

A Novel DNA Damage Response Network Associated with the CTD of RNA
Polymerase II

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Biochemistry in the Graduate School
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ABSTRACT

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Abstract

Since RNA Polymerase II (RNAPII) transcribes much of the genome, it is well situated to encounter and initiate a response to various types of DNA damage. However, to date very little is known about any response of RNAPII to DNA damage outside of Transcription Coupled Nucleotide Excision Repair (TC-NER). A link between DNA damage response mechanisms and the C-terminal domain of RNAPII (CTD) is suggested by an overlap between proteins that bind the CTD and genes required for resistance to DNA damaging agents. In this thesis, I show that proper deployment of CTD associated proteins is required to respond to DNA damaging agents. Furthermore, I show that a CTD associated protein (Set2) is required for response to DNA damage, but its catalytic activity is not. Finally, I show that the recombinational ability of strains lacking the CTD kinase, Ctk1, is deficient. Based on these lines of evidence, I propose a novel CTD Associated DNA Damage Response (CAR) system of proteins that is required for proper response to DNA damaging agents.

Dedication

In memory of Craig Bennett whose optimism in life and passion for science taught me more than I could ever learn by attending seminars or reading papers.

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1. Introduction

1.1 RNA Polymerase II and its C-Terminal Domain

RNA Polymerase II (RNAPII) is responsible for the bulk of transcription in eukaryotes, including synthesis of all mRNAs and most non-coding RNAs such as miRNA precursors, lnc RNAs, and some small nuclear and nucleolar RNAs. One key feature of RNAPII is an unusual C-terminal domain (CTD) on the core subunit of the polymerase (Rpb1). The CTD is an unstructured domain that extends from the body of the polymerase allowing coordination of other co-transcriptional processes by binding to factors involved in those processes (Egloff et al., 2012; Phatnani and Greenleaf, 2006; Bartkowiak et al., 2011; Chapman et al., 2008; Buratowski, 2009). It consists of tandem heptad repeats of the consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$ (Corden et al., 1985). This domain is highly conserved throughout all eukaryotes and the number of repeats roughly correlates with genomic complexity; for example, the mammalian CTD (52 repeats) is twice as long as the yeast CTD (26 repeats) whereas the *Drosophila* CTD has an intermediate length (44 repeats) (Allison et al., 1988; Corden, 1990).

Although all eukaryotes contain a CTD, conservation of the consensus repeats varies. *Saccharomyces cerevisiae* has a uniform CTD consisting primarily of consensus repeats whereas the *Drosophila* CTD is quite degenerate with only 2 repeats exactly matching the consensus sequence. The mammalian CTD on the other hand, consists primarily of consensus repeats on the N-terminal half and largely of degenerate repeats on its distal or C-terminal half. Although the CTD is dispensable for transcription *in vitro*, it plays an essential role and cells in which the CTD has been completely deleted are inviable (Zehring et al., 1988;

Nonet et al., 1987). Furthermore, in yeast, the CTD cannot be truncated below 10 repeats without severe growth defects and truncation beyond 8 repeats is completely lethal (West and Corden, 1995). The required functional unit of the CTD seems to be two contiguous repeats as a CTD that is interrupted by insertion of alanine between every two repeats is tolerated while insertion of alanine between each repeat is not (Stiller and Cook, 2004).

1.1.1 CTD regulation via posttranslational modifications

Given the abundance of potentially modifiable amino acids in the CTD, it is not surprising that the CTD is subject to posttranslational modifications. Indeed, early analysis of the CTD revealed extensive phosphorylation of serine and threonine residues. A later analysis also found some phosphorylation of tyrosine residues although the function of the latter modification has not been ascertained (Zhang and Corden, 1991; Baskaran et al., 1993).

Current research suggests that it is phosphorylation of Ser2 and Ser5 of the heptad that are the major targets for transcriptionally regulated phosphorylation. Recent results also suggest that Ser7 plays an important role (Bartkowiak et al., 2011; Egloff and Murphy, 2008; Egloff et al., 2007; Chapman et al., 2007). The CTD is thought to undergo a series of modifications depending on the phase of transcription. RNAPII with an unphosphorylated CTD is recruited to promoters. After formation of the preinitiation complex, the CTD is phosphorylated on Ser5 and Ser7. During elongation, phosphorylation is added at Ser2 as well. It has been appreciated for some time that CTDs doubly phosphorylated on Ser2 and Ser5 are important during elongation and recent information about Ser7 phosphorylation suggests that in many cases it may also

be important during elongation although less is known about this modification (Phatnani and Greenleaf, 2006; Chapman et al., 2007; Glover-Cutter et al., 2009; Kim et al., 2009). As RNAPII approaches the 3' end of the gene, Ser5 levels drop leaving a CTD enriched for phosphorylation at Ser2 and Ser7 (Kim et al., 2009).

This general model is likely a fairly severe oversimplification. Recent papers suggest that the precise pattern of CTD phosphorylation across the length of a gene is largely gene specific (Drogat and Hermand, 2012; Egloff et al., 2012). For example, this general model is much more likely to hold true for protein coding genes; Ser7 phosphorylation is thought to remain high throughout transcription on many protein-coding genes but seems to drop towards the 3' end of non-coding genes. Other gene-specific deviations from this general model are also common.

Another area of study intimately connected to the pattern of phosphorylation on the CTD across a gene is that of the kinases and phosphatases responsible for placing these marks. As the preinitiation complex forms, the Ser5 and Ser7 marks are placed on the CTD by the CTD kinase Kin28 in yeast and CDK7 in metazoans (Akhtar et al., 2009; Glover-Cutter et al., 2009). This kinase is part of the general transcription factor TFIIF.

As RNAPII enters productive elongation, the Ser2 mark is placed on the CTD. Two kinases are known to place this mark in yeast, Ctk1 and Bur1 (Sternier et al., 1995; Lee and Greenleaf, 1989). The primary target of phosphorylation by Bur1 may be the transcription elongation factor Spt4/Spt5 (Keogh et al., 2003; Pei and Shuman, 2003); however, it has been shown to also be important in Ser2 phosphorylation as it binds to a Ser5 phosphorylated CTD and contributes Ser2 phosphorylation early on. These initial Ser2 phosphorylation events may

stimulate Ctk1 activity, causing Ctk1 to catalyze the bulk of Ser2 phosphorylation as productive elongation continues.

For many years, it seemed that higher eukaryotes had only one Ser2 kinase, P-TEFb, and this kinase was thought to perform in metazoans the functions of both Ctk1 and Bur1 in yeast. However, it has recently been shown that this is not the case. Another kinase, CDK12 has been identified as an elongation phase kinase responsible for the bulk of Ser2 phosphorylation and the functional counterpart of Ctk1 (Bartkowiak et al., 2010; Blazek et al., 2011). Therefore, the model of Ser2 phosphorylation in higher organisms has been updated to include P-TEFb (CDK9) as the functional counterpart of Bur1 and CDK12 as the counterpart of Ctk1.

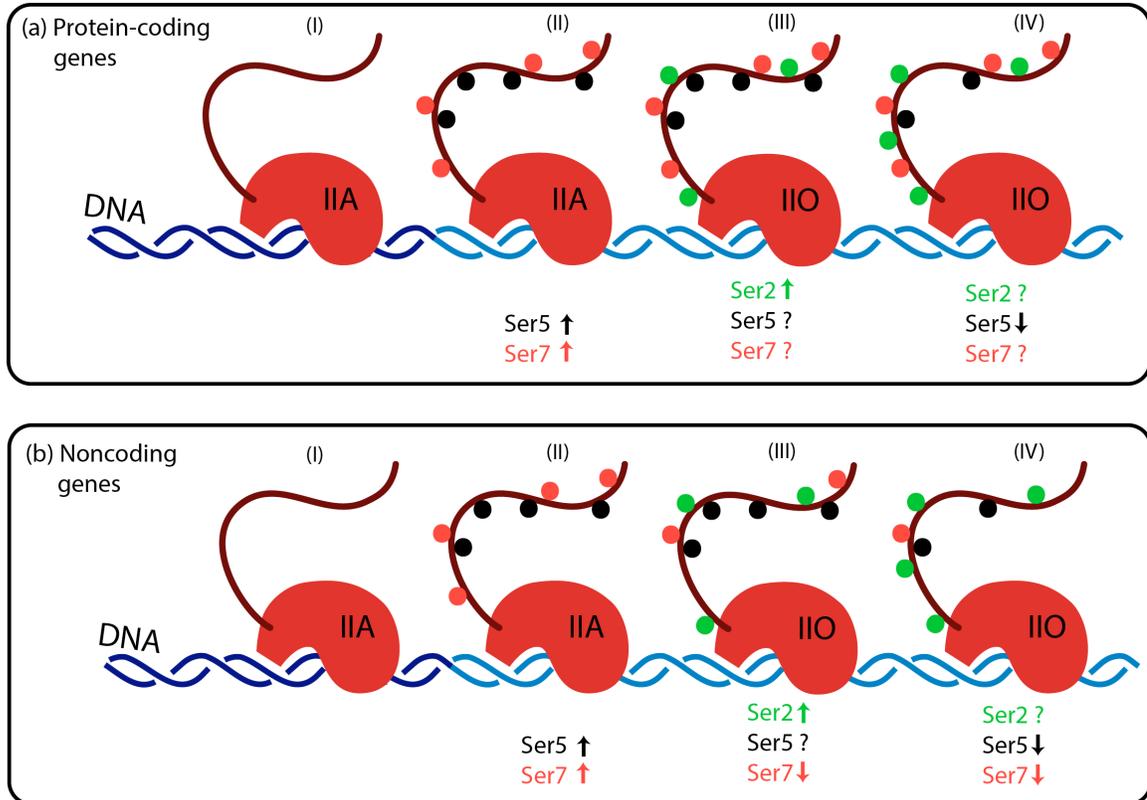


Figure 1: Cycle of CTD phosphorylation

(a) (I) At protein coding genes, unphosphorylated RNAPII is recruited to the promoter (IIA form). (II) After the pre-initiation complex is formed, the CTD gets phosphorylated at Ser5 (black) and Ser7 (red). (III) The hyperphosphorylated form of the CTD occurs during elongation as Ser2 (green) gets phosphorylated. (IV) At the 3' end of the gene, phosphatases decrease the levels of Ser5 phosphorylation. (b) (I) At non-coding genes, unphosphorylated RNAPII is also recruited to the promoter. (II) As with coding genes, following formation of the pre-initiation complex, the CTD is phosphorylated at Ser5 and Ser7. (III) During elongation, Ser7 phosphorylation levels decrease while Ser2 phosphorylation levels increase. (IV) Towards the 3' end of the gene, phosphatases cause a decrease in Ser5 levels.

While many questions remain to be answered about the kinases responsible for CTD phosphorylation, even less is known about the phosphatases responsible for their removal. Several phosphatases capable of removing phosphates from the CTD have been identified. In yeast, Ssu72 and Rtr1 have both been shown to dephosphorylate Ser5 (Ssu72 can also remove Ser7 phosphates) and future studies are needed to determine more about their individual roles during transcription (Zhang et al., 2012; Werner-Allen et al., 2011; Xiang et al., 2010; Mosley et al., 2009). A third phosphatase in yeast, Fcp1, can remove Ser2 phosphates making RNAPII available for subsequent rounds of transcription (Archambault et al., 1997).

Another post-translational modification of the CTD that has received attention is the isomerization of prolines. The peptidyl proline isomerase, Ess1, is known to bind a phosphorylated CTD and then catalyze isomerization of the prolines at positions 3 and 6 of the CTD repeat (Morris et al., 1999). Recent studies have shown that CTD phosphatases may require either the trans or cis configuration prior to associating with the CTD and catalyzing the removal of phosphates (Kops et al., 2002; Werner-Allen et al., 2011; Xiang et al., 2010). Thus, it is likely that proline isomerization allows for fine-tuning of phosphate removal and is an additional level of regulation on CTD phosphorylation.

Additional post-translational modifications of the CTD continue to be discovered. For example, Arg methylation in the mammalian CTD appears to affect expression of some small nuclear RNAs and small nucleolar RNAs (Sims et al., 2011). Also, the mammalian CTD contains seven Lys residues in its distal half, and these appear to be subject to acetylation (K. Adelberg and colleagues, in

press). Finally, O-linked glycosylation has been detected in the mammalian CTD (Ranuncolo et al., 2012)

1.1.2 Why CTD phosphorylation is important: co-transcriptional binding of specific factors

The changes in CTD phosphorylation throughout the transcription cycle allow different proteins to bind the CTD during different phases of transcription. For example, capping of the 5' end of mRNAs occurs co-transcriptionally due to recruitment of capping enzyme to the 5' end of a gene via a CTD phosphorylated on Ser5 (Schroeder et al., 2000). In the case of capping, the enzyme has been shown to be capable of binding to both Ser2P and Ser5P synthetic peptides; however, only the Ser5P peptide caused the capping enzyme to be allosterically activated (Ho and Shuman, 1999). Furthermore, transcripts produced in a system made by a CTD-less RNAPII are not capped properly (Cho et al., 1997). Thus, proper capping of mRNAs is completely dependent on the ability of capping enzyme to bind the CTD and be stimulated by Ser5 phosphorylation.

Capping of mRNAs is not the only CTD-dependent event that happens co-transcriptionally. Rather, many different proteins associate with the CTD in a transcription phase dependent manner. Other phosphorylation patterns have also been shown to bind specifically to other proteins. For example, Pcf11 is involved in 3' end formation and has been shown to associate with Ser2 phosphorylated CTD repeats (Licatalosi et al., 2002). The recruitment of each of these proteins fits nicely into the canonical view of the CTD; capping enzyme is needed at the 5' end of the gene and is therefore recruited by Ser5 phosphorylation which predominates at the 5's end; Pcf11 is needed at the 3' end of the gene and therefore is recruited by Ser2 phosphorylation which

predominates at the 3' end. While these two proteins fit nicely into a standard RNAPII CTD model, they don't answer the question of what is happening along the CTD while the polymerase is in the middle of a gene, during the bulk of elongation. Recent studies investigating highly phosphorylated forms of the CTD thought to predominate during elongation have uncovered some exciting and unexpected results.

Several proteins have been shown to bind CTD repeats phosphorylated on both Ser2 and Ser5, a phosphorylation state that is believed to predominate during the bulk of elongation. In fact, a large number of CTD associated proteins were discovered several years ago when our lab, led by Phatnani *et al* (2004), undertook an effort to systematically identify proteins that associated with the elongating form of the CTD in yeast. Yeast extracts were fractionated and fractions that contained potential Phospho CTD Associating Proteins (PCAPs) were applied to affinity columns that contained either a recombinant yeast CTD hyperphosphorylated by CTDKinase-I (CTDK-I) or synthetic peptides each containing three heptad repeats that were phosphorylated on Ser2 and Ser5 of each repeat. Proteins that bound to either of these affinity columns, but not to control columns, were identified by mass spectrometry. This study identified over 100 PCAPs as associating with this elongating form of the phosphoCTD. Several of these proteins have been shown in this study and subsequent studies to have a direct binding interaction with CTD repeats phosphorylated on Ser2 and Ser5 including the splicing factor Prp40, the histone methyltransferase Set2, the yeast export factor Yra1, and the kinase Hrr25 (Phatnani et al., 2004; Kizer et al., 2005; MacKellar and Greenleaf, 2011).

