Title

Co-localization of Sensors is Sufficient to Activate the DNA Damage Checkpoint in the Absence of Damage

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Publication Date

2008-08-15

Peer reviewed|Thesis/dissertation

Co-Localization of Sensors is Sufficient to Activate the DNA Damage Checkpoint in
the Absence of Damage

by

Carla Yaneth Bonilla

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

With the approval of my dissertation committee members, material previously published in Molecular Cell, Vol 30, 267-276, is being used as part of the requirement for completion of this thesis. Chapter 2 contains the text, figures, and legends of the article published in Molecular Cell titled "Co-Localization of Sensors is Sufficient to Activate the DNA Damage Checkpoint".

Acknowledgements

Many years ago my mom, Delia, and dad, Carlos, left El Salvador hoping to give my brother and I a future. And I can say that they did a great job. If there was one thing they instilled in me, it was the dedication for school. I don't know if it was to please them or to stay out of trouble, but I always stayed in school. So, in retrospect their persistence paid off. It would be impossible to sum up all the things they have done to help me, but I certainly wouldn't have come this far without them. They remain my inspiration to keep going. I want to make them proud and ultimately pay back all the effort and sacrifices they made for me. Thank you mom and dad.

Trying to thank and acknowledge everyone who has ever helped me in my journey through grad school would be an impossible task, but I will try to start with the one person without whom I wouldn't be finishing my thesis today. That person is Dave T. a.k.a. David Toczyski. I remember being very scared of starting something new, but with Dave's guidance and patience I have accomplished many feats. I never thought I would have had such a wonderful experience in grad school, but being in this lab made it easy. Dave always fosters a cooperative environment in the lab. Every member seems to be handpicked to be supportive, friendly and really smart. I believe that is a reflection on Dave and the energy that he exudes. I was very lucky to have worked on an amazing project but also have had Dave's support through every step, from experiment conception to going over my very rough manuscript drafts; multiple drafts. Dave was not the only one who held my hand initially; Justine was my first hands-on mentor in the lab. I learned

my yeast genetics from her and she taught me to be thorough with my experiments. Everyone else in the lab has also brought me joy, from happy hour at the Fish Bowl to cake time on birthdays.

Hite and Wendell were my thesis committee and meeting every year with them to talk about my project was great help. They have given me advice on my science as well as career wise and I have relished those words of wisdom and experience. I aspire to be like them and take a little of their tenacity and creativity in my next phase at MIT.

I have been very fortunate to have many mentors and one of my first mentors is Leti, Dr. Marquez-Magana. Leti and I reunited on my senior year at UC Berkeley and every since she has been instrumental in my development as a scientist. I aspire to be like her in many ways, but mostly in the way that she embodies political activism and science. I can only hope to be that strong Latina scientist one day.

Now, just because they are last does not mean they don't count as much as my PIs, but my friends are a group of people I want to thank now. Where can I start to sum up over a decade of support and friendship. Dave, Paula, Vero, Leila, Larry, Lucia, Joyce, Audrey and Noe are only a handful of the loving people who surround me. They have brought me laughter when I was crying, and reminded me that getting this Ph.D. was bigger than me and I needed to see it through the end. But before pursuing my Ph.D. they were always there from Community College to Cal, to State and now UCSF. I could never ask for a better group of friends. They are not just friends; they are my family.

Co-Localization of Sensors is Sufficient to Activate the DNA Damage

Checkpoint in the Absence of Damage

Carla Bonilla

Abstract

Previous work on the DNA damage checkpoint in *Saccharomyces cerevisiae* has shown that two complexes independently sense DNA lesions: the kinase Mec1-Ddc2 and the PCNA-like 9-1-1 complex. To test whether co-localization of these components is sufficient for checkpoint activation, we fused these checkpoint proteins to the LacI repressor and artificially co-localized these fusions by expressing them in cells harboring Lac operator arrays. We observed Rad53 and Rad9 phosphorylation, Sml1 degradation and metaphase delay, demonstrating that co-localization of these sensors is sufficient to activate the checkpoint in the absence of DNA damage. Our tethering system allowed us to examine checkpoint activation in the absence of damage processing and checkpoint protein recruitment. Thus, we were able to establish that CDK functions in the checkpoint pathway downstream of damage recognition. We found that CDK-dependent phosphorylation of Rad9 is required for efficient checkpoint activation. Lastly, we began analysis of the regulation by CDK on Rad9 in its ability to activate Rad53. We found that the Rad9 CDK mutant was not phosphorylated by Mec; and was not able to find Rad53 efficiently after DNA damage.

