *overnight*. Yeast lysis and DNA extraction was then carried out as described in materials and methods section and the resultant DNA extract, suspended in 50 μl TE (PH 7.5) and incubated at room temperature *overnight*. The PCR was performed using ctp1ext – F+R primers on 1 μl of RNase-A treated DNA extract, 2x My *Taq* red PCR mix and sterile water. PCR products were loaded on agarose gel and the image showed that the amplified DNA sequence was of about 1.8kb as predicted (Figure 3.8) for all products.

Afterwards 20 μl of each PCR product was then treated with 2 μl SAP and 0.5 μl *ExoI,* and sent for sequencing using both ctp1int – F & ctp1int2 – R primers, for forward and reverse sequence result that should span the entire region of ctp1 gene.

Analysis was made on the sequenced data using Snapgene, Mega5 and Finch TV software packages to edit and annotate sequence results. Figure 3.9 shows an image of ctp1 gene map aligned with some of the sequence results. Highlighted in the figure are the two reading frames of ctp1, which has in between it a 44 bp intron. Analysis on 14 sequenced result using bio-informatics tools (Table 3.7) revealed that DNA sequences of three of the strains did not contain any mutation within ctp1 gene sequence. It most likely indicates that these colonies would have been erroneously picked, and the presumed phenotypes shown were as a result of poor manual replication of the colonies onto the drug plates. Furthermore, of the eleven that showed mutations within the reading frame of ctp1, three of the strains produced poor reads on the DNA sequencer chromatogram. This resulted in eight strains that showed interesting phenotypes, of which had actual mutations in their genes.

Further investigations were required to be carried out on the library of mutants. Higher concentration of CPT and phleomycin were prepared and fresh MMS was obtained for screening the library of mutants. Stored library of mutants in YEL + glycerol media was thawed at room temperature, transferred to fresh YE liquid media with the aid of a sterile 96 prong replicator and incubated at 30℃. These were to be used as transfer plate for the screen, and drug plates prepared contained the following concentrations; CPT (8 μM & 12 μM), MMS (0.004% & 0.008%) and phleomycin (3.5 μg/ml & 4.5 μg/ml) in 50 μl of YEA. The *S. pombe* library of mutants was replicated onto the drug plates and control plate. They were allowed to grow at 30℃ for four days, after which they were observed. It was evident that sensitivity of the cells to the drug agents reduced tremendously, irrespective of the increased concentration. Compared to the initial data, fewer colonies showed sensitivity and the result of this screen was inconsistent with the initial result. The concentrations of drug agents were increased the third time using the same library of mutant from storage. Target plates containing 60 μl of YEA were mixed with drug agent to achieve concentration of; CPT (12 μM & 16 μM), MMS (0.008% & 0.012%) and phleomycin (4.5 μg/ml & 5.5 μg/ml). The Rotor HAD robot was used to accurately replicate library of mutants onto each drug plate and control plate. However, in spite of the high concentration of drug agents, result was still inconsistent with fewer colonies sensitive to drug agents. This could be due to trans-contamination across 96 well plates, while handling the titre plates. Due to time constraint, further investigation could not be carried out in the laboratory.

Table 3.6: Scanned agarose gel image of screened library of mutants on selected plates containing CPT, MMS and phleomycin.

Control plate	CPT	MMS	Phleomycin

Plate 1

B3, B6, B7, B9 & E9 showed phenotype on one or more drug agents.