Table 1: PCAPs required for DNA damage resistance

Cellular Function	PCAPs Found
Transcription	Cdc73^{IR} , Cka1 , Cka2 , Ctr9 , Ess1 , Hog1^{DX} , Hsf1 , Not5^{IR&DX} , Pho2^{DX} , Rap1 , RDS2^{DX} , Rtg2 , Spt15 , Ume6^{IR}
RNA Processing	Brx1 , Cbf5 , Cbp2 , Ctl1 , Emg1 , Enp1 , Glc7 , Hca4 , Kem1 , Mrt4^{IR&DX} , Pus1^{DX} , Rai1^{IR} , Rat1 , Reg2 , Rpf2 , Rrp5 , Snu56 , Ssd1 , Ssf1 , Ssf2 , Tsr1 , Utp20 , Vip1 , Yra1
Chromatin Structure	Gcn5^{IR&DX} , Has1 , Hat1 , Hta1/2 , Htb1/2 , Htz1^{DX} , Isw1 , Nhp6a/b , Nnf1 , Set2^{IR&DX} , Spt7^{DX}
DNA Metabolism	Cdc14 , Chl1^{IR} , Hrr25 , Jem1^{IR} , Pms1 , Stm1 , Tah18 , Top1 , Ypr078c
Protein Synthesis & Turnover	Cdc33 , Cic1 , Fun12^{IR&DX} , Gcd10 , Gcd14 , Lia1^{DX} , Mis1 , Mrp7 , Mrp18 , Pus2 , Sis1 , Ssb1 , Ssb2 , Tef1 , Tef2 , Trm1 , Trm8 , Trm82 , Tys1 , Yer087w , Ygr054w , Zuo1^{IR&DX}
Other/Unknown	Abf2 , Arh1 , Fmt1 , Fox2 , Msw1 , Myo3 , Myo5 , Nfs1 , Noc2 , Osh2 , Qri2 , Rvs161^{IR} , Sac1^{DX} , Tes1 , Vps16 , Ygr210c , Ygr273c , Yil105c , Yjr149w , Ylr455w , Ymr265c , Ypl260w^{DX}

List of PCAPs identified by Phatnani et al (2004) and sorted by cellular function. Proteins in red are products of genes identified by Bennett and colleagues as required for normal resistance to ionizing radiation (IR)(Bennett et al., 2001) or doxorubicin (DX) (Westmoreland et al., 2009). **Bold** = non-essential; light = essential; underlined = binds directly to PCTD; *italics* = does not bind directly to PCTD

Research in the coming years will likely identify even more of these PCAPs as having a specific and direct association with doubly phosphorylated CTD repeats.

Perhaps even more remarkable than the number of PCAPs discovered by Phatnani *et al*, is the diversity among them. As can be seen in Table 1, many proteins with a wide variety of known functions associate with the elongating form of RNAPII. Why would so many different classes of proteins associate with elongating polymerase and is there any connection between them? While we were pondering this and other questions related to the assortment of PCAPs that we had discovered, Bennett and colleagues (2001) had screened the diploid version of the yeast deletion collection for genes that were required for ionizing radiation (IR) resistance in yeast. They discovered, 169 genes that were not previously known to be required for resistance to IR in yeast (Westmoreland *et al.*, 2004; Bennett *et al.*, 2001). Intriguingly, several of these genes encoded proteins that we had identified as PCAPs associated with the elongating form of RNAPII. Indeed, the correlation between these two data sets is extremely significant. In order to make sure that this was indeed statistically significant and not just a random event, we analyzed the overlap between Bennet's IR data and the non-essential PCAPs found in our lab (our screen for PCAPs was able to observe essential proteins, while Bennett was unable to examine essential genes, so all genes/proteins required for cell viability were left out of our statistical analysis for consistency). Table 1 illustrates this overlap by showing all PCAPs found in the screen by Phatnani *et al* (2004) and highlighting those that were also found to be required for DNA damage resistance in red. Chi Squared analysis of these two data sets gives a P value of <0.0001. Or in other words, if

there was no association between these two data sets, the likelihood of observing this overlap as a random event is <0.01%.

Indeed, the more we examined our list of PCAPs for proteins which when deleted may exhibit sensitivity to DNA damaging agents, the more significant this observation seemed to be. Including another screen for sensitivity to doxorubicin (DX) further expanded the overlap between PCAPs and DNA damage (Westmoreland et al., 2009). Also, although we were unable to include PCAPs that were essential for cell viability in our statistical analysis, some essential PCAPs had been previously shown to be linked to DNA damage events (see next section for more discussion on PCAPs previously known to be involved in DNA damage related responses).

1.2 Transcription and DNA Damage Responses

The idea that transcriptional events may intersect with DNA damage responses is not new. In fact, more than two decades ago, it was discovered that nucleotide excision repair (NER) of UV-induced pyrimidine dimers in mammalian cells occurs faster on an actively transcribed strand of DNA than on the non-transcribed strand or in the genome overall (Mellon et al., 1987). Since that time, the pathway of “transcription-coupled repair” (TCR, or TC-NER), has been extensively studied (Hanawalt and Spivak, 2008; Tornaletti, 2009). A great deal is now known about the role of RNA polymerase II (RNAPII) and its coupling factors in TC-NER (Lagerwerf et al., 2011). More recently RNAPII has been implicated in responses to other types of DNA damage, and extant evidence now suggests a much broader role for RNAPII in DNA damage responses (Svejstrup, 2010).

It has been appreciated for some time that the remarkably slow off-rate for RNAPII that has encountered elongation-blocking DNA lesions may provide the polymerase great sensitivity in DNA damage recognition (Lindsey-Boltz and Sancar, 2007). With the recent understanding that RNAPII in fact transcribes almost the entire genome at some level (ENCODE Project Consortium et al., 2007), it has become clear that RNAPII could play a major role in detecting and signaling the presence of many types of DNA damage in a large fraction of the total DNA of a cell. Given the stability of stalled RNAPII elongation complexes, it is probable that polymerase does not have to specifically recognize DNA damage to detect it efficiently; the RNAPII merely needs to be blocked by it. This mode of finding DNA damage has been referred to as “recognition by proxy” (Sancar et al., 2004).

In view of the above, the fate of RNAPII stalled within a transcription unit for any reason is of great interest. A number of studies have contributed to current ideas in this area, but there is some debate over what happens to RNAPII after its movement is blocked. One well established fate for stalled RNAPII is ubiquitylation, which can eventually target the enzyme for degradation (Bregman et al., 1996). Rsp5 (in yeast) has been known for some time as an E3 ligase targeted to RNAPII (Huibregtse et al., 1997; Somesh et al., 2005). Rsp5 binds to the CTD of RNAPII (Morris and Greenleaf, 2000), and this binding is required for the Rpb1 ubiquitylation process (Somesh et al., 2007). Recently, it was shown that two ubiquitin ligases, Rsp5 and the Elongin-Cullin complex, act in succession to mark the Rpb1 subunit for degradation (Harreman et al., 2009). In mammals, which contain several homologs of Rsp5, the functional counterpart of Rsp5 appears to be Nedd4. The two-step ubiquitylation process acting on

Rpb1 is thought to also function in mammalian cells, with Nedd4 acting first and elongin-cullin 5 acting second (Harreman et al., 2009). It is important to note that results presented by Anindya *et al* (2007) indicate that simply stalling or blocking the RNAPII, even in the absence of DNA damage, also induces the ubiquitinylation response.

In addition to ubiquitinylation, it has been reported that RNAPII can also be sumoylated when elongation is impaired by a DNA lesion or an inhibitor (Chen et al., 2009a). Although the role of this modification has yet to be determined, it is not involved in checkpoint activation or RNAPII ubiquitinylation and degradation; nor is it required for proper TC-NER. Thus, sumoylation likely represents yet another transcriptional response to DNA damaging conditions (Chen et al., 2009a).

In mammalian cells, stalling of RNAPII has also been shown to initiate the p53-dependent checkpoint pathway (Derheimer et al., 2007). One effect of inhibiting transcription (initiation or elongation) is a buildup of p53 in the nucleus. This likely occurs because under normal circumstances p53 is shuttled to the cytoplasm by a process that is coupled to RNA export. Inhibiting transcription leads to reduced RNA export causing p53 to accumulate in the nucleus. When elongation is blocked, generating stalled RNAPIIs, the accumulated p53 becomes phosphorylated. The trigger for this phosphorylation (and thus activation) of p53 appears to be the stalled polymerase itself. Since p53 phosphorylation causes checkpoint initiation, one mechanism of checkpoint activation can thus be directly linked to the physical stalling of RNAPII (Derheimer et al., 2007).

1.3 DNA Damage Responses and the CTD: another process co-transcriptionally linked to RNA Polymerase II via the CTD?

Since the CTD coordinates many transcription-related processes, it is sensible to predict that it may also help coordinate DNA damage response pathways involving RNAPII. Indeed, proper damage responses are dependent on RNAPII's having a functional full-length CTD (Wong and Ingles, 2001), and the phosphorylation state of the CTD can be affected by transcription-blocking DNA damage (Kleiman et al., 2005; Heine et al., 2008; Sordet et al., 2008). Furthermore, the CTD kinase, CTDK-I, is required for normal levels of resistance to HU, UV, MMS, 4NQO, IR and doxorubicin (Ostapenko and Solomon, 2003; Jeong et al., 2005; Westmoreland et al., 2009). Moreover, individual deletions of any one of the three components that comprise CTDK-I (Ctk1, Ctk2 or Ctk3) have been found to be synthetically lethal with individual deletions in a large number of well characterized genes required for DNA repair, notably genes in repair pathways involving homologous recombination (HR), such as *rad50*, *rad51*, *rad52*, *rad6*, *rad27*, *rad54*, *rad55*, and *mre11* (see the Saccharomyces Genome Database, SGD <<http://www.yeastgenome.org>>).

Even before we recognized an extensive overlap between our PCAPs and sensitivity to DNA damaging agents (see section 1.1.2), there was evidence that several PCAPS may be involved in DNA damage responses. As previously mentioned Rsp5, an E3 ligase in the RNAPII ubiquitinylation pathway, binds to the enzyme via the CTD, linking the CTD to RNAPII degradation following DNA damage (Morris and Greenleaf, 2000). Furthermore, several PCAPs had been shown to be required for resistance to DNA damaging agents or are

otherwise involved in genome stability; these include yeast PCAPs Ess1, Hrr25, Cdc14, Chl1, Pms1 and Topo1 (Morris et al., 1999; Ho et al., 1997; Phatnani et al., 2004; Jeong et al., 2005), as well as mammalian PCAPs PARP1, Topoisomerase I, and RecQ5 (Carty and Greenleaf, 2002; Bouchard et al., 2003; Dantzer et al., 2006; Li, 2005; Kanagaraj et al., 2010; Wu et al., 2010).

Given the accumulating evidence that RNAPII plays a role in several DNA transactions that affect genome stability, the extensive range of already-known CTD functions, and the significant overlap between PCAPs and genes required for resistance to DNA damaging agents, we propose that the CTD plays a much larger role in orchestrating RNAPII-dependent DNA damage responses than is generally recognized. In the remainder of this thesis, I will present further evidence that RNAPII indeed participates in cellular responses to DNA damage and that the CTD plus its associated factors are components of an expansive genome integrity-maintenance system.

2. Depletion of CAR proteins from elongating RNAPII causes sensitivity to DNA damage

2.1 Introduction

As discussed in Chapter 1, many factors that act co-transcriptionally are recruited to RNAPII at the proper time via reversible phosphorylation marks placed on the CTD coincident with the appropriate phase of transcription. As a flexible binding platform that continually oscillates between phosphorylation states during the transcription cycle, the CTD is the perfect instrument to recruit a wide variety of factors to RNAPII. Therefore, any process that must occur co-transcriptionally could potentially require essential factors to be recruited to RNAPII via the CTD. In chapter 1, I described current evidence that suggests a role for transcription by RNAPII in DNA damage and repair mechanisms extending beyond TC-NER. I also discussed data from our lab that suggests there may be a CTD specific role in such a mechanism. Based on these observations, we propose that proteins associated with the highly phosphorylated form of the CTD present during elongation may function in a DNA damage repair network. Such a system does not need to be composed entirely of proteins dedicated solely to the detection and repair of regions of damaged DNA. Rather, we believe it is reasonable for biology to make use of proteins already recruited to the CTD for other purposes. Simply obstructing smooth functioning transcription may be enough to trigger a DNA damage response. Such a response could be initiated by a stalled RNAPII with the end result of triggering established DNA repair pathways. We refer to such PCAPs that have a DNA damage response phenotype as CTD Associating DNA damage Response proteins or CAR proteins.

In this chapter, I make use of a system that disrupts proper deployment of PCAPs to the CTD in an effort to examine what happens when the endogenous RNAPII

is deprived of a proper arrangement of CAR proteins. As will be shown in section 2.3, I was able to characterize this system to show that it does indeed disrupt PCAPs as anticipated. Furthermore, I explore the DNA damage phenotype of cells that are experiencing such a disruption. Finally, I briefly examine potential synthetic lethal phenotypes between disrupting proper PCAP deployment to the CTD and deletion for genes of interest.

2.2 Materials and Methods

2.2.1 Strains and plasmids

We used diploid strains from the BY4743 background. Deletions were obtained from the yeast deletion collection (Winzeler et al., 1999).

The nucCTD and cytoCTD plasmids were derived from pTCM-RA and pTCM-RR (Moreland et al., 1985) by restriction digestion of a β galactosidase-CTD fusion construct at the Sst1 site internal to the LacZ gene and an EcoRI site downstream of the CTD sequence, followed by ligation into the corresponding sites of the pTCM plasmid (J.M. Lee, unpublished). To construct the nuc β Gal plasmid we used Pfu DNA Polymerase (Invitrogen) to amplify the portion of the LacZ gene used in the nucCTD plasmid from pTCM-RA. The resulting PCR product was then purified using a PCR clean-up kit (Qiagen) and incubated for 10 minutes at 72 degrees with taq polymerase and dNTPs to add adenine overhangs on the 3' ends of the PCR product. We then used the Gateway system (Invitrogen) and TOPO cloning to clone this product into an ENTRY vector. The resulting ENTRY vector was used in an LR recombination reaction to transfer the nuc β Gal sequence to pYES DEST52. All cloning reactions were performed according to the manufacturer's instructions (Invitrogen).

2.2.2 Localization of fusion proteins

Plasmid-containing cells were grown to confluency in complete medium minus

uracil with glucose as the carbon source (CM+glu-ura) and then were washed in water and resuspended in complete medium minus uracil with galactose as the carbon source (CM+gal-ura) to induce expression of the β Gal-CTD fusion protein. Cells were allowed to grow for six hours and were then fixed in formaldehyde. Cells were prepared as previously described (Pringle et al., 1991) and stained with both DAPI and mouse anti- β galactosidase (Promega). A goat anti mouse secondary conjugated to Alexa Flour 488 (Invitrogen) was used for visualization of the primary antibody.

2.2.3 IR sensitivity assay

Irradiations were performed as described in Bennett et al. (2001). Briefly, for dilution plating assays, plasmid-containing cells were grown for two days in a 96 well dish in CM+gal-ura. Five-fold serial dilutions were made, plated to CM+gal-ura plates, and irradiated. For survival curves, cells were pre-grown for 24 hours in liquid CM+gal-ura, diluted in water, and irradiated. Irradiated cells were then plated to CM+gal-ura plates and colony counts were compared to plates from the same dilution where the cells had not been irradiated.

2.2.4 Doxorubicin Sensitivity

Strains containing the indicated plasmids were grown two days in complete medium lacking uracil. Serial 5-fold dilutions were plated to either CM+gal-ura or CM+glu-ura plates that contained either no doxorubicin or 25 μ g/mL doxorubicin.

2.2.5 Antibodies and Western Blotting

Extract Preparation:

Plasmid-bearing cells were grown in 20 mL of CM+glu-ura to saturation. Cells were then washed in H₂O and split into two equal portions that were resuspended in 50 mL of either CM+glu-ura or CM+gal-ura and incubated approximately 14 hrs. Cells

were then pelleted and washed one time in H₂O. Cell pellets were flash frozen by extruding cell pellets into liquid N₂. Approximately 0.1 g of frozen cells were weighed out and placed in a 2-mL screw cap microvial (Sarstead). Five hundred μ L of 95% ethanol was added to each tube and the samples were broken open by shaking for 5 minutes with zirconia beads in Mini-Beadbeater 16 (Biospec). The entire contents of each microvial (including beads) were placed under vacuum (SpeedVac) until dry. 300 μ L 2.5X SDS sample buffer without dye was added to each tube, followed by heating 10 minutes at 98°C, with intermittent vortexing. The resulting SDS sample was collected through a hole that was made in the bottom of the tube by centrifugation into another tube. This spin-through was then centrifuged at max speed in a microfuge (ca. 16,000 X g) for 10 minutes to precipitate any unsolubilized material. The SDS-solubilized portion (supernatant) was placed in another tube, and bromophenol blue was added to 0.02% prior to SDS-PAGE.