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CHAPTER 1

Introduction

Cells utilize signaling pathways called checkpoints to control the accurate and timely segregation of the genome. Endogenous and exogenous DNA damage can cause double strand breaks that may lead to the loss of genetic information if not repaired in time. The DNA damage checkpoint ensures cells arrest in the G2/M phase of the cell cycle and that repair of the break occurs (Weinert and Lydall 1993). When these checkpoints fail, cell viability may decrease; or if mutations are propagated they may lead to carcinogensis. Therefore, specialized proteins carry out the sensing and signal transduction to ensure genome stability.

DNA damage checkpoint signaling proteins vary in their functions and enzymatic activities. They can be thought of as sensors, adaptors and transducer proteins. One type of sensor proteins belong to the family of PI3 kinase-like kinases and are localized to sites of damage. A PCNA like checkpoint clamp represents the other sensor complex. The adaptors are large BRCT containing proteins that may serve as platforms to recruit the transducer kinases. Finally, the kinases responsible for the amplification of the checkpoint signal are transducer serine, threonine kinases that have targets in the cell cycle machinery, thereby inhibiting cell cycle progression (Reviewed in Melo and Toczyski). Sensor kinases, the PCNA-like clamp, adaptors and effector kinases are conserved from yeast to mammals, however, the relative function of some diverge.

The vertebrate sensor proteins are ATM and ATR, Mec1 and Tel1 in

Saccharomyceis cerevisiae, and they initiate a phosphorylation cascade that leads to the

eventual arrest of the cell cycle as well as repair of the damage. Both sensors localize to

sites of damage, but their mode of recruitment varies. ATM is recruited to double strand

breaks via its interaction with the MRN (Mre11-Rad50-Nbs1) complex; this mechanism

is conserved with the yeast Tel1, recruited via the yeast MRX (Mre11-Rad50-Xrs2)

(You, Chahwan et al. 2005). The MRN complex has multiple activities, such as

exonuclease activity in the Mre11 subunit, which has been suggested to aid in the

creation of ssDNA at a double strand break (Nakada, Hirano et al. 2004). ATM has been

shown to autophosphorylate on S1981 and undergoes a change from inactive dimer to an

active monomer. This transition is aided by Nbs1 (Bakkenist and Kastan 2003; Lee and

Paull 2005; You, Chahwan et al. 2005; Dupre, Boyer-Chatenet et al. 2006). Once ATM is

activated, it is responsible for the phosphorylation of MRN as well as downstream targets

of the checkpoint, such as the histone variant H2AX.

S. cerevisiae Mec1, the homologue of the ATR sensor kinase, is in a complex

with Ddc2 (ATRIP, ATR Interacting Protein). Both Ddc2 and ATRIP are recruited to

single stranded DNA (ssDNA) through its interaction with RPA, bringing Mec1 and ATR

to sites of DNA damage or replication stress respectively (Paciotti, Clerici et al. 2000;

Rouse and Jackson 2002; Zou and Elledge 2003). ssDNA forms after resection of the 5'

occurs with the activity of exonucleases, such as Exo1 (Nakada, Hirano et al. 2004).

Once Mec1/Ddc2 are recruited to the DNA break, Mec1 also phosphorylates H2A at Ser

129, serving as a marker of damage and recruitment site for adaptor proteins with affinity

to phosphorylated Ser129 (Redon, Pilch et al. 2003). This site is important for foci

formation of adaptor proteins, yet loss of it does not have a significant effect on checkpoint signaling in the yeast checkpoint (Toh, O'Shaughnessy et al. 2006). In mammals, the damage-dependent phosphorylation occurs on the histone variant H2AX at serine 139 (Celeste, Fernandez-Capetillo et al. 2003).

The heterotrimeric clamp sensor complex, 9-1-1 (hRad9-hHus1-hRad1) localizes to DNA breaks early in the checkpoint pathway and it is required for full checkpoint function. It shares structural similarity to the replication clamp PCNA, suggesting that it may also encircle the DNA at damage sites (Thelen, Venclovas et al. 1999). Its recruitment requires the action of a clamp loader complex Rad17-RFC (Kondo, Wakayama et al. 2001; Melo, Cohen et al. 2001; Bermudez, Lindsey-Boltz et al. 2003). Similar to PCNA, 9-1-1 is thought to be loaded onto DNA, encircling it, as it is opened by the RFC-like complex Rad17-RFC. This clamp loader is composed of 4 of the 5 RFC subunits, substituting RFC1 for Rad17 (Green, Erdjument-Bromage et al. 2000; Ellison and Stillman 2003). The yeast homolog of the clamp and clamp loader, Ddc1-Mec3-Rad17 and Rad24 respectively, are also recruited to DNA breaks at the ssDNA (Kondo, Wakayama et al. 2001; Melo, Cohen et al. 2001). The clamp must contain all the subunits in order for it to localize to ssDNA. Mutations in the subunits of the 9-1-1 complex render the checkpoint defective, probably due to the inability of the clamp to load as a partial complex. Additionally, Rad24-RFC may not be able to bind the incomplete 9-1-1 clamp.