CPT 4 μM

CPT 8 μM

MMS 0.004%

MMS 0.008%

Phleomycin 2.5 μg/ml

Phleomycin 3.5 μg/ml

Plate 2

F5, G7, C8, & A10 showed phenotypes to one or more drug

CPT 4 μM

CPT 8 μM

MMS 0.004%

MMS 0.008%

Phleomycin 2.5 μg/ml

Phleomycin 3.5 μg/ml

Plate 3

D4 & D12 showed phenotypes

CPT 4 μM   **MMS 0.004%**   **Phleomycin 2.5** μg/ml

CPT 8 μM   **MMS 0.008%**   **Phleomycin 3.5** μg/ml

Plate 4

F8, D9 & H9 showed phenotype

CPT 4 μM   **MMS 0.004%**   **Phleomycin 2.5** μg/ml

CPT 8 μM   **MMS 0.008%**   **Phleomycin 3.5** μg/ml

Table 3.6 shows agarose gel image of library of mutants screened on various drug plates against a control, which is used as a reference plate to analyze phenotypes shown by colonies on the drug plate. Four sets of 96 well plates carrying mctp1 were replicated onto the target plates, of which 14 colonies showed phenotype to the drug agents. Finger print smear on plate one control plate occurs while trying to scan pictures of plate and did not affect outcome of screen.

Plate 2 isolate F5 was the only colony strain that showed sensitivity to CPT but did not show any sensitivity to other drug agents. Other plate isolates showed sensitivity to two or more genotoxins.

Figure 3.8: Agarose gel image of PCR products of mctp1 extracted from library of mutants that showed sensitivity to drug agents
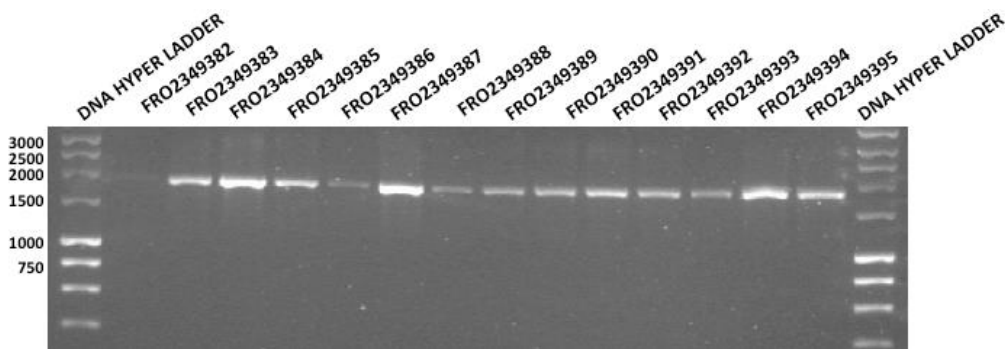
Figure 3.8 shows bands of DNA PCR product suspected to be mctp1; The bands appear to have a molecular weight of 1.8 kb, of which the respective products sent for sequenced.

Figure 3.9: map showing section of *S. pombe* genomic DNA that region of ctp1 aligned with some sequence results of mctp1 extracted from library of mutants
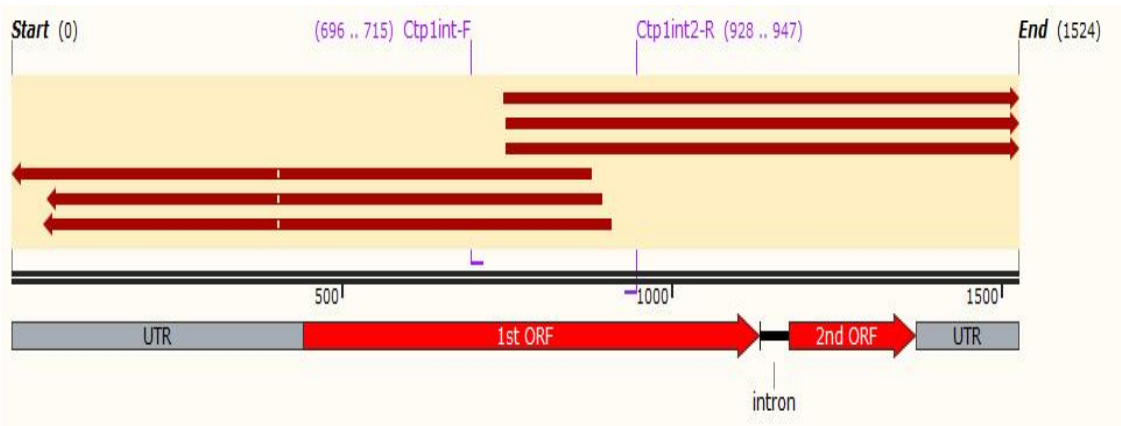
Table 3.9 shows map of *S. pombe* ctp1 featuring its untranslated region (UTR), and intron between its ORF. It is aligned with sequence results of mctp1 extracted from library of mutants created. The mctp1 gene was sequenced using ctp1int – F (sequence to the right) and ctp1int2 – R (sequence to the left) primers. Map shows entire gene of ctp1 were covered bt the sequence results of mctp1.