Antibodies used in western blotting include: “anti-2,5P,” a rabbit polyclonal antibody made against a CTDK-I phosphorylated yeast CTD fusion protein and affinity purified on a two repeat CTD peptide column with serines 2 and 5 phosphorylated in each repeat; “anti-5P,” rat mAb 3E8 (Chapman et al., 2008); “anti-2P,” rat mAb 3E10 (Chapman et al., 2008); mouse anti- β Gal, (Promega Z378B); anti-Pgk1, mouse anti-3-phosphoglycerate kinase (Invitrogen A6457).

2.2.6 Synthetic Dosage Lethal Interactions

The plasmids nucCTD and cytoCTD were introduced into WT and isogenic diploid deletion strains by transformation and selection on CM+glu-ura. Deletion strains containing NucCTD or CytoCTD plasmids were grown overnight in CM+glu-ura. Cells were then diluted in water and equal numbers were plated for single colonies to either CM+glu-ura or CM+gal-ura. The plates were allowed to grow at 30 C until

colonies appeared and survival was determined by comparing colony-forming units on CM+glu-ura vs. CM+gal-ura.

2.2.7 Immunoprecipitations

Strains containing either the nucCTD plasmid or the nuc β Gal plasmid were grown in raffinose-containing medium to an A600 of 0.6-0.8. At that point, 40% galactose was added to a final concentration of 2% and the cells were grown for 6 more hours. Cells were collected by centrifugation and frozen in liquid nitrogen as described by Rout and colleagues (Oeffinger et al., 2007). Extraction buffer (50mM HEPES pH 8.0, 100 mM NaCl, 0.5% NP40, 0.1% PMSF, 1% protease inhibitor [Sigma p8215], 1% phosphatase inhibitor [Sigma p2850]) was also frozen and added to the frozen cells for a total mass of approximately 5g (about 2.5g cells + about 2.5 g frozen buffer). Cells+buffer were homogenized in the frozen state using a Retsch MM400 Ball Mill, essentially as described (Oeffinger et al., 2007), and the resulting powder was stored at -80°C. Approximately 1g aliquots of cell powder were resuspended in 1.5 mL of extraction buffer in the cold room (all subsequent steps at 4°C unless otherwise noted) and then clarified by centrifugation at 14,000Xg for 10 min at 4°C. Meanwhile, 40 uL of magnetic Protein A/G bead slurry (Dynabeads, Invitrogen) per IP were incubated with 100 uL IgG (0.2 mg/mL anti β Galactosidase affinity purified by ALG)+ 100 uL PBST for 10 min at room temperature while they rotated end over end. This was then added to the supernatant (WCE) and rocked for 1 hr. The beads were then removed from the slurry using a magnet and were washed three times using 1 mL of wash buffer (50 mM HEPES pH 8.0, 100 mM NaCl, 0.5% NP40). Proteins were eluted off the beads by boiling in 50 uL of 1XSDS sample buffer for 5 minutes.

2.3 Results

To examine whether association of CAR proteins to the CTD of elongating RNAPII was required for resistance to DNA damage, the endogenous RNAPII CTD was depleted of its associated CAR proteins. To provoke such a disruption, I made use of two recombinant expression constructs in which a full-length CTD is fused to the C-terminus of a portion of β -galactosidase (Figure 2A). The constructs encode a β Gal-CTD fusion protein that either does or does not contain a nuclear localization signal (“nucCTD,” and “cytoCTD,” respectively), and expression of these proteins is regulated by a *GAL* promoter. I characterized this expression to make sure the proteins were induced by galactose and properly localized (Figure 2). Immunofluorescence comparing a DAPI stain for nuclei with an anti- β -galactosidase antibody showed that indeed, the nucCTD protein was mostly located in the nucleus and the cytoCTD protein was predominately in the cytoplasm, thus confirming proper localization of each fusion protein (Figure 2C). Further characterization by western blot using antibodies directed against specific forms of the phosphorylated CTD also demonstrated that both fusion proteins are induced by growth on galactose and are highly phosphorylated (Figure 2B). Phosphorylation patterns known to be present on elongating RNAPII CTDs were detected by specific antibodies, including monoclonal antibodies specific for either Ser2 or Ser5, and a polyclonal antibody to a hyper-phosphorylated CTD as generated by CTDK-I (see Materials and Methods for details). Detection by these antibodies suggests that these fusion proteins have at least some CTD repeats that are phosphorylated *in vivo* in a manner similar to that of the CTD on an elongating polymerase (Figure 2B). Because of the similarity of these repeats to an elongating RNAPII CTD, we expect that our fusion protein is able to bind many of the same proteins that the CTD binds as RNAPII progresses through the transcription cycle.

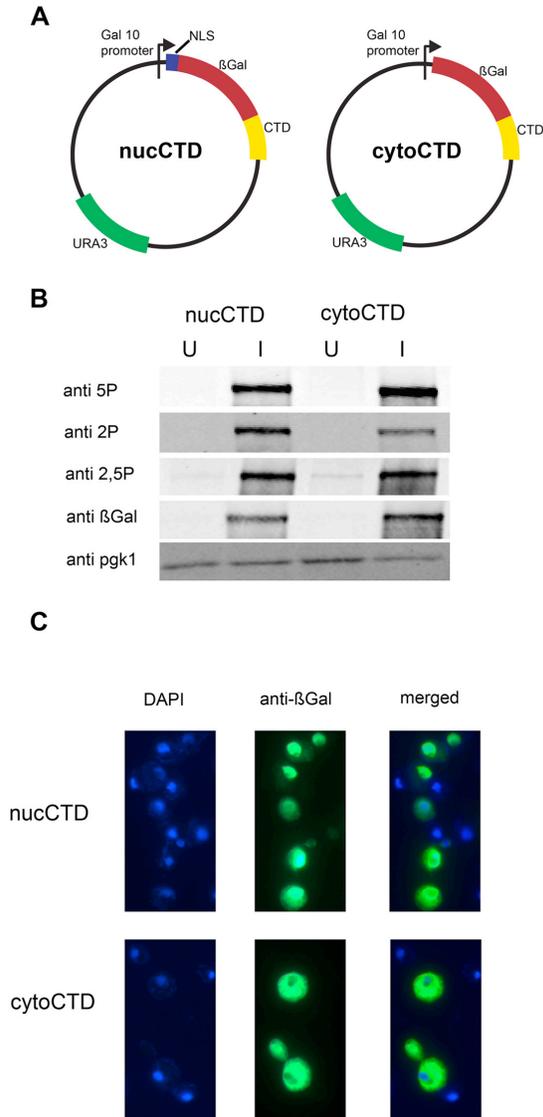


Figure 2: Characterization of CTD fusion proteins

(A) Plasmids for expression of CTD fusion proteins. NLS, Nuclear Localization Signal; β Gal, N-terminal 2/3 of β -Galactosidase. (B) Western Blots of uninduced (U) or induced (I) CTD fusion proteins. Antibodies against Ser5 phosphorylation (anti 5P), Ser2 phosphorylation (anti 2P), hyper-phosphorylated CTD (anti 2,5P), β -Galactosidase (anti β Gal), and a loading control (anti pgk1), show that the fusion proteins are phosphorylated. (C) Immunofluorescence of stains expressing the fusion proteins. Comparing nuclear staining (DAPI) with fusion protein expression (anti- β Gal) shows that the fusion proteins are properly localized.

To confirm the ability of the nucCTD fusion protein to bind CAR proteins, I probed for a specific CAR protein following immunoprecipitation of the β -galactosidase tag on nucCTD. This was compared to immunoprecipitation of a CTD-less nuclearly expressed portion of β -galactosidase (nuc β Gal) equivalent to the tag on nucCTD (Figure 3). Following immunoprecipitation of nucCTD and nuc β Gal using an antibody directed against β -galactosidase, I examined the resulting immunoprecipitate by western blot. Antibodies against Set2 (a CAR protein whose direct binding to the RNAPII CTD has been previously examined and is well known (Kizer et al., 2005; Li, 2005)) were able to detect Set2 co-precipitating with the nucCTD fusion protein but not with nuc β Gal (Figure 3). The ability of the nucCTD construct to bind Set2 indicates that nucCTD is capable of attracting regular binding partners of the elongating RNAPII CTD. Given the observation that the nucCTD fusion protein can bind CAR proteins, we used the expression of this protein as a molecular magnet to attract CAR proteins and presumably cause the depletion of CAR proteins from elongating polymerases (see Figure 4 for model). Expression of nucCTD should cause improper deployment of CAR proteins to the CTDs of transcribing polymerases.

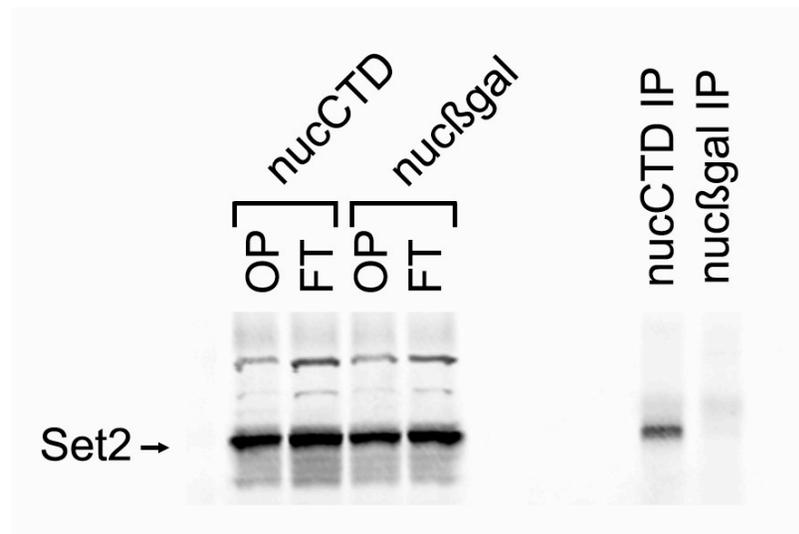


Figure 3: Set2 associates with nucCTD

Fusion proteins were expressed in WT yeast cells and pulled down by immunoprecipitation (IP) with an anti β -Galactosidase antibody. Co-IP of Set2 is illustrated via western blot using an antibody against Set2. Onput (OP) and Flow Through (FT) show Set2 is present in the extract. IP from extract where nucCTD is expressed show that Set2 is present; however, Set2 is absent from extract expressing only nuc β gal (without a CTD fusion).

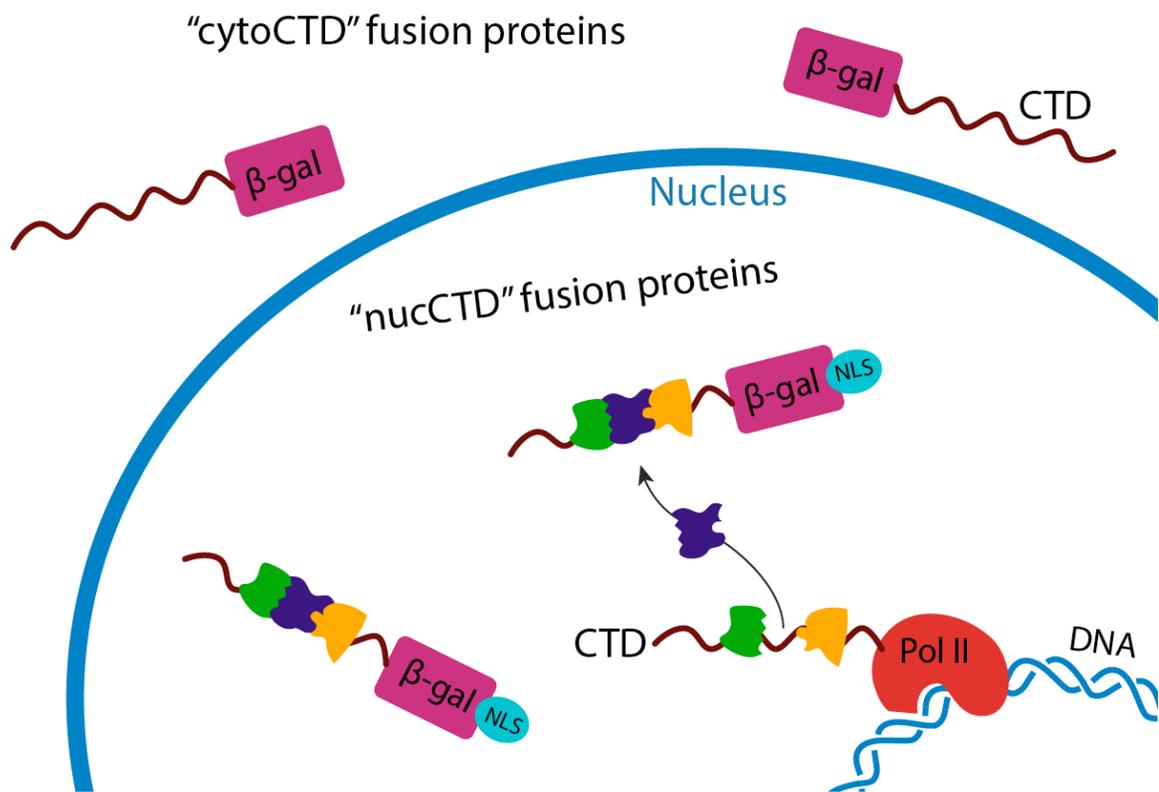


Figure 4: nucCTD titrates proteins away from elongating RNAPII

Model of nucCTD induced depletion of proteins from elongating RNAPII. Over-expression of the NucCTD causes binding of PCAPs to the fusion protein instead of the elongating RNAPII. The cytoCTD on the other hand, does not have access to the nucleus and therefore does not have the same effect.

We used this expression to ask whether proper association with CAR proteins is required for resistance to DNA damaging agents (Figure 5 and Figure 6). Expression of nucCTD alone does cause slow growth even in the absence of any DNA damaging agents (Figure 5 compare top two rows). Colony counting assays show that while nucCTD does cause slow growth, under normal conditions it does not decrease survival when compared to WT cells expressing either empty vector, cytoCTD, or nuc β Gal (data not shown). However, following exposure to ionizing radiation (IR), expression of the nucCTD protein reduces survivability of WT cells by about 10 fold (Figure 5). This is seen most clearly by colony counting (Figure 5B).

To see if nucCTD expression had a sensitizing effect for other DNA damaging agents, we also exposed cells to the chemotherapeutic agent doxorubicin (DX) following induction of either nucCTD, cytoCTD or an empty vector (Figure 6). DX is known to damage DNA through intercalation between DNA bases and inhibition of topoisomerase II (Fornari et al., 1994; Frederick et al., 1990). Survival was significantly reduced for cells expressing nucCTD when compared to cells expressing either cytoCTD or an empty vector control (Figure 6). We note that after induction of fusion protein expression, cells expressing cytoCTD grow at least as well as controls (empty vector) whereas cells expressing nucCTD grow more slowly even in the absence of drug (compare colony sizes in Figure 6, lower left panel). Despite this disparity in growth, there are still colonies at all cell dilutions tested. However, as seen in the lower right panel, presence of doxorubicin reduces survival selectively of the +nucCTD strain, as no colonies are present in its last 2 dilution spots. The increased sensitivity to IR and doxorubicin caused by expressing the nucCTD is consistent with our proposal that normal PCAP associations with the PCTD are required for cells to properly handle the damage caused by these agents.

As another means of observing interplay between the exogenously expressed nucCTD and putative CAR proteins, we tested for synthetic genetic effects of expressing the nucCTD (or cytoCTD) fusion proteins in 17 different strains each deleted for a PCAP, IR resistance gene, or both (CAR gene). As shown in Table 2, about one-third of the deletions tested showed synthetic lethality with nucCTD expression, and of these, about one-third were deletions of a known CAR gene. Whether these fractions would change if all PCAPs were tested is a question for the future. In any event, these results demonstrate synthetic dosage lethal interactions between a subset of PCTD-associated proteins and an over-expressed nuclear CTD fusion protein.