Both sensors complexes co-localize at break sites and are required for complete activation of the checkpoint, yet they are not required for each other's recruitment (Melo, Cohen et al. 2001; Zou and Elledge 2003). It has been proposed that the 9-1-1 clamp may activate ATR when they are in proximity on the DNA either directly or indirectly through another protein. The idea that there is another checkpoint protein that activates ATR has recently been reinforced with the work in Xenopus and mammalian cells identifying TopBP1 as an ATR activating protein (Kumagai, Lee et al. 2006). TopBP1 is an essential replication protein that contains eight BRCT domains (Yamane, Wu et al. 2002). An ATR Activating domain (AAD) was mapped between BRCT domain VI and VII. This region can bind ATR and is sufficient to activate it and induce phosphorylation of ATR substrates (Kumagai, Lee et al. 2006). Both ATR and ATRIP contain domains responsible for binding TopBP1 (Mordes, Glick et al. 2008) and any single interaction was not sufficient to activate ATR, suggesting both protein interactions, ATR-TopBP1 and ATRIP-TopBP1 are required for full activation. Mutations in conserved domains of ATRIP and Ddc2 were found to affect the strength of the checkpoint signal (Mordes, Glick et al. 2008). These are thought to disrupt the interaction between ATRIP and TopBP1 and Ddc2 with Dpb11, although the Ddc2-Dpb11 interaction has not been documented.

The initial TopBP1 recruitment to ATR seems to happen through the 9-1-1 clamp. The Rad9 (yeast Ddc1) subunit of the 9-1-1 complex interacts directly with the a region on TopBP1 between BRCT domains I and II (Delacroix, Wagner et al. 2007; Lee, Kumagai et al. 2007). In fission yeast, *S. pombe*, the TopBP1 orthologue, Cut5 has been

shown to interact with the Rad9 subunit of the 9-1-1 complex (Furuya, Poitelea et al. 2004). Recently, the *S. cerevisiae* Dpb11 protein was found to bind the Ddc1 subunit of the 9-1-1 clamp, similarly to the Cut5-Rad9 interaction. The damage dependent phosphorylation of Ddc1 was required for its interaction with Dpb11 (Puddu, Granata et al. 2008). Even though Dpb11 lacks the conserved ADD, it may still activate Mec1 through an analogous mechanism. This conservation in mechanism argues that an interaction between TopBP1 and the 9-1-1 clamp recruits TopBP1 to the proximity of ATR, where it can bind and activate it.

TopBP1 may not be the only way that the 9-1-1 clamp aids in activating ATR. Majka et al. showed that in an in vitro assay for Mec1 kinase activity, purified 9-1-1 clamp could activate Mec1 on a subset of substrates (Majka, Niedziela-Majka et al. 2006). They went on to show that the single subunit Ddc1 (hRad9) could also induce Mec1 kinase activity, although it required non-physiological salt conditions. While this result does not definitively show that Ddc1 is a Mec1 activating protein, it points to a mechanism whereby Ddc1 can activate Mec1 when TopBP1 is not around.

Checkpoint adaptor proteins rely on the sensor kinases to be recruited. They are often large proteins composed of multiple BRCT domains that bind phospho-proteins. The BRCT domains of adaptor proteins have been shown to be involved in oligomerization, protein-protein interactions, and H2AX binding (Hammet, Magill et al. 2007). Some examples of adaptors are the metozoan MCD1, Claspin, 53BP1, and the yeast Mrc1, Rad9 and Crb2. Most adaptors have the ability to interact with phospho H2AX, which may help in their maintenance

at DNA damage sites. The phosphorylation of H2AX by checkpoint kinases promotes an interaction between the adaptors and phosho H2AX through the BRCT domains on the adaptors. The BRCT domains on Rad9 are important for its binding to phospho H2A (Hammet, Magill et al. 2007). The vertebrate adaptor 53BP1 also binds phospho H2AX and this interaction is important for 53BP1 damage dependent foci formation (Ward, Minn et al. 2003)