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Table 3.7: Analysis of mctp1 sequence results from screening

Plate: Isolate	Barcode # + Primers	Region of ctp1 sequenced	Point mutations	Amino acid change
P1: B3	FRO2349382 + ctp1int – F FRO2349328 + ctp1int2 – R	0 - 929	174 A → T 664 T → *	CAG → CTG = Gln → Leu 58 Frame shift
P1: B6	FRO2349383 + ctp1int – F FRO2349329 + ctp1int2 – R	0 - 929	61 A → G 225 T → C 664 T → A	NOT CLEAN
P1: B7	FRO2349384 + ctp1int – F FRO2349330 + ctp1int2 – R	0 - 929	364 T → A 829 T → C	AAT → AAA = Asn → Lys 121 TGT → CGT = Cys → Arg 261
P1: B9	FRO2349385 + ctp1int – F FRO2349331 + ctp1int2 – R	0 - 929	126 A → G 165 A → G	NOT CLEAN
P1: E9	FRO2349386 + ctp1int – F FRO2349332 + ctp1int2 – R	0 - 929	NONE	--
P2: F5	FRO2349387 + ctp1int – F FRO2349333 + ctp1int2 - R	0 - 929	88 A → G 305 T → C 378 T → C 482 T → C 620 T → G 672 A → G	GAA → GAG = Glu → Glu 29 TCA → CCA = Ser → Pro 102 GTC → GCC = Val → Ala 126 TTA → CTA = Leu → Leu 161 TTT → GTT = Phe → Val 207 TAC → TGC = Tyr → Cys 224

P2: G7	FRO2349388 + ctp1int – F FRO2349334 + ctp1int2 - R	0 - 929	52 T → C 201 T → C 478 G → C 848 T → A 900 T → A	CGT → CGC = Arg → Arg 17 GTT → GCT = Val → Ala 67 ATG → ATC = Met → Ile 159 GTG → GAG = Val → Glu 267 AAT → AAA = Asn → Lys 284
P2: C8	FRO2349389 + ctp1int – F FRO2349335 + ctp1int2 – R	0 - 929	NONE	--
P2:A10	FRO2349390 + ctp1int – F FRO2349336 + ctp1int2 – R	0 - 929	53 C → T 425 T → C 464 T → C	CAG → TAG = Gln → stop* 18 TTG → CTG = Leu → Leu 142 TCC → CCC = Ser → Pro 155
P3: D4	FRO2349391 + ctp1int – F FRO2349337 + ctp1int2 – R	0 - 929	491 A → G 658 A → G 740 T → A	AGG → GGG = Arg → Gly 169 AAA → AAG = Lys → Lys 219 TTC → TAC = Phe → Tyr 231
P3:D12	FRO2349392 + ctp1int – F FRO2349338 + ctp1int2 – R	0 - 929	NONE	--
P4: F8	FRO2349393 + ctp1int – F FRO2349339 + ctp1int2 – R	0 - 929	18 A → T 464 T → C 728 T → A	NOT CLEAN
P4: D9	FRO2349394 + ctp1int – F FRO2349340 + ctp1int2 - R	0 - 929	45 T → G 355 T → A 658 A → T	GTT → GGT = Val → Gly 15 NOT CLEAN NOT CLEAN

P4: H9	FRO2349395 + ctp1int – F FRO2349341 + ctp1int2 - R	0 - 929	376 T → C 806 T → G 849 G → A 866 A → G 888 A → T	TTT → TTC = Phe → Phe 125 NOT CLEAN NOT CLEAN CAT → CGT = His → Arg 273 AAA → AAT = Lys → Asn 280

Table 3.7 shows analysis on DNA sequence using Snapgene software package to discover point mutation accrued on the selected plate isolates. Of the 14 DNA sequence isolated and sequenced, only eight carried mutations on its gene with the remainder either having no mutations across its open reading frame or having contaminated chromatogram readings.