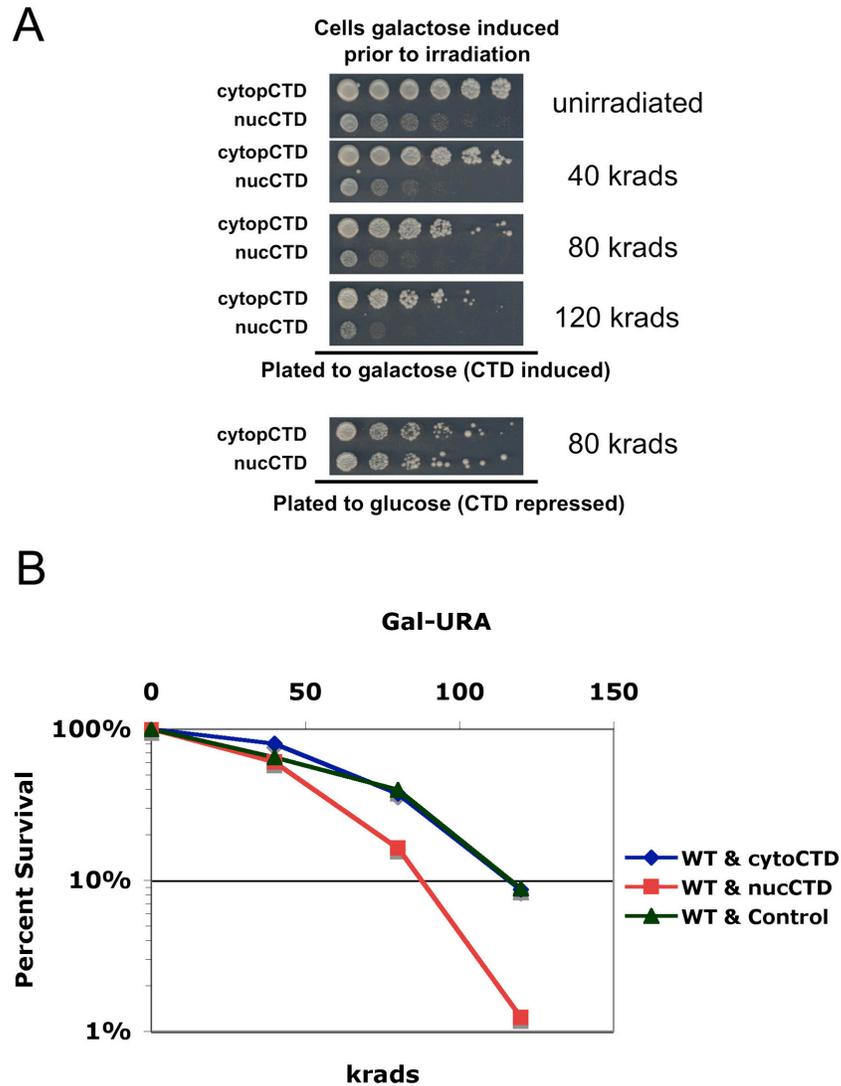


Figure 5: nucCTD is sensitive to IR

(A) IR plating assay. Stationary phase cells that had been grown in either galactose (CTD fusion proteins expresses) or glucose (CTD fusion proteins repressed) were plated in 5-fold serial dilutions and exposed to various doses of IR. NucCTD expression causes slow growth even in the absence of IR (Second row from top). However, IR does increase lethality of this strain (compare the change in growth between unirradiated cytoCTD and IR exposed cytoCTD with the same change for nucCTD). (B) Survival of fusion protein expressing strains following exposure to IR. Following expression of the fusion proteins, cells were exposed to IR and survival was determined by colony counting. Expression of nucCTD causes a 10-fold decrease in survival compared to cytoCTD or an empty vector control.

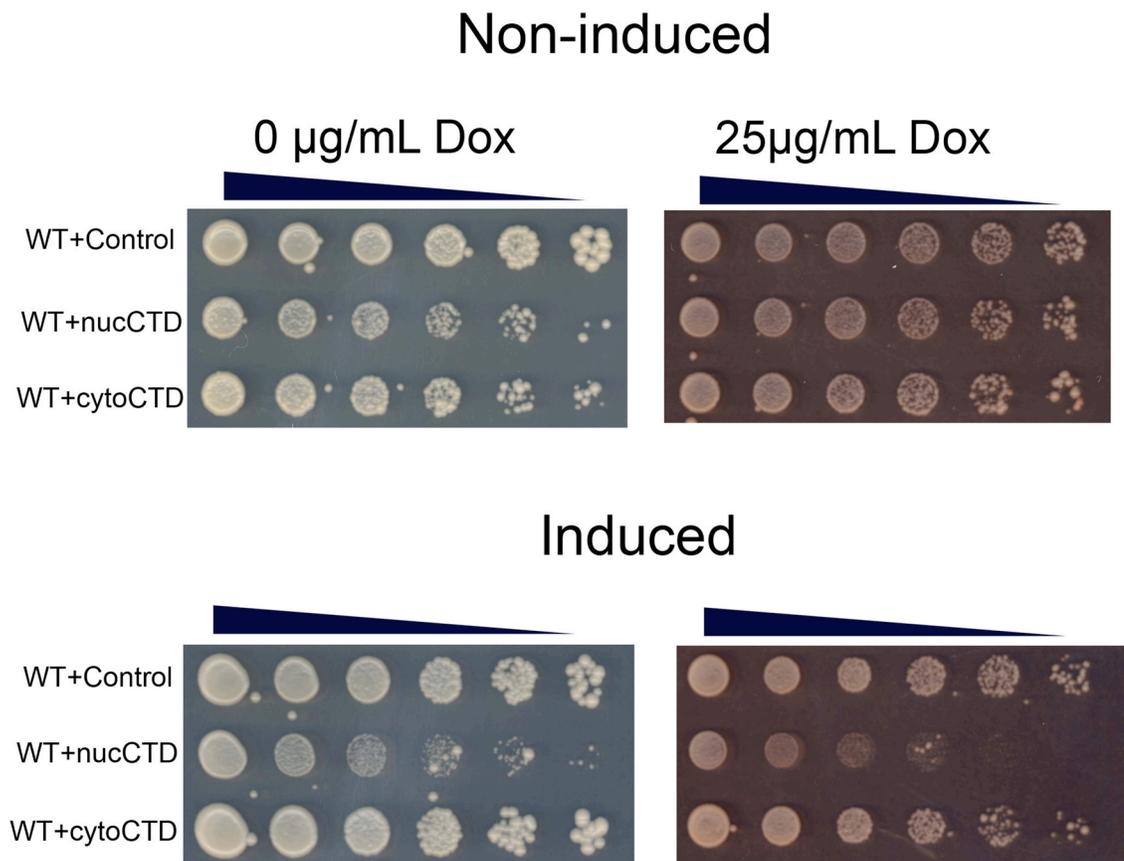


Figure 6: nucCTD is sensitive to doxorubicin

Cells grown on glucose (Non-Induced) or galactose (Induced) were diluted in 5-fold serial dilutions and plated to plates with and without doxorubicin. When nucCTD is expressed, exposure to doxorubicin decreases survival (compare the ratio of nucCTD middle row on bottom left panel with the same row on bottom right panel).

Table 2: nucCTD synthetic lethal interactions

Function	Gene deleted	PCAP?	IR sensitive?	Synthetic Lethal?
associated with Paf1 complex	cdc73	✓		
	paf1		✓	
	rtf1		✓	
histone variant	htz1	✓		
histone modification	set2	✓	✓	
RNA processing	rai1	✓	✓	✓
	hpr1		✓	✓
	mrt4	✓	✓	
	trm8	✓		
	trm9		✓	
	ssd1	✓		
	spt4		✓	
associated with Ccr4/Not complex	not4		✓	✓
	not5	✓	✓	✓
	ccr4		✓	✓
	pop2 = caf1		✓	✓
	dhh1		✓	
	dbf2		✓	

2.4 Discussion

From my characterization of the nucCTD fusion protein, I conclude that this system does perturb proper deployment of PCAPs to elongating RNAPII CTDs. As illustrated by the model in Figure 4, when nucCTD is expressed and phosphorylated, it binds PCAPs with the effect of titrating them away from the endogenous polymerase. However, based on the data presented in this chapter, it is impossible to know exactly which PCAPs are “captured” by the nucCTD protein, which remain associated with the elongating polymerase, and which are depleted but still present at some lower level. Since nucCTD is actively attracting PCAPs and thereby depleting the endogenous RNAPII from a full complement of PCAPs, it seems likely that this would disrupt transcription either by slowing it down, causing an increase in faulty or improperly processed transcripts, or both. Thus, it is not surprising that such an effect might decrease the ability of the cell to grow and divide, which would account for the slow-growth phenotype. The decrease in survival of strains exposed to DNA damaging agents following PCAP depletion of RNAPII suggests that PCAPs may be key in a DNA damage response. An inability to recover from DNA damage indicates not only a slow-down in transcription as seen by mere expression of nucCTD, but that these transcriptionally active proteins are also *required* for proper response to damaging events, linking CTD associated proteins and damage responses. This is consistent with our initial observation that there is an extensive overlap between PCAPs and genes required for resistance to IR in yeast. Furthermore, it also suggests the need for PCAPs in DNA damage responses and indicates that some PCAPs may indeed function as CAR proteins.

In addition to our observation that proper association of CAR proteins with the CTD is required for resistance to DNA damaging agents, we also found that expression of nucCTD is lethal in some strains that were also deleted for a gene of interest. While we don't know exactly what is causing this synthetic dosage lethality, it may be that the combination of titrating PCAPs away from the endogenous RNAPII in combination with the absence of another protein may destabilize a complex of proteins on the CTD to the point that it can no longer function at all. While the function of individual members of a complex may not be completely required for cell survival, function of the complex as a whole may be absolutely required. If association with the CTD is required for the function of such a complex, titrating away members of the complex from the endogenous RNAPII in conjunction with deletion of a member of the complex would be a lethal event.

Titration of PCAPs from the endogenous polymerase allows us to make a compelling argument that PCAPs are important in responses to DNA damage. However, while this broad based approach suggests that there is an important phenomenon to observe, it does little to actually describe it or parse apart anything specific about what is going on. Rather, it is a blunt instrument designed only to indicate that *something* significant is occurring without the ability to discover *what* or *how* these things might be happening. Further experiments are needed to discover what pathways these PCAPs may be involved in and how they may be participating in a DNA damage response.

3. DNA damage sensitivity of a single CAR mutant depends on its proper association with the RNAPII CTD

3.1 Introduction

While many proteins are known to associate with the elongating RNAPII CTD, whether that association is direct or not is only known for a few proteins. Of those proteins that are known to associate directly with the phosphorylated CTD, the details of binding are known for fewer still. However, one PCAP for which detailed information is known regarding its CTD binding is the histone methyltransferase, Set2 (Kizer et al., 2005; Li, 2005).

Set2 methylates histone H3 on lysine 36 and in yeast is capable of placing mono-, di-, and tri-methyl marks. Methylation by Set2 occurs co-transcriptionally during elongation by RNAPII and is thought to play a role in preventing spurious intragenic transcription (Carrozza et al., 2005). Proper methylation of H3 K36 by Set2 depends on Set2's interaction with the CTD of RNAPII (Li et al., 2003). Set2 preferentially binds CTD repeats phosphorylated at positions 2 and 5, and this interaction is dependent on proper phosphorylation of the CTD by CTDK-I (Li, 2005; Xiao et al., 2003; Kizer et al., 2005). The portion of the Set2 protein that binds the CTD is known as the Set2 Rpb1 Interacting domain, or SRI domain (Kizer et al., 2005). The ionizing radiation screen by Bennett, *et al* (2001) did not originally pick up *SET2* as a gene required for radiation resistance in yeast, but we later examined it and found it to be required for IR resistance (Figure 7). Based on this observation, it was added to our list of CAR genes.

Given that we have such a large list of CAR genes, most of which are known to have a biological function that would seem unrelated to DNA damage responses, we preferred a model which used the organization or structure on the CTD for proper DNA damage response over a model that required each CAR protein to have a specific,

unique, damage response function. Since Set2 is a CAR protein that has a well-described enzymatic function that is currently not known to be involved in DNA damage response, and since the CTD binding domain is known, it seemed like the ideal candidate to ask the question, “Is Set2 important in DNA damage responses because of its unique enzymatic function, or is it more likely playing a more generic or structural role as a CTD associated protein?”

3.2 Materials and Methods

3.2.1 Strains and Plasmids

All SET2 mutant strains were diploids of BY4743 background. The WT and set2 Δ were obtained from the yeast deletion collection. The Δ SRI strain was constructed by mating a BY4742 Set2 Δ SRI::KanMX strain with a BY4741 Set2 Δ strain. Both haploids were a gift from Brian Strahl. See table in Appendix B for full genotype.

The histone mutant strains were constructed using LRY1443 and LRY1444. These strains both have histones H3 and H4 knocked out of the genome and they are covered with the pDM9 plasmid (which contains HHT1 and HHF1 as well as a URA3 marker, and also known as pLR450). This plasmid was then replaced using a standard plasmid shuffle assay with another plasmid containing TRP1, HHT1, and either HHF1 (+WT H3 strain) or hhf1 K36A (+H3 K36A strain). The haploid strains were then mated and mated structures were picked using a dissection microscope to yield diploid strains. Strains and plasmids were a gift from Laura Rusche. Plasmids are originally from the lab of Fred Winston (Duina and Winston, 2004). See table in Appendix B for full genotype of yeast strains.

3.2.2 Ionizing radiation

Ionizing radiation assay was performed essentially as described in Bennett *et al* (Bennett et al., 2001). Briefly, cells were grown in a 96 well plate in YPD for 2 days.

Stationary phase cells were then diluted in sterile H₂O by 5-fold serial dilutions and plated. Plates were exposed to a Cs source until the desired exposure was reached.

3.2.3 MMS sensitivity

Cells were grown in a 96 well plate in either YPD (for genomic mutants and histone mutants) or complete medium lacking uracil (CM-URA) for 2 days. Stationary phase cells were then diluted in sterile H₂O by 5-fold serial dilutions and plated to YPD or CM-URA plates that either did or did not contain methyl methanesulfonate (MMS). For plates containing MMS (Sigma, 129925), 0.02% MMS was added to warm agar media immediately before plates were poured.

3.2.4 Western Blots

Extract preparation was performed as described in Chapter 2. Antibodies used were as follows: Anti-Histone H3 (trimethyl K36) antibody (Abcam ab9050), Anti-Histone H3 antibody (Abcam ab1791), Monoclonal ANTI-FLAG M2 antibody (Sigma F3165).

3.3 Results

Deletion of *SET2* was not originally picked up as a mutation that conferred sensitivity to ionizing radiation (Bennett et al., 2001). However, since it is likely that some genes were missed in the original screen, and Set2 is a well-described CTD binding protein, we tested this deletion specifically for its sensitivity to ionizing radiation (Figure 7) and MMS (Figure 8). We found that indeed, *SET2* is required for resistance to these DNA damaging agents and so we added it to our list of CAR genes.

After confirming the IR sensitivity phenotype of *set2Δ* cells, we simplified our analysis by using chemical mutagens. As can be seen in Figure 8, deletion of Set2 causes slow growth and lethality in the presence of 0.02% MMS when compared to the growth

of WT cells. Furthermore, to see whether this MMS-sensitive phenotype was dependent on proper association of Set2 with the RNAPII CTD, we examined a mutant (Δ SRI) that was missing only its CTD binding domain (the “Set2 Rpb1 Interacting” domain or SRI domain) but had its catalytic domain intact. In fact, although deletion of the SRI domain has been shown to eliminate binding of Set2 to the CTD, thereby destroying proper H3 K36 methylation *in vivo*, its catalytic activity *in vitro* remains unaffected (Kizer et al., 2005). Indeed, we found that resistance to DNA damage was dependent on proper association of the protein to the CTD (Figure 8, compare Δ SRI to WT and *set2* Δ). In fact, deletion of the SRI domain causes cells to be as sensitive to MMS as complete deletion of the protein.

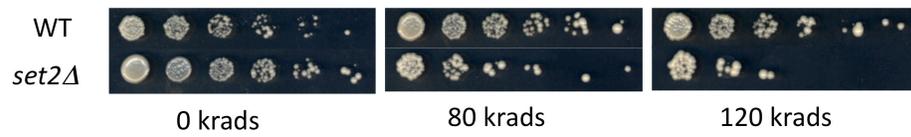


Figure 7: Set2 is required for resistance to ionizing radiation

5-fold serial dilutions of either WT or *set2Δ* cells were exposed to 0, 80, or 120 krad of IR. Survival of *set2Δ* cells is significantly decreased.

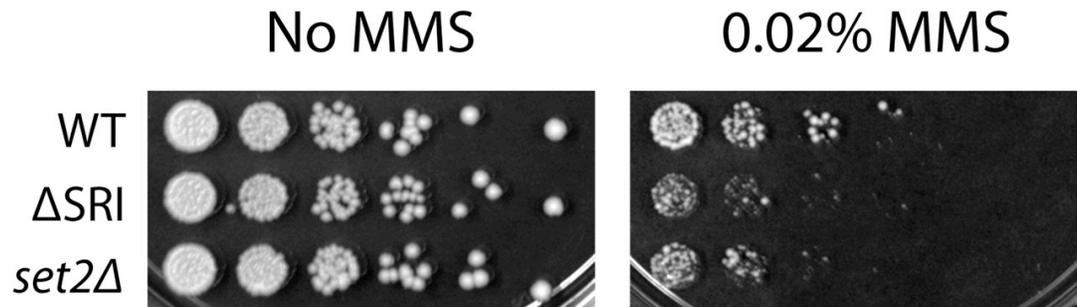


Figure 8: SRI domain is required for resistance to MMS

5-fold serial dilutions were plated to plates with and without 0.02% MMS. Growth of the Δ SRI strain (which is lacking the CTD binding domain of Set2) on MMS is as poor as in the *set2* Δ strain, indicating that the CTD binding domain is required for resistance to DNA damaging agents.

Although the enzyme present in the Δ SRI strain is capable of performing catalysis, it is not recruited to the CTD and may not have the opportunity to methylate H3 K36. To discover whether the catalysis of the enzyme is required, we examined two Set2 point mutants that had previously been reported as catalytically inactive (Strahl et al., 2002) for their ability to grow in the presence of 0.02% MMS. As can be seen in Figure 9, covering a *set2* Δ strain with either Set2 point mutant restores the same growth ability as covering the *set2* Δ strain with a WT *SET2* allele. However, expression of an empty vector in the *set2* Δ strain shows sensitivity to MMS. Thus, the catalytic activity of Set2 does not appear to be required for resistance to DNA damage.

Although the *set2* C201A and *set2* R195G point mutants had previously been reported to be completely catalytically inactive (Strahl et al., 2002), I examined the *set2* Δ strains covered by these mutants for the presence of tri-methyl K36 using an antibody specific to this histone mark. Since Set2 is the only enzyme in yeast that catalyzes methylation of H3 K36, *set2* Δ strains are completely absent for this mark, and a truly catalytically dead mutant should also have no K36 methylation. Although tri-methylated K36 was greatly reduced (Figure 10), the residual K36 methylation indicated that there was some *in vivo* catalytic activity present in both point mutants. It is not clear why my results differ from those previously published, but it may be because I was using a different antibody to detect K36 methylation, or because of differences in extract preparation.

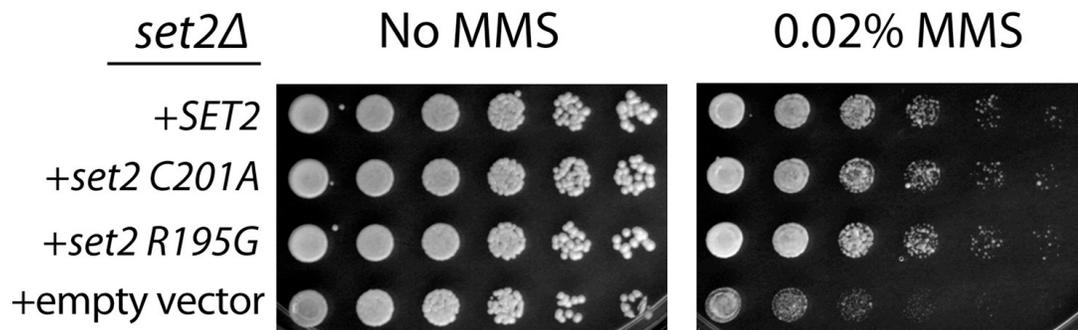


Figure 9: Catalytic activity is not required for resistance to MMS

5-fold serial dilutions of *set2*Δ strains covered by various mutants of Set2 and plated with or without 0.02% MMS. Catalytically dead point mutants (C201A and R195G) are as resistant to MMS as the WT allele.

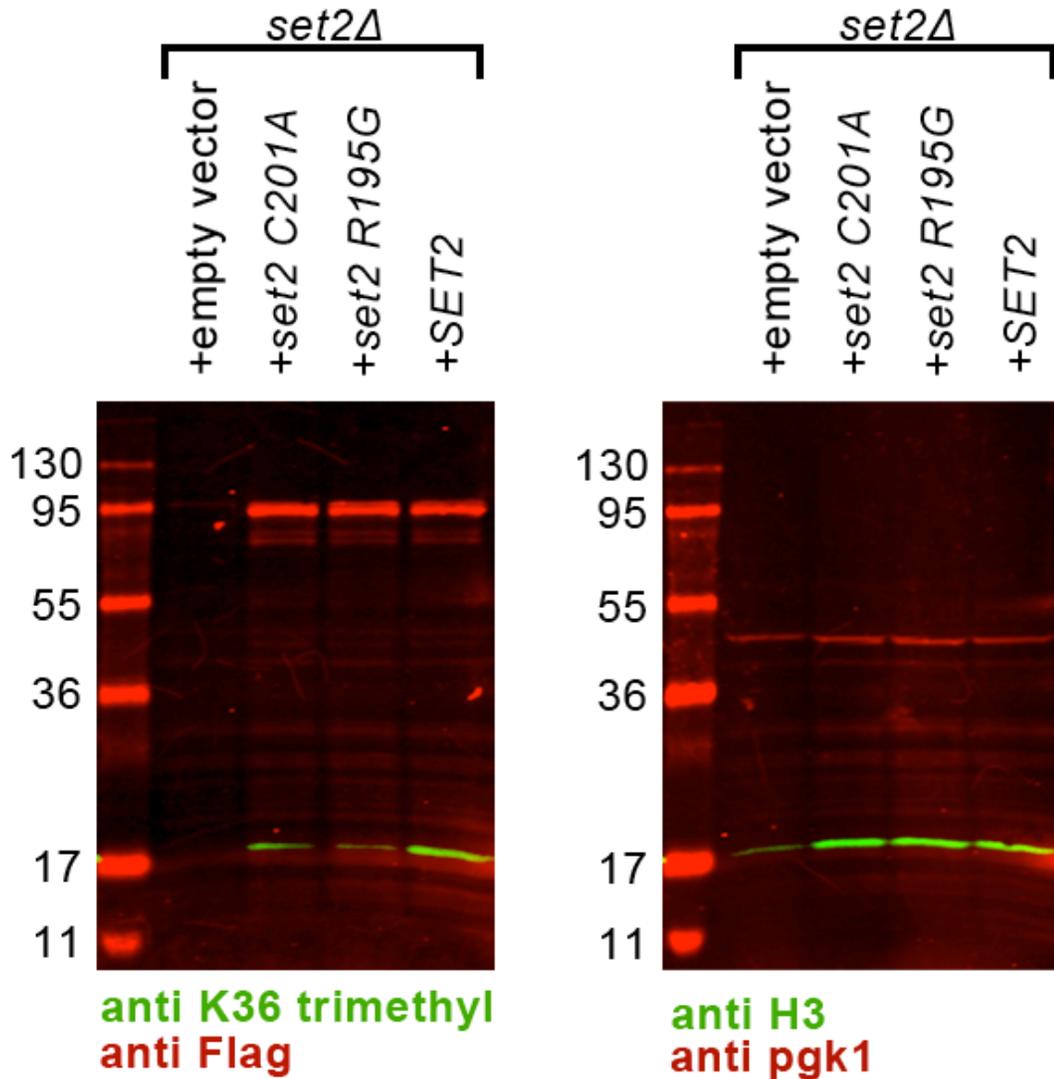


Figure 10: Set2 point mutants retain some catalytic activity

Western Blots from extracts of cells expressing Set2 mutants. All strains show histone H3 (green bands, right panel) and pgk1n (red bands, right panel) as loading controls. Although K36 methylation is decreased, both point mutants previously reported as catalytically dead (C201A and R195G) show some K36 trimethylation (green bands, left panel) and therefore retain some catalytic activity. Set2 is present at normal levels in all extracts covered with a version of Set2 (red bands, left panel are to a Flag tag on Set2).

Given that there was some activity in these point mutants, I needed to find a way to more robustly determine if K36 methylation was actually required for resistance to DNA damage. Since these mutants had some activity, we were concerned that if damage resistance was dependent on K36 methylation, a small amount of activity would still be able to rescue the sensitivity phenotype. Thus, merely testing these mutants was not sufficient to determine if it was Set2's association with the RNAPII CTD alone that is required for DNA damage resistance. As a second way to test for the requirement of K36 methylation, I tested a strain in which all copies of histone H3 had been deleted from the genome and in which this deletion was covered by H3 on a plasmid. Using a standard plasmid shuffle protocol, I replaced the H3 plasmid covering this deletion with a plasmid that had either a WT or K36A histone H3. The K36A mutant would prevent methylation at K36 on the histone, and allow me to test for DNA damage sensitivity / resistance without any K36 methylation present. As can be seen in Figure 11, a mutant strain with a genomic mutation for H3 grew as well on 0.02% MMS whether it was covered by a WT H3 allele, or an H3 with a K36A mutation. This result strongly suggests that methylation on K36 of histone H3 is not required for resistance to DNA damage and that the DNA damage sensitive phenotype we have seen for *set2Δ* is due to the lack of Set2 on the RNAPII CTD and not due to the catalytic activity of the enzyme.

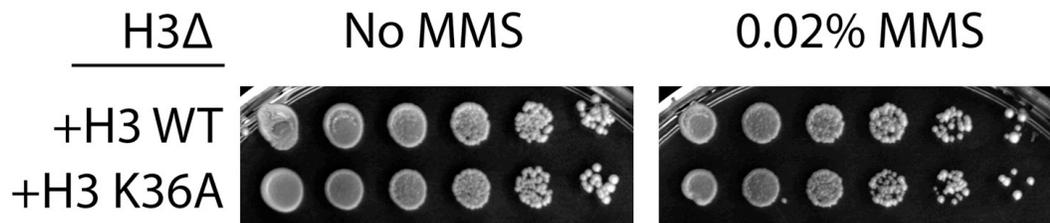


Figure 11: Methylation of H3 K36 is not required for resistance to MMS

5-fold serial dilutions of strains in which histone genes are deleted from the genome but covered by either a plasmid with WT histones (top row) or a plasmid where H3 has a K36A point mutation (bottom row). These dilutions were plated to plates with and without 0.02% MMS. There is no observable difference in survivability between the WT strain and the mutant strain when exposed to MMS.

3.4 Discussion

We used Set2 as a case study to examine whether CAR proteins each have their own, specific function in DNA response or whether some of them might play a more general or structural role. Our data suggest that while Set2 is required for resistance to DNA damaging agents, it is Set2's association with the RNAPII CTD that is important for DNA damage response and not its catalytic activity. This is a very important discovery in explaining the large number of CTD associated proteins that we have found to be required for DNA damage resistance. Given that many of these proteins have other known functions, that seem completely unrelated to DNA damage responses, it makes sense that their role may be more related to their structure on the CTD than their other, historical roles.

The idea that an enzyme might play an important structural or scaffolding role independent of its catalytic activity is not unprecedented. In fact, such a role has already been suggested for the CTD kinase, Ctk1 (Ahn et al., 2009). Ahn et al. demonstrated that Ctk1 is required for proper dissociation of basal transcription factors following initiation. However, this does not seem to depend on Serine 2 phosphorylation of the CTD or even the ability of Ctk1 to catalyze phosphorylation. Rather, the presence of the protein alone is required, suggesting a structural or scaffolding role for Ctk1 in this instance and not a catalytic role. Likewise, we believe that Set2 is required for DNA damage resistance in yeast not because of its ability to methylate K36 on histone H3 but rather because its binding to the CTD may be structurally important to facilitate a DNA damage response when a transcribing RNAPII encounters damage. Indeed, given the vast number of CAR proteins that have other, seemingly unrelated functions, a model where the arrangement of CTD associated proteins allows for proper damage response

seems much more likely than a model where the individual function of each CAR protein is independently significant.

4. Mitotic recombination decreases in the absence of CTK1 or a CAR protein

4.1 Introduction

In yeast, a major method of repairing the genome is through homologous recombination. Although recombination in mitosis is generally a rare event, it plays a key role in repairing DNA lesions. Double-strand breaks (DSB) are known to be efficiently repaired in yeast via homologous recombination (HR). Traditionally, a DSB induced by IR has been thought of as the lethal lesion unless repaired, and in yeast DSBs are efficiently repaired through recombination events. Therefore, it is thought that spontaneous mitotic recombination is likely the result of a cell repairing a spontaneously occurring DSB (Prado et al., 2003; Symington, 2002; Pâques and Haber, 1999). However, it has also been reported in the literature that DNA nicks and single-stranded gaps may be even more relevant to spontaneous HR in mitotically growing yeast cells than a DSB (Lettier et al., 2006). Whether due to a DSB, or single-stranded nicks and gaps, HR seems to be an extremely efficient method that yeast use to repair their DNA.

Several different previous observations led us to wonder if the CAR system influenced mitotic recombination. First, deletion of any of the three subunits of CTDK-I (*ctk1Δ*, *ctk2Δ*, or *ctk3Δ*) is synthetic lethal with deletion of a number of DNA integrity genes, including several members of the *RAD52* group of genes known to be involved in recombination (Pan et al., 2006). Second, as described in Chapters 1 and 2, deletion of a CAR gene or titration of CAR proteins away from the CTD induces sensitivity to IR. Since much IR damage is believed to be repaired by HR, it would make sense for the CAR system to play a role in HR.

This observation that deletion of CTDK-I genes was synthetic lethal (SL) with DNA integrity genes, led us to wonder whether any CAR genes showed similar interactions. A paper by Pan et al (2006) screened 78 DNA integrity genes for SL interactions, and 38 of them were SL with *ctk1* Δ , *ctk2* Δ , and / or *ctk3* Δ . As many CAR proteins are likely dependent on CTDK-I for proper CTD binding, a similar synthetic lethality profile would not be unexpected. Therefore, we checked these 38 DNA integrity genes in SGD (see the Saccharomyces Genome Database, SGD, <<http://www.yeastgenome.org>>) for SL interactions with CAR proteins. As shown in Table 3, 8 CAR genes also exhibited SL profiles with one or more of these DNA integrity genes (note that not all CAR genes have been subjected to genome-wide SL analysis). Many of these genes seem to cluster such that several of them are SL with a subset of the DNA integrity genes.

In this chapter, we examine the ability of strains deleted for select CAR proteins that are also involved in this SL network (*rvs161* Δ and *set2* Δ) to undergo spontaneous mitotic recombination. We also discover that deletion of the catalytic subunit of CTDK-I (*ctk1*) causes a severe decrease in mitotic recombinational ability.