A total of 25 mutations were discovered across eight plate isolates, which resulted in one frameshift, a nonsense mutation, six silent mutations and 17 missense mutations. Every plate isolate either carried a mutation that changed transcript of the ctp1 gene and ultimately impaired function of its protein.

Table also shows amino acid residues in which the changes in codons were discovered.

DISCUSSION

Fission yeast also known as S. pombe are in a family of ascomycete that reproduce sexually through formation of spores. Fission yeast is a genetically amenable eukaryotic model organism, with a studied cell growth and cell division and a fully sequenced genome. In addition, the specie has been shown to experience high rates of HR, which allows precise manipulation of the genome to be used in genetic and molecular tools (Forsburg, 2001; Watson et al., 2007). Eukaryotes have two main pathways in repair of DNA DSB ends; they are NHEJ and HR, and the components of these pathways are evolutionary conserved from yeast to humans (Limbo et al., 2007; Yasuhiro Tsutsui et al., 2011). Fission yeast has a haploid life cycle that facilitates recovery of recessive mutations, which is useful in determining gene functions, and subject to carrying out random insertion of mutations. This helps in identification of gene of interests tagged by inserted cassettes. The results show that I was able to insert mutated ctp1 (mctp1) into its endogenous gene location in *S. pombe,* and perform screening with dosages of CPT, MMS and phleomycin to predict likely phenotypes of mutants that showed sensitivity to the drug agents. Mutants defective in ctp1 in *S. pombe* were identified because of their inability to form colonies on growth media that contained dosages of these genotoxins.

The protein coding ctp1 gene in *S. pombe,* has been shown to possess limited homology with SAE2 in *S. cerevisiae* and CtIP in *H. sapiens* at its C – terminal domain. The ctp1 is implicated in DSB end resection where it interacts with Nbs1 to activate endonuclease activity of Mre11, in which CtIP has been shown to carry out same function. The ctp1 gene being a CtIP – related endonuclease makes it the ideal tool to study the role of CtIP in providing resistance to CPT. The ctp1/CtIP are responsive to IR and studies have shown that they are both highly sensitive to dosage of CPT (You et al., 2010). However, although two CDK dependent phosphorylation sites (S-327 & T-847) have been discovered in CtIP, ctp1 phosphorylation site is still unknown. According to studies by Yasuhiro Tsutsui et al., CtIP/ctp1 possess homologous RHR and CxxC motifs of which are maintained across diverse eukaryotic species. Limbo et al. investigated this functional relevance of the conserved CxxC motif in ctp1, in which the motif was changed, and mutant

strains grew slowly and were hypersensitive to genotoxins. Result of my study in screening mutated ctp1 gene on CPT, MMS and phleomycin, produced colonies that were sensitive to the genotoxins. This implies that ctp1 is an essential in DSB repair through HR, and it plays important roles in removing covalently attached topoisomerases from 5' and 3' end of the DNA ends.