Table 3: Synthetic Lethal interactions with DNA integrity genes

DNA Integrity Genes	CAR genes that are synthetic lethal (SL) with DNA integrity genes						
ARD1	CTK2, 3	CDC73		RVS161	SAC1	SET2	
ASF1	CTK1, 2, 3	CDC73		RVS161		SET2	UME6
BRE1	CTK1, 2, 3				SAC1	SET2	UME6
CCR4	CTK1	CDC73	NOT5	RVS161	SAC1		
CSM1	CTK1, 2, 3						
CTF18	CTK1, 2, 3		CHL1				
CTF8	CTK1, 2		CHL1				
DCC1	CTK1, 2		CHL1			SET2	UME6
DUN1	CTK1, 2, 3	CDC73					
ELG1	CTK1		CHL1				
HEX3/SLX5	CTK1, 2, 3					SET2	
HIR1	CTK1, 2, 3	CDC73					
HIR2	CTK1, 2, 3						
HPR5/SRS2	CTK1, 2, 3		CHL1				
LGE1	CTK1, 2, 3				SAC1	SET2	UME6 ZUO1
LRS4	CTK1, 2, 3						
LYS7/CCS1	CTK1, 2, 3						
MDM39/GET1	CTK1, 2, 3	CDC73	NOT5	RVS161	SAC1		UME6
MMS22	CTK3	CDC73					ZUO1
MRE11	CTK1, 2, 3						
NAT1	CTK2	CDC73		RVS161	SAC1	SET2	
POL32	CTK3			RVS161			
POP2/CAF1	CTK1		NOT5	RVS161	SAC1		
RAD18	CTK1, 2, 3	CDC73					
RAD27	CTK1, 2, 3	CDC73	CHL1		SAC1		UME6
RAD50	CTK1, 2, 3						
RAD51	CTK1, 2, 3						
RAD52	CTK1, 2, 3	CDC73					
RAD53	CTK3						
RAD54	CTK1, 2, 3						
RAD55	CTK1, 2, 3						
RAD6	CTK1, 2, 3			RVS161	SAC1	SET2	UME6 ZUO1
RMD7/GET2	CTK1, 2, 3		NOT5	RVS161	SAC1		UME6
RPN4	CTK1, 2, 3			RVS161			UME6
RPN10	CTK3						
SLX4	CTK1, 3						
TSA1	CTK2, 3						
XRS2	CTK1, 2, 3	CDC73					

4.2 Materials and Methods

4.2.1 Yeast strains

The WT haploid yeast strains were obtained from Kevin Lewis at the University of Texas, but were originally constructed by Robert Malone (Malone, 1983). M7 and M53 haploids (see Table 4 in Appendix B for genotypes) were allowed to mate and mated structures were picked. To avoid sporulation, mated cells were grown on YPD media where the dextrose had been increased to 4% (YP4%D). As soon as colonies were large enough to pick, 3 mL cultures were started and frozen down as glycerol stocks while still in log phase. Mutants were made by knocking out the gene of interest in each haploid with the KanMX cassette prior to mating (Wach et al., 1994).

4.2.2 Mitotic Recombination Assays

Mitotic Recombination assays were performed based on the protocol in Malone and Hoekstra (1984). Two days prior to starting the liquid cultures, we streaked them to YP4%D and allowed colonies to grow up. These colonies were resuspended in sterile H₂O and counted on a hemocytometer. A small number of cells (about 100 cells/mL) was added to liquid YPD and cultures were allowed to grow to a final cell density of approximately 1×10^7 cells/mL. Various dilutions were plated to YPD; YPD containing cyclohexamide; drop out media lacking leucine, uracil, or histadine; and drop out media lacking argenine but containing canavanine. Plates were incubated at 30 °C and then scored.

4.2.3 Mutation Rate Calculations

We performed fluctuation analysis using the Lea-Coulson Method of the Median (Lea and Coulson, 1949) using the FALCOR website (Hall et al., 2009).

4.3 Results

As spontaneous mitotic recombination is a rare event, it has traditionally been measured using genetic techniques where auxotrophic strains with a given heteroallele can gain prototrophy only after undergoing a recombination event. The frequency of this event can be measured by selecting for prototrophs and comparing the number of recombinants to the total number of viable cells.

We used diploid yeast strains originally constructed by Malone (Malone, 1983; Malone and Esposito, 1981; Malone and Hoekstra, 1984) to assay recombinational ability at several loci. Alleles available for recombination are illustrated in Figure 12

M7:	<i>MATα</i>	<i>lys2-1</i>	<i>his7-2</i>	<i>can1^R</i>	<i>ura3-13</i>	<i>trp5-d</i>	<i>leu1-12</i>
M53:	<i>MATα</i>	<i>lys2-2</i>	<i>his7-1</i>	<i>CAN1</i>	<i>ura3-1</i>	<i>trp5-c</i>	<i>leu1-c</i>

Figure 12: Alleles available for recombination assays

M7 (*MAT α*) and M53 (*MAT α*) haploids were mated together and diploid cells were picked using a dissection scope. These diploids were used to inoculate a YPD culture and grown to approximately 1×10^7 cells per mL. At this point we plated the cultures to various selection media to select for cells that had gained prototrophy. We then performed fluctuation analysis, using the Lea and Coulson (Lea and Coulson, 1949) method of the median to determine the rates of recombination. We did this with both the WT strain (*WT* with respect to the gene being tested) as well as M7 and M53 strains that had been deleted for a gene of interest.

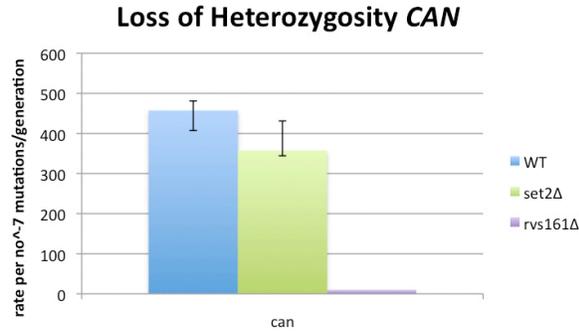
To examine the effect of deleting a CAR protein on recombination events, we looked at mitotic recombination rates for two CAR protein deletions, *set2 Δ* and *rvs161 Δ* ,

that exhibit some of the same SL interactions to DNA integrity genes as deletion of *ctk1Δ* cells (see Table 3). We employed two basic assays. The first one looked simply at loss of heterozygosity (LOH). In this assay, loss of the dominant *CAN1* gene yields resistance to the chemical canavanine (or in other words the *CAN1* allele makes cells unable to grow in the presence of canavanine). Here, any event that causes loss of this one allele will make cells sensitive. This includes the occurrence of a reciprocal recombination event anywhere between the *CAN1* gene (towards the end of chromosome V) and the centromere. Because there are several events that can cause LOH, as well as a large region where recombination can occur to yield resistant cells, total recombination rates are much higher in this assay. As can be seen in Figure 13A, *set2Δ* cells do not experience LOH at significantly different rates than WT cells. On the other hand, we were not able to observe any resistant *rvs161Δ* cells, indicating that either LOH is extremely low in this strain, or there is some additional issue in using canavanine resistance to examine genome stability in this strain. As we did not see any colonies when we plated *rvs161Δ* to canavanine in our LOH assay, we need to further examine this strain to verify our lack of colonies is not an artifact. However, initial testing of the haploid strains used to make the *rvs161Δ* show that the haploid with the *can1^R* allele grows fine in the presence of canavanine while the haploid with the *CAN1* gene does not. This result suggests that the alleles are acting as they should in the haploids, even following deletion of *rvs161Δ* and so it seems unlikely that the diploid would not behave as expected in this assay. Thus, we think it is likely that deletion of *RVS161* genuinely causes a large decrease in LOH and we simply need to investigate further to get a more accurate assessment of exactly how low LOH may be in this strain.

We also examined these two strains in a mitotic recombination assay. In this assay, two heteroalleles are present, neither of which confer upon the cells the ability to grow without a select nutrient. To gain prototrophy, a recombination event must occur

in the gene such that the two mutant alleles recombine to yield a new intact allele. Because this event must occur in a fairly small region, we observed lower recombination rates than with the LOH assay. However, for the same reason, this assay is probably an even better measure of a potential CAR system because the recombination must be within the gene where active transcription is occurring. As can be seen in Figure 13B, neither *set2*Δ cells nor *rvs161*Δ cells exhibit a significant change in recombination when compared to WT cells.

A



B

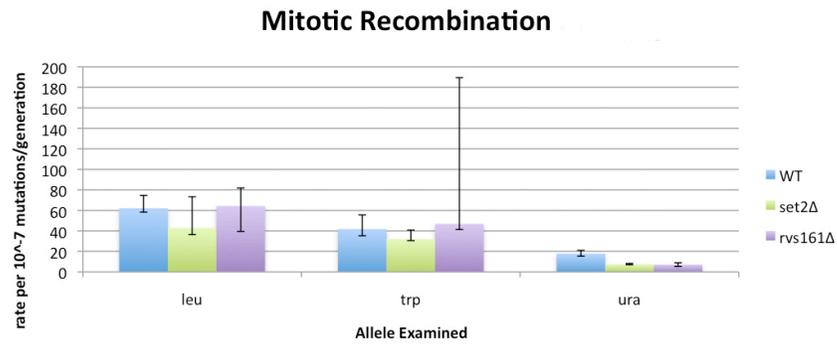
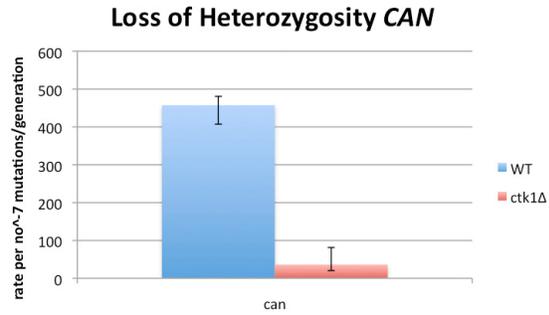


Figure 13: Recombination of strains deleted for CAR genes

(A) Loss of Heterozygosity (LOH) assay. Error bars illustrated are 95% confidence levels. *set2Δ* cells are not significantly decreased for LOH when compared to WT cells. Note that assays performed on *rvs161Δ* cells yielded 0 colonies, and thus recombination rates with accompanying errors could not be calculated. (B) Mitotic Recombination assay. Error bars illustrated are 95% confidence levels. For recombination at all alleles tested (*leu*, *trp*, and *ura*), neither *set2Δ* nor *rvs161Δ* strains exhibit a significant change in recombination rates when compared to the WT strain. The 95% confidence interval upper limit for *rvs161Δ* strains is high and this assay should be repeated to get a more precise mutation rate.

A



B

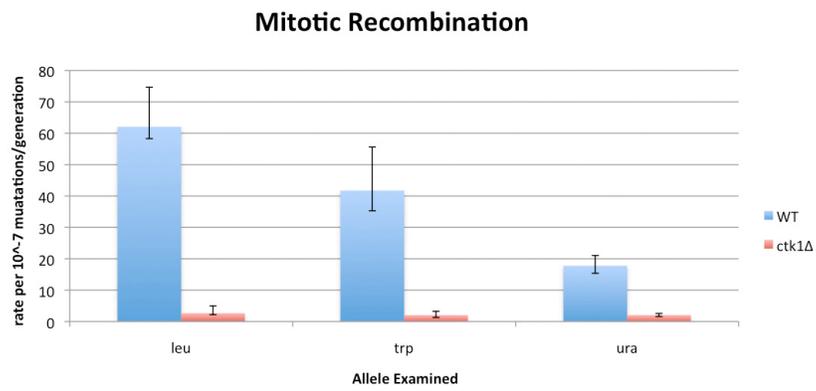


Figure 14: Recombination of *ctk1Δ* strains

(A) LOH assay with *ctk1Δ* strain. Error bars illustrated are 95% confidence levels. Following deletion of *CTK1*, strains exhibit a 10-fold decrease in LOH. (B) Mitotic Recombination assay with *ctk1Δ* strain. Error bars illustrated are 95% confidence levels. Deletion of *CTK1* causes a 5-10 fold decrease in recombination at three different heteroalleles.

These same assays were performed on *ctk1Δ* cells with drastically different results. Figure 14 illustrates the remarkably different recombination rates we see following deletion of *CTK1*. The LOH assay (Figure 14A) shows a 10-fold decrease in LOH for the mutant strain. Likewise, mitotic recombination rates are down 5-20 fold, depending on the allele (Figure 14B). This result suggests that Ctk1 is required for recombinational proficiency, implying that in the absence of Ctk1, recombinational repair is unable to occur properly. Furthermore, the fact that this recombinational event must occur in the region of an actively transcribed gene is consistent with the idea of a transcriptional response initiating repair by HR. We believe that the requirement for *CTK1* is dependent on the initiation of a direct DNA damage response and not due to decreased transcription of repair genes because gene expression comparisons between *CTK1* and *ctk1Δ* strains, as assessed by Affimetrix genome array analysis of mRNAs, do not reveal significant perturbations for DNA repair or recombination genes in *ctk1Δ* strains (D. Skaar and A. Greenleaf, unpublished).

4.4 Discussion

Analysis of mitotic recombination in *ctk1Δ* cells gives us insight into the ability of the cells to respond to endogenous damage. As *ctk1Δ* cells are severely deficient in recombination levels, we expect that they are also deficient in their ability to respond to DNA damage via recombination. Since Ctk1 is an elongation phase CTD kinase, we expect that lack of Ctk1 would alter the ability of CAR proteins to properly associate with the CTD and therefore disrupt the CAR response. Interestingly, deletion of a single CAR gene does not have the same effect as deletion of *CTK1*. While it is impossible to know exactly why this is the case, we would expect *CTK1* deletion to have a broad affect on the CTD, causing impaired CTD binding for a variety of CAR proteins. We do not know directly how this may affect the CAR response, however it seems likely to have

far-reaching implications. On the other hand, deletion of a single CAR protein would be expected to have a much more limited effect. Thus, this result is reasonable although unexpected.

Mitotic HR levels may vary depending on deletion of individual CAR genes. As it is not known what role an individual CAR protein may be playing, it is reasonable to suppose that there will be individual differences in their CAR response. In fact, the differences between *set2* Δ and *rvs161* Δ speak to this possibility. Although deletion of *set2* Δ does not seem to alter a cell's LOH profile, deletion of *rvs161* Δ may cause a severe decrease in LOH. Thus, further investigation into specific CAR proteins may uncover multiple mechanisms of DNA damage response.

5. Expanding our understanding of how transcription interacts with DNA damage response pathways- Conclusions and Future Directions

5.1 Conclusions

As discussed in Chapter 1, there are several different ways in which transcription is known to influence DNA damage response mechanisms. In addition to the well-studied TC-NER, known responses of RNAPII to DNA damage include ubiquitinylation of the polymerase, and initiation of the p53 checkpoint after blockage of the polymerase. In this thesis, I have shown several lines of evidence for a novel CTD associated transcriptional response to DNA damage. We have termed this response the CAR system. These lines of evidence include a significant overlap between factors required for resistance to DNA damaging agents and PCAPs that bind the CTD. Furthermore, disruption of proper PCAP association on the CTD also causes sensitivity to DNA damaging agents. The association of CAR proteins with the CTD is vital as in the case of Set2, where CTD association is required but catalytic activity of the enzyme is not. Finally, proper phosphorylation of the CTD plays an important role as HR does not occur properly when the CTD kinase, CTDK-I is disrupted.

5.2 CAR System Model

While these results strongly suggest that the CAR system does exist, we still know very little about how it might work. Although there may potentially be several ways to explain these data, we favor a model that depends on the proper arrangement of CAR proteins on the CTD. A model based on the structural integrity of CTD associated proteins goes far to explain many of our observations such as the requirement for Set2 on the CTD, but not for its catalytic activity. Other observations may also make more sense if seen from the perspective of a CAR system highly dependent on CAR protein

arrangement. For example, why are so many proteins with other known, non-DNA damage related functions involved? Why would general depletion of proteins from the endogenous RNAPII make a difference? And finally, why would HR be dependent on a CTD kinase?

Figure 15 illustrates this type of model. Panel A shows an example of what the CAR system may look like when it is functioning properly. When the RNAPII is stalled at a site of damage, changes take place in a group of CAR proteins. While the nature of such changes is not known, they may include conformational changes, dissociations, covalent modifications and/or activity changes. We assume that at some point this type of response triggers a DNA damage repair mechanism. However, if something disrupts the ability of these CAR proteins to organize on the CTD properly, the system will not work. Panel B illustrates what may happen if a CAR protein was not bound to the CTD (for example, the Set2 mutant deleted for its SRI domain). Here, the signal is not allowed to channel through the system of CAR proteins and the resulting response is incomplete. In panel C, a single CAR protein is missing from the CTD. This may be due to deletion or the protein being titrated away by a CTD fusion protein. As in the case illustrated in Panel B, a proper CAR signal is not initiated.

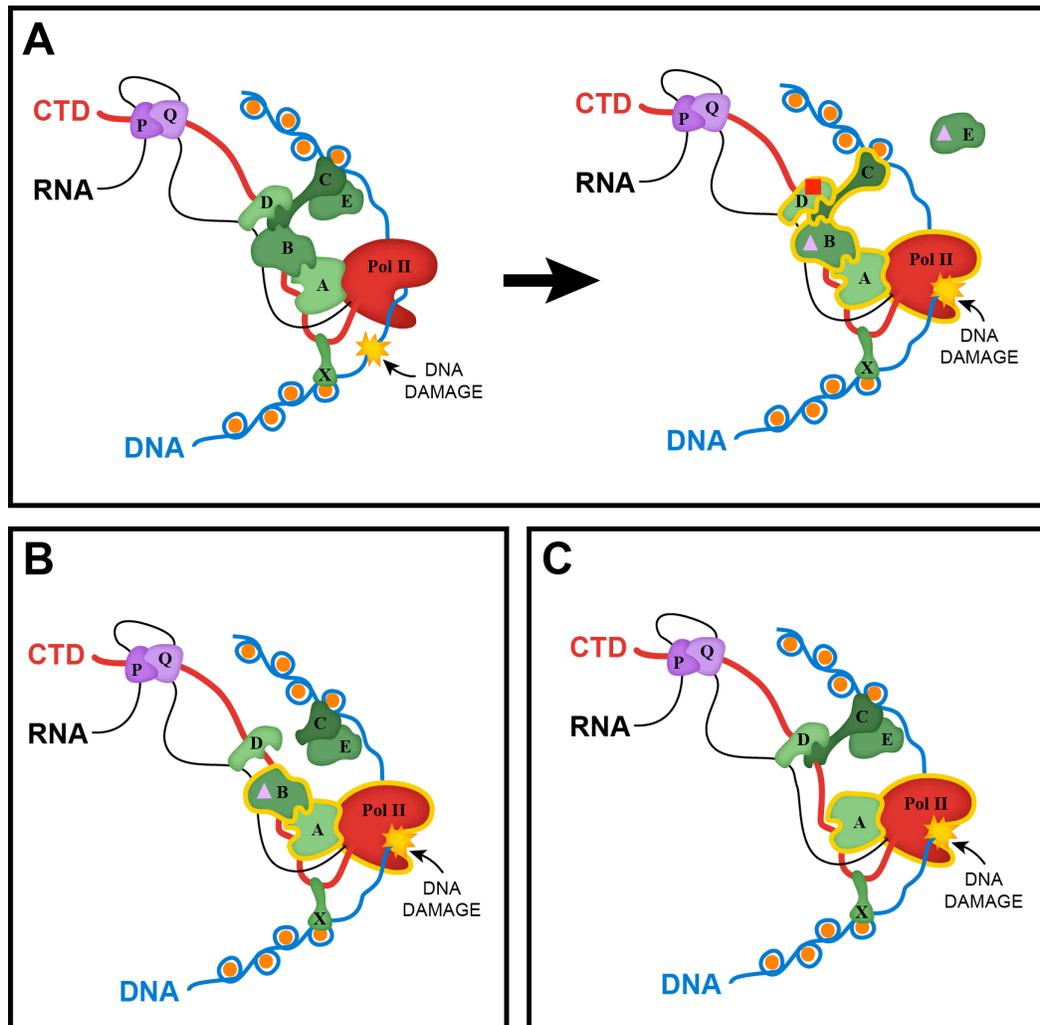


Figure 15: CAR System Model

(A) As RNAPII is transcribing, it encounters a site of DNA damage along with its associated proteins. A subset of these proteins (A-E) is affected by the stalling of the polymerase. Stalling of the RNAPII complex could initiate one or more potential responses such as conformational changes (yellow outline), dissociations (protein E), activity changes (purple triangle), or covalent modifications (red square). (B) In this example, the CTD binding domain of the C protein is removed. This prevents any of the potential signaling responses associated with proteins C-E from occurring. (C) In this example, protein B is absent, preventing any of the potential signaling responses associated with proteins B-E from occurring.

5.3 Future Directions

Although we have compelling evidence for the existence of a network of CAR proteins, much more work needs to be done and many questions remain to be answered. For example, although we expect that this network would be dependent on transcription by RNAPII, we do not yet have direct evidence for such a dependency. We plan to explore this idea through a genetic assay, in collaboration with Nayun Kim. Kim and Jinks-Robertson (2011) have already generated a system designed to examine recombination between ectopic alleles on an actively transcribed gene. This system is controlled by a tetracycline-regulatable promoter and therefore, transcription at the gene of interest can be turned on or off as needed. We plan to introduce a *ctk1Δ* mutation into these strains and examine whether or not recombination rates are substantially lower than the WT strain both before and after induction of transcription. If the CAR network is truly dependent on transcription as we think it is, we would expect recombination levels in the *ctk1Δ* strain to be lower than in WT when transcription is activated, but not much different than WT when transcription is repressed. Such a result would strongly suggest that recombination signaled via the CAR system is indeed dependent on active transcription.

We would also like to examine the CAR response by a more direct biochemical method. Ideally, we would set up an assay with a known site of damage in the middle of a transcribed gene. Addition of a regulatable promoter would allow us to examine the difference between repair at a site of damage before and after transcription is initiated. Once such a system was established, we could use it to examine the fate of RNAPII and associated CAR proteins after encountering a site of DNA damage (e.g. via chromatin immunoprecipitation).

Our initial characterization of a CTD associated response to DNA damage gives compelling evidence that the CTD and associated factors do play a role in responding to DNA damage in the cell. However, we know very little about how this response may occur. Future studies will be needed to determine exactly how the CAR system works and how it fits into current models of DNA repair and recombination.

Appendix A: Spt4 as a potential binding partner for BRCT domain containing proteins

A.1 Introduction

A.1.1 Can BRCA1 be playing a role in transcriptional responses to DNA damage?

Several connections previously reported in the literature between RNAPII and responses to DNA damage were discussed in Chapter 1. In Chapters 2-4, I showed data from our lab and argued that the RNAPII CTD coordinates a novel response system to DNA damage. Given that transcription seems to be a good way of recognizing and responding to DNA damage events, it is possible that still other intersections between transcription and DNA damage responses will be uncovered. In the following section, I introduce some preliminary evidence for a transcriptional response to DNA damage events that connects the breast cancer susceptibility gene BRCA1 to the transcription factor Spt4. Although further evidence is required to prove such a connection, there is enough compelling evidence that this line of inquiry seems worthy of further pursuit.

A.1.2 BRCA1 and its BRCT domain

BRCT domains are known to bind phosphorylated sites on their interacting partners, and these interactions are thought to be modulated by kinases such as ATM and ATR that respond to DNA damage (Manke et al., 2003). The BRCT domain of BRCA1 is specific for peptides of the pattern pSer-X-X-Phe (Yu et al., 2003; Williams et al., 2004). Interactions of the BRCA1 BRCT domains with peptides of this recognition sequence have been extensively studied for several proteins including the DNA helicase BACH1 and ACC1.

There is no known BRCA1 homolog in yeast, and expression of BRCA1 induces lethality in both haploid and diploid yeast strains (Humphrey et al., 1997; Westmoreland

et al., 2003). One possible reason for this lethality is that when BRCA1 is expressed in yeast, it is able to interact with an evolutionarily conserved pathway and ends up disrupting it. To identify conserved genes that may be interacting with BRCA1, Bennett and colleagues screened the yeast deletion collection for deletion strains that were able to survive expression of BRCA1 (Bennett et al., 2008). Interestingly, the two best suppressors of BRCA1 lethality were the deletion of the CTD kinase, Ctk1, and deletion of the transcriptional regulator, Spt4. Furthermore, Bennett was able to illustrate protein-protein interactions between Spt4/5 and BRCA1 using co-immunoprecipitation. Spt4 was shown to co-IP with BRCA1 in yeast and Spt5 co-IPed with BRCA1 in human cells. These observations lead to the possibility that in higher organisms, BRCA1 may interact with the Spt4/Spt5 complex and when BRCA1 is expressed in yeast, it maintains the ability to interact with this complex and does so in a manner that is detrimental to survival.

A.1.3 What is Spt4/5?

Spt4 and Spt5 (DRB sensitivity-inducing factor or DSIF complex (Bennett et al., 2008; Wada et al., 1998)) are known to both positively and negatively influence RNAPII elongation (Chen et al., 2009b; Wu et al., 2003). The Spt4/5 complex increases the processivity of RNAPII and is required for transcription of long G/C rich regions in yeast (Rondón et al., 2003). Recent structural studies of the archaea homolog of Spt4/5 illustrate that this complex is able to bind the RNA polymerase clamp and encapsulate the DNA in a manner reminiscent of a DNA helicase clamp (Klein et al., 2011; Martinez-Rucobo et al., 2011). As illustrated by these structural studies, association of Spt4/5 with RNAPII allows the polymerase to clamp around the DNA. Such an association explains the previous observation that Spt4/5 increases the processivity of the polymerase. In these studies, Spt4 was also shown to bind the side of Spt5 that is not associated with the

polymerase and would otherwise be exposed, explaining previous observations that Spt4 can protect Spt5 from degradation (Malone, 1983; Ding et al., 2010).

A.2 Materials and Methods

A.2.1 BRCT domain purification

A plasmid (called pGEST15-BRCT) containing the BRCT domains from BRCA1 fused to a GST was constructed in the Bennett lab. GST-BRCT fusion protein was expressed in BL21-RIPL cells (Stratagene) following a 3 hour IPTG induction. Cells were then pelleted by centrifugation (yielding approx. 4.5 g wet cell pellet) and frozen at -80 °C. Pellet was thawed and resuspended in 50 mL cold cell extraction buffer (1XPBS, 1:1000 PMSF, protease inhibitor (Sigma p2714) 1:1000). This suspension was then cracked open in the cell cracker. Following cell cracking, Triton X was added to 1%. Then the extract was spun at 12,000Xg for 10 minutes. The supernatant was then passes through a 0.45 μ M filter. This flow through became the cell extract. It was applied to 1.5 mL washed glutathione sepharose beads, and incubated for approx. 1.5 hrs. Beads were spun and the FT was taken off the top. Beads were then applied to a column and washed with 30 mL of PBS. Following washing, seven 750 μ L elution fractions of elution buffer (10mM Glutathione, 50mM Tris-HCl, pH 8.0) were collected. Samples from the purification were then run on an SDS-PAGE gel and stained with coomassie. Elutions 2 and 3 were combined and dialized into storage buffer (25 mM HEPES, pH 7.6, 100 mM NaCl).

A.2.2 Peptide Columns

Peptide column binding was performed essentially as described in MacKellar *et al* (2011) . Briefly, synthesized peptides containing N-terminal biotin tags were bound to Avidin beads and washed. Purified BRCT fusion protein was applied to beads and

extensively washed. Elutions were then performed in buffer containing 300 mM NaCl followed by a secondary buffer containing 1 M NaCl.

A.2.3 Strains

A FY2205 strain in which Spt4 had been genomically tagged with a Flag tag was a gift from Fred Winston.

A.2.4 Western Blotting

Cell extracts were made essentially as described in Section 2.2.5. Antibodies to a Flag tag fused to Spt4 were used at a final dilution of 1:5,000.

A.3 Results

Based on the observations made in Bennett et al (2008) and the known preference for BRCT repeats to bind pS-X-X-F sequences, I examined Spt4 for possible BRCT binding domains. Indeed, Spt4 does contain a potential BRCT binding site. This region of Spt4 is highly conserved and the essential S and F binding residues are completely conserved throughout all organisms I examined (Klein et al., 2011; Martinez-Rucobo et al., 2011). Recent structural studies of the Spt4/5 complex bound to polymerase show that this S-X-X-F motif resides on an exposed loop on Spt4 (Klein et al., 2011; Martinez-Rucobo et al., 2011).

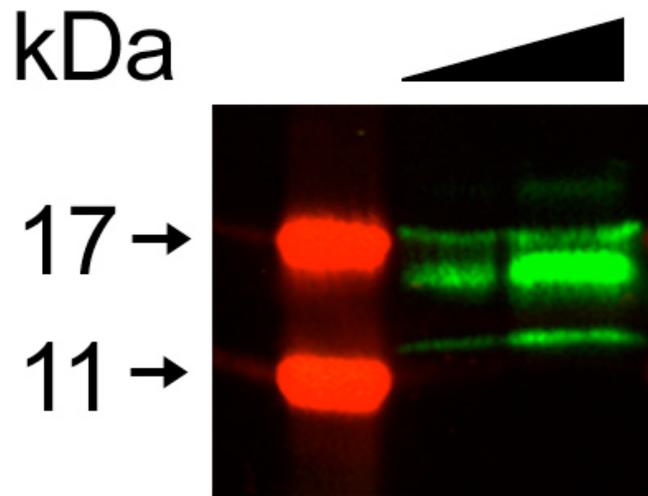


Figure 17: Multiple forms of Spt4

Western blot of extract from a Flag tagged Spt4 strain. An increasing amount of Spt4 (green bands) extract is loaded and visualized using an anti-Flag antibody. Red bands are from 17 and 11 kDa markers.

Western blot analysis of a tagged version of Spt4 shows that multiple forms of the protein are present *in vivo* (Figure 17). While we do not know what may be causing these various versions of Spt4, post-translational modifications, including serine phosphorylation could explain these variations. Furthermore, because this region is on an exposed loop of the protein, it would be accessible for regulation by post-translational modifications. Based on these two facts, the idea that this serine might be a target of phosphorylation seems reasonable.

In an effort to determine whether the BRCT domain can actually bind to this sequence, we had peptides of this region synthesized for both the phosphorylated and the non-phosphorylated Spt4 sequences from both yeast and humans. These peptides were then immobilized through a biotin tag to an Avidin resin. We purified a BRCT domain recombinant protein, comprising the two BRCT repeats from BRCA1 (BRCT domain purification in Figure 18), and we examined its ability to bind to the Spt4 peptide columns (yeast peptides Figure 19, human peptides Figure 20). The unphosphorylated peptide was unable to bind any of the BRCT protein, which all came through in the flow through and washes. However, when the essential serine was phosphorylated, the BRCT domain bound tightly and did not elute in the flow through, washes, or 300 mM NaCl step, but did elute once 1M NaCl was added to the column. This result shows that the BRCT domain can bind a Spt4 peptide and that this binding is dependent on phosphorylation of the Serine in the Ser-X-X-Phe motif.