4.1 CRE-LOX RMCE SUFFICIENTLY CARRIES OUT RECOMBINATION OF CASSETTES BETWEEN LOX SITES

The cre enzyme derived from bacteriophage P1 is a characterized recombinase that influences site-specific recombination systems, thereby providing important tools for genetic manipulation (Watson et al., 2007). The reaction relies on hetero-specific sites Lox – P & Lox – M3, which recombine inefficiently with each other. Its recognition site located at the Lox – P is a 34 bp element consisting of two 13 bp repeat and an 8 bp spacer region in between. The mutant Lox – M3 carries a mutation between its spacer region that ensures compatibility and directionality of the recombination event and a stable product (Watson et al., 2007). The cre-lox RMCE were carried out *in vitro* to exchange cassettes containing mctp1 and ccdb, both flanked by lox – P and lox – M3 sites using commercially available cre enzyme. The aim was to incorporate mctp1 gene into cre expression plasmid pAW8 – ccdb in preparation for transformation into *E. coli* cells, and subsequent recombination into S. pombe base strain.

The *in vitro* cre reaction was carried out between linearized pAW8 – ccdb plasmid carrying a cre expression marker and mctp1 insert. The pAW8 – ccdb plasmid digested by *SpeI* close to its lox - M3 site, generated a linearized plasmid and this promoted the recombination event. Luxuriant growth of transformed E. coli cells carrying the recombinant plasmid was expected but this wasn't always the case. There were several situations when the cre – reaction did not lead to any transformed colonies after transformation into *E. coli* cells. Not carrying out the correct calculation of the molar concentration at the initial stage was one of the reasons for this, as a 1:4 ratio of plasmid vector to mctp1 was required. Also, poor pipetting practice was also a factor, along with use of excess cre buffer for the reaction mix with the thought that it would optimize the reaction to yield more products. Nonetheless, it is important that for efficient RMCE to occur, the lox sites must not be compatible with each other to prevent inversion of cassette or deletion of GOI in the presence of cre recombinase. On the other hand, *in situ* cre RMCE was performed after transformation of cre expression plasmid carrying mctp1 gene into *S. pombe* base strain. This site specific RMCE was more dependable as it efficiently

carried out the recombination event *in situ* by expression of cre marker on the plasmid. This resulted to numerous colonies growing in the media as expected.

4.2 TRANSFORMATION BY ELECTROPORATION PRODUCES MORE EFFICIENT OUTCOMES.

The product of *in vitro* cre reaction to incorporate mctp1 into cre - expression plasmid was then to be transformed into competent E. coli cells. In order to optimize transformation condition, the transformations were carried out on four different cell types to find out which transformation protocol would generate a more desired outcome. Electro-competent commercially available NEB 5-alpha and laboratory made DH5-alpha *E. coli* cells were used alongside chemically competent commercially available NEB 5-alpha and laboratory made DH5-alpha *E. coli* cells. After their respective transformation protocols were carried out, the transformants were mixed with SOC media and incubated for an hour to express beta – lactamase to break down Ampicillin used for selection on solid media. Result of transformation reaction showed Laboratory made electro-competent cells produced the best outcome for a single transformation reaction, with transformation efficiency 61.5 transformants/ng of DNA. This was followed closely by NEB electro-competent cells, which had 60.7 transformants/ng of DNA. Transformation efficiency postulates ability of cells to be transformed. On the other hand low yield were seen in the chemical transformation reaction, which is consistent with the fact that chemical transformation produces as much as three fold reductions in transformation efficiency. Other factors that could have led to the low yield would have been performing the heat shock with the hot water bath, which often had conflicting temperature readings.

Nevertheless, transformations into competent E. coli cells were often carried out successfully, however that amount yielded was not always at a maximum as shown in the result section. This could be as a result of handling of the cells, although cells were always kept on ice. It is a presentable fact that even when cells stay frozen, drop in temperature may lead to loss of its efficiency. This I learnt as I was guilty of using the cells on ice and returning it to storage at an apparent loss of efficiency only to use the remainder cells at a different time. This could be attributed to the reason laboratory made electro-competent cells, which had been under relatively constant temperature produced better transformation outcome than commercial NEB competent cells that may experience minute change in temperature during the course of packaging and transportation.