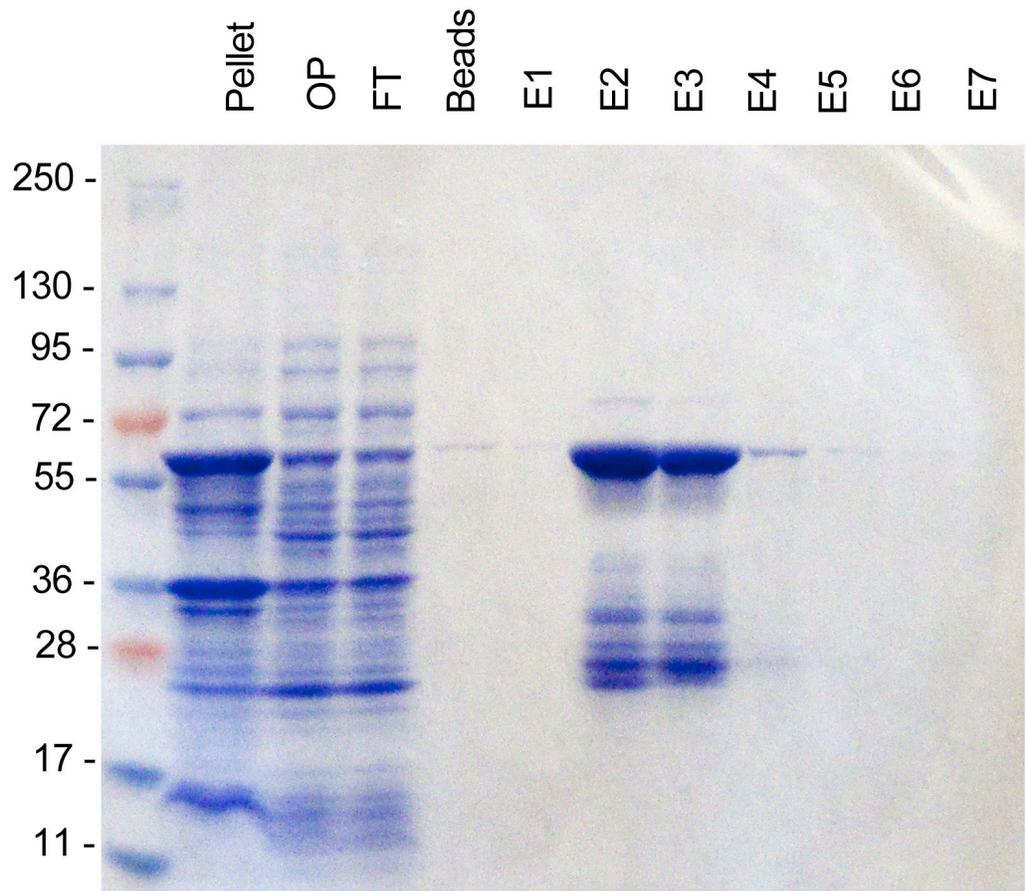


Figure 18: BRCT domain purification

Coomassie stained gel of fractions from the purification of the GST-BRCT fusion protein. A portion of the pellet remaining from extract preparation was solubilized in SDS sample buffer and loaded onto gel. I also loaded a portion of the extract or output (OP), flow through (FT), glutathione beads boiled in SDS sample buffer to see what remained on the column (beads) and elution fractions 1-7 (E1-E7). Most of the purified protein eluted in fractions E2 and E3.

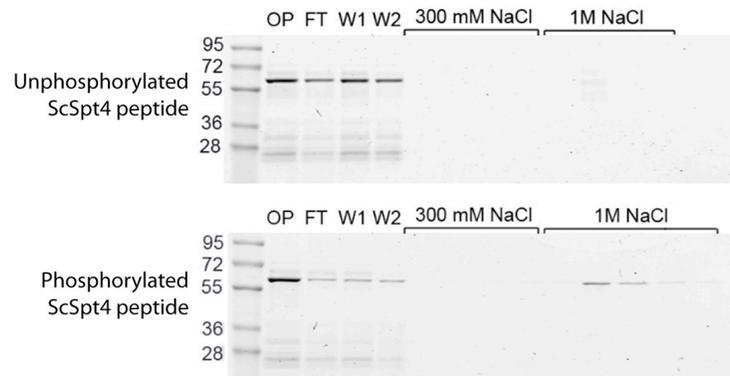


Figure 19: BRCT domain binds to phosphorylated ScSpt4 peptides

BRCT domain binding to both phosphorylated and unphosphorylated ScSpt4 sequences. Onput (OP), flowthrough (FT), and washes (W1, W2) show how the column was loaded. For the unphosphorylated peptide (top panel), all of the GST-BRCT fusion protein comes off in the FT and washes. For the phosphorylated peptide, fusion protein remains bound to the column even through a 300 mM NaCl wash and does not elute until a 1M NaCl wash is added.

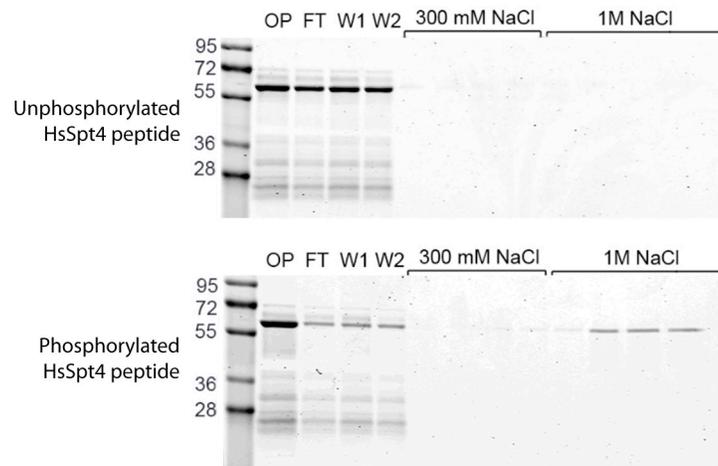
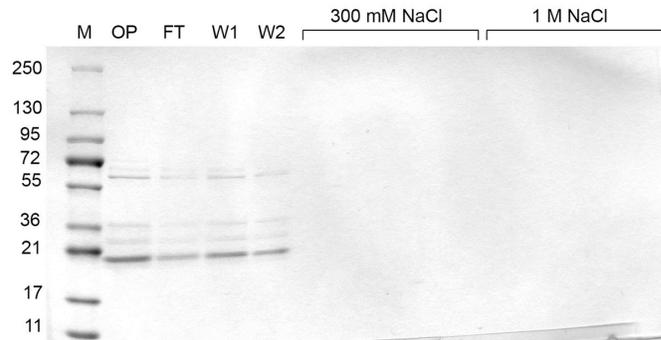


Figure 20: BRCT domain binds to phosphorylated HsSpt4 peptides

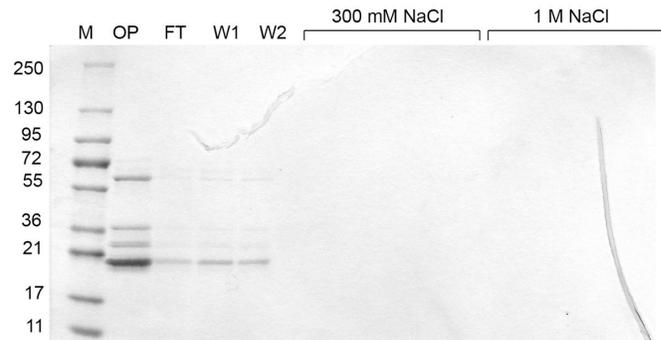
Same as Figure 19, except column contains the HsSpt4 peptide sequence. Again, BRCT domain does not bind the unphosphorylated Spt4 sequence, but binds strongly to the phosphorylated Spt4 sequence, remaining bound through a 300 mM NaCl wash and only eluting in 1 M NaCl.

Because the sequence of the potential BRCT binding domain in *Saccharomyces cerevisiae* Spt4 I (TSPSF) is very similar to the canonical RNAPII CTD repeat sequence (TSPSYSP), one might expect the CTD peptide phosphorylated at the analogous Ser residue (the Ser5 position on the CTD) would also bind the BRCT domain peptide. Therefore, we tested binding of the BRCT domain to CTD peptides that are phosphorylated on either Ser2 (2P), Ser5 (5P) or both Ser2 and Ser5 (2,5P) of each repeat for three total CTD repeats. However, as shown in Figure 21, the BRCT domain is not able to bind any of these CTD peptides, including the ones that are phosphorylated on the Ser2 position. Thus, having the conserved phenylalanine in the Spt4 peptides is very important, and it cannot even be substituted with a tyrosine. Further examination of structural studies published on the BRCT binding domain suggest that there may not be room for the hydroxyl on a tyrosine in the tight pocket where the phenylalanine binds to the canonical BRCT binding domain is (Williams et al., 2004; Botuyan et al., 2004). This would explain the ability of the BRCT binding domain to bind the Spt4 peptide but not any of the CTD peptides and suggests that while BRCA1 may associate with the transcription complex via Spt4, it does not associated with RNAPII via binding to the RNAPII CTD.

A. 2P Column



B. 5P Column



C. 2,5P Column

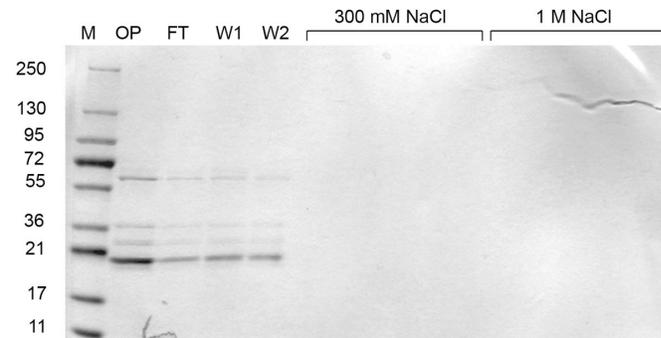


Figure 21: BRCT domain does not bind CTD peptide columns

GST-BRCT domain was tested for binding to CTD peptide columns with various phosphorylation patterns. Fusion protein did not bind to any of the 3-CTD repeat peptide columns. (M, Marker; OP, output; FT, flowthrough; W1, wash 1; W2, wash2; 300mM NaCl, low salt elution; 1M NaCl, high salt elution)

To determine if Spt4 was phosphorylated *in vivo* as we predicted, and to study this phosphorylation further, we had antibodies generated to phosphorylated peptides from both Sc Spt4 and Hs Spt 4 in collaboration with Active Motif. While further testing still needs to be done, initial results suggest that antibodies may have resulted that will allow us to look at phosphorylated forms of Spt4 in both yeast and human cells.

A.4 Discussion

Further research must be done to establish a connection between BRCA1 and the DSIF complex in human cells. However, the data I have presented in this section including *in vitro* binding of the BRCT domain of Spt4 and western blotting showing that several potential forms of the protein exist *in vivo*, are consistent with such a connection. Since BRCA1 is thought to associate with a processive RNAPII complex during elongation (Lane, 2004), having it associate via Spt4 binding makes a lot of sense. I propose a model that would account for my data, literature reports that BRCA1 associates with the elongating form of RNAPII, and the screen by Bennett et al (2008) that found deletion of either *spt4* or *ctk1* suppresses lethality of BRCA1 expression in yeast. In my model, a version of Spt4 phosphorylated by CDK12 (the mammalian homolog of Ctk1) recruits BRCA1 to the elongating form of RNAPII where it fulfills a role in DNA damage recognition and/or response. This model simply and elegantly explains all of the previously described data. However, much needs to be done to prove such a model. First, *in vivo* association of Spt4 and the BRCT domain of BRCA1 must be established. Furthermore, as Spt4 is known to be important for processivity and transcription of long genes, it must be shown that *spt4*Δ doesn't suppress BRCA1 lethality simply because it reduces BRCA1 expression. This possibility was examined

briefly in Bennett et al (2008), but it may be wise to revisit it more robustly. Finally, Ctk1 / CDK12 must be shown to phosphorylate Spt4 at the relevant position. Although much remains to be done to prove a connection between Spt4 and BRCT domain of BRCA1, the preliminary evidence is intriguing and warrants more research.

Appendix B: Yeast Strains Used

Table 4: Yeast Strains Used

Strain	Genotype	Origin
BY4743	MAT a/ α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0</i>	yeast deletion collection
BY4743 <i>set2Δ</i>	MAT a/ α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 set2Δ::KanMX/set2Δ::KanMX</i>	yeast deletion collection
BY4743 <i>set2ΔSRI</i>	MAT a/ α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 set2Δ::KanMX/set2ΔSRI::KanMX</i>	haploids used in construction from Brian Strahl
BY4743 <i>SET2/set2Δ</i> (WT <i>SET2</i> strain in Chapter 3)	MAT a/ α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 set2Δ::KanMX/SET2</i>	haploids used in construction from Brian Strahl
LRY1443/LRY1444	MAT a/ α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11/his3-11 leu2-3,112/leu2-3,112 myc-SUM1/myc-SUM1 trp1-1/trp1-1 ura3-1/ura3-1 p^{GAS2}-HIS3/ p^{GAS2}-HIS3 hht1-hhf1Δ::NatMX hht2-hhf2::HygMX</i>	haploids used in construction from Laura Rusche
M7/M53 WT recombination strain	MAT a/ α <i>lys2-2/lys2-1 tyr1-2/tyr1-1 his7-1/his7-2 CAN1/can^r ura3-1/ura3-13 cyh2^r/CYH2 ADE5/ade5 ade2-1/ade2-1 ade6/ADE6 leu1-c/leu1-12 trp5-c/trp5-d met13c*/met13-d</i>	haploid used in construction made by Robert Malone
M7/M53 <i>ctk1Δ</i>	MAT a/ α <i>lys2-2/lys2-1 tyr1-2/tyr1-1 his7-1/his7-2 CAN1/can^r ura3-1/ura3-13 cyh2^r/CYH2 ADE5/ade5 ade2-1/ade2-1 ade6/ADE6 leu1-c/leu1-12 trp5-c/trp5-d met13c*/met13-d ctk1Δ::KanMX/ctk1Δ::KanMX</i>	derived from M7/M53
M7/M53 <i>rvs161Δ</i>	MAT a/ α <i>lys2-2/lys2-1 tyr1-2/tyr1-1 his7-1/his7-2 CAN1/can^r ura3-1/ura3-13 cyh2^r/CYH2 ADE5/ade5 ade2-1/ade2-1 ade6/ADE6 leu1-c/leu1-12 trp5-c/trp5-d met13c*/met13-d rvs161Δ::KanMX/rvs161Δ::KanMX</i>	derived from M7/M53
M7/M53 <i>set2Δ</i>	MAT a/ α <i>lys2-2/lys2-1 tyr1-2/tyr1-1 his7-1/his7-2 CAN1/can^r ura3-1/ura3-13 cyh2^r/CYH2 ADE5/ade5 ade2-1/ade2-1 ade6/ADE6 leu1-c/leu1-12 trp5-c/trp5-d met13c*/met13-d set2Δ::KanMX/set2Δ::KanMX</i>	derived from M7/M53
FY2205 Spt4-Flag	Mat α <i>his4-912δ lys2-128 δ leu2Δ ura3-52 trp1Δ63 RPB3-HA::LEU2 SPT4-3XFLAG::KANMX</i>	Fred Winston
All other deletion strains mentioned are diploids from the yeast deletion collection and of the BY4743 background. Therefore, they are isogenic to BY4743 as listed above except the gene of interest is eliminated from both alleles using the KanMX marker.		

Appendix C: List of Abbreviations Used

Table 5: Abbreviations Used

Abbreviation	Meaning
4NQO	4-Nitroquinoline 1-oxide
BRCT domain	BRCA1 C Terminus domain
CAR	CTD Associating DNA damage Response
CM	complete media
CTD	Carboxy-terminal Domain of RNAPII
CTDK-I	CTD Kinase-I
cytoCTD	cytoplasmically expressed β -Galactosidase-CTD fusion protein
DSB	Double Strand Break
DSIF	DRB sensitivity-inducing factor
DX	doxorubicin
HR	Homologous Recombination
HU	Hydroxy Urea
IR	Ionizing Radiation
IR	Ionizing Radiation
LOH	Loss of Heterozygosity
MMS	methyl methanesulfonate
NER	Nucleotide Excision Repair
nucCTD	nuclearly expressed β -Galactosidase-CTD fusion protein
PCAP	Phospho-CTD Associated Protein
PCTD	Phosphorylated CTD
RNAPII	RNA Polymerase II
SRI domain	Set2-Rpb1 Interacting domain
SL	Synthetic Lethal
TC-NER	Transcription coupled-NER
UV	Ultra Violet Radiation
WT	Wild Type

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Biography

Tiffany Sabin Winsor was born on September 1, 1980 in Provo, UT to John and Terry Sabin. Tiffany grew up primarily in the Washington, DC Metro Area and upon graduation from high school, she returned to Provo, UT to pursue a BS in Biochemistry at Brigham Young University. Upon completion of her undergraduate work in April, 2004, she came to Duke to pursue her PhD. While at Duke, Tiffany participated in the publication of several manuscripts that have either been published or are currently in preparation. Articles Tiffany participated in that are currently available in the scientific literature include:

Bennett,C.B. et al. (2008) Yeast screens identify the RNA polymerase II CTD and SPT5 as relevant targets of BRCA1 interaction. *PLoS ONE*, **3**, e1448.

Westmoreland,T.J. et al. (2009) Comparative genome-wide screening identifies a conserved doxorubicin repair network that is diploid specific in *Saccharomyces cerevisiae*. *PLoS ONE*, **4**, e5830.