4.3   SCREENING FOR PHENOTYPES ON SELECTED DRUG AGENTS

Screens on *S. pombe* library of mutant carrying mutations on ctp1 were performed on varying concentrations of CPT, MMS and phleomycin. The general aim being to identify mutations on ctp1 that led to sensitivity to the drug agents, and more importantly discover mutations on ctp1 that showed sensitivity on CPT but not the other DNA damaging agents. Sequenced DNA data showed eight of the plate isolates were sensitivity to at least one of the drug agents, with isolate P2: F5 showing clear sensitivity to CPT but not to the other genotoxins. A total of one frameshift, a nonsense mutation, six silent mutations and 17 missense mutations were verified on the ORF of all mctp1 gene sequenced (Table 3.7). This is consistent with the fact that CPT sensitivity of mctp1 mutant cells, reflects defects in topoisomerase removal, of which validates the hypothesis that ctp1 plays distinct role in removing covalently linked topoisomerase from DSB end (Hartsuiker et al., 2009). This results in resistance of CPT to maintain it's covalent attachment on DSB ends and prevent replication and transcription. However it is worthy of note that CPT attachment to DNA DSB ends does not lock topoisomerase on the strand permanently, but only increases the half-life of topoisomerase – DNA covalent interaction thereby stalling replication and transcription process (Hartsuiker et al., 2009; Deshpande et al., 2009).

The ctp1 open reading frame encodes 294 aa, while human CtIP is a 897 aa protein sequence, and both proteins collaborate with MRN complex through motifs on its Nbs1 domain to activate mre11 that generate clean 3' ssDNA tails for invasion of homologous sister strands. However, screening for phenotypes is a statistical effort that requires generation of libraries to continuously create original mutations that could lead to more data for screens and more possible outcome. Nevertheless, the experiment would shed more understanding and extensively display more unique sites that bring about sensitivity to CPT.

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APPENDIX I

CRE-RECOMBINASE REACTION

Molar Ratio of Plasmid to Insert Ratio

Purified DNA	Concentration (C)
Concentration of mutagenized pAW8-ctp1 insert	50ng/μl
Concentration of wild-type pAW8-ctp1 insert	50ng/μl
Concentration of *Spe1* restricted pAW8-ccdb plasmid vector	91.4ng/μl

Purified DNA	Molecular Weight (MW)
Length of Spe1 restricted pAW8-ccdb plasmid vector	8867bp
Length of pAW8-ctp1 insert	1200bp

Molar ratio of plasmid:insert ratio of 1:4 was calculated using the formulae

For a 1:1 volume weight of insert = length of plasmid/length of vector x 170ng

$$= 23ng$$

Taking 170ng to be weight of plasmid vector

For plasmid:insert ratio of 1:4 volume of insert = 23ng x 4 **/** 50ng/μl

$$= 1.8μl$$

Volume of plasmid vector = 170ng **/** 91.4ng/μl

$$= 1.9 μl$$

Volumes Used for Cre – Reactions

Total Cre reaction volumes for each reaction is 10μl

Plox2 Positive control	Negative control	Mutagenized ctp1	Wild-type ctp1
Linearized plox2 – 1μl	ccdb Plasmid – 1.9μl	Ccdb plasmid – 1.9 μl	Ccdb plasmid – 1.9 μl
25% PEG soln. – 2μl	25% PEG soln - 2 μl	Mctp1 insert – 1.8 μl	Wctp1 insert – 1.8 μl
10 x Cre-buffer - 1μl	10 x Cre-buffer - 1 μl	25% PEG soln - 2 μl	25% PEG soln - 2 μl
Cre recombinase – 0.5μl	Cre recombinase – 0.5μl	10 x Cre-buffer - 1 μl	10 x Cre-buffer - 1 μl
Sterile water – 5.5μl	Sterile water – 4.6μl	Cre recombinase – 0.5μl	Cre recombinase – 0.5μl
		Sterile water – 2.8 μl	Sterile water – 2.8 μl

All reactions were placed in a thermo-cycler at $37°c$ for 30 minutes and $70°c$ for 10 minutes and transformation into *DH5 alpha* competent *E.coli* cells.