Besides binding to phosphorylated histones, adaptor proteins also bind methylated histones. The checkpoint proteins Rad9/Crb2/53BP1 are recruited to damage sites by histone methylations. In mammalians, methylation of lysine 79 on H3 is important for localization of 53BP1 (Huyen, Zgheib et al. 2004). Cells deficient in Dot1, the histone methyl transferase (HMT) responsible for lysine 79 methylation, are unable to form 53BP1 foci after damage. The requirement for K79 methylation in 53BP1 foci formation is most likely due to a direct interaction between H3 and 53BP1, since 53BP1 can bind H3 *in vitro* (Huyen, Zgheib et al. 2004). *S. cerevisiae* seems to share this mechanism. Mutants deleted for *DOT1* or unable to be methylated at lysine 79 (H3K79A) show a decrease in the kinetics of radiation-induced Rad53 phosphorylation after DNA damage (Giannattasio, Lazzaro et al. 2005). As with 53BP1, Rad9 binds H3 *in vitro* (Huyen, Zgheib et al. 2004). Similar to the loss of γ- H2AX, deletion of *DOT1* does not entirely eliminate the checkpoint, indicating that an independent mechanism for the recruitment of Rad9 must exist (Giannattasio, Lazzaro et al. 2005).

S. pombe also uses histone methylation to recruit the adaptor protein Crb2 to damage. While methylation-directed recruitment appears to be conserved, *S. pombe* uses a different methylation site, lysine 20 on histone H4, to recruit Crb2 (Sanders, Portoso et al. 2004). The methylation on H4K20 requires the HMT Set9, and studies with DNA damaging agents showed that set9 deleted cells were more sensitive than wild type to several types of damage. However, *crb2* mutants are much more damage sensitive than *set9* (or H2A S129A) mutants, consistent with a model in which an alternative mechanism exists for Crb2 recruitment. As with the loss of mammalian *DOT1,* which causes a decrease in the number of 53BP1 foci (Huyen, Zgheib et al. 2004), *set9* deleted cells show a reduction in the number of cells with multiple Crb2 foci (Sanders, Portoso et al. 2004). Thus, the loss of H3K79 or H4K20 methylation is not sufficient to abolish the roles of Rad9/53BP1 or Crb2, respectively, in the DNA damage checkpoint. Despite the fact that these methylation sites are not conserved, the domain of the adaptor checkpoint protein that is thought to bind the methylated histone, called the Tudor domain, is conserved (Huyen, Zgheib et al. 2004). Tudor domains have been characterized in several proteins that recognize methylated proteins, and have structural and sequence similarities to other methyl-binding domains, such as Chromo domains. The similarities between Tudor and Chromo domains coincide at the methylated-histone binding region. Huyen et al. showed that mutants in the Tudor domain of 53BP1 eliminate its ability to form damaged-induced foci and bind H3K79 containing chromatin *in vitro* (Huyen, Zgheib et al. 2004).

Once recruited to a break site, the adaptors aid in the phosphorylation and activation of the transducer kinases. For example, *S.cerevisiae* Rad9 interacts with the transducer kinases Rad53 and Chk1. Rad9 is phosphorylated by Tel1 and Mec1 at multiple sites (Emili 1998; Vialard, Gilbert et al. 1998). These phosphorylations are responsible for the interaction between Rad53 and Rad9. Of the many phosphorylations on Rad9, one particular cluster of amino acids is heavily phosphorylated by Mec1. These sites are particularly important for Rad9's ability to serve as a Rad53 adaptor. Rad9 undergoes a change in oligomerization state as part of its Rad53 activating function (Gilbert, Green et al. 2001). Rad9 is found in a large molecular weight complex that undergoes an oligomerization change to yield smaller complex calculated to contain two Rad9 molecules. This happens when cells are stressed with DNA damage and requires two protein chaperones Ssa1 and Ssa2. The interaction with the chaperones is damage-independent, suggesting they may aid in maintaining Rad9 in a state ready for activation following DNA damage.

The transducer kinases Rad53 and Chk1 phosphorylate substrate proteins involved in repair processes and cell cycle arrest. Rad53 becomes activated by first being phosphorylated by Mec1 or Tel1 (Sanchez, Desany et al. 1996). This activates Rad53, which leads to autophosphorylation easily seen in an in situ autophosphorylation assay. The autophosphorylation of Rad53 is thought to happen while it is bound to Rad9, since addition of Rad9 to an in vitro kinase reaction containing Mec1 and Rad53 makes Rad53 a better substrate for Mec1 (Sweeney, Yang et al. 2005). The interaction between Rad53 and Rad9 is thought to happen through Rad53's FHA domains. (Sun, Hsiao et al. 1998).

FHA domains function to facilitate protein-protein interactions and in the case of the

checkpoint aid in regulating the interaction between Rad53 by allowing it to bind Rad9

once Rad9 has been phosphorylated by Mec1. Yeast Chk1 activation is less well

understood, yet we know that it is phosphorylated by Mec1/Tel1 and that it requires the N

terminus of Rad9 (Blankley and Lydall 2004). The best studied role of Chk1 in the yeast

checkpoint is its ability to stabilize Pds1 (Wang, Liu et al. 2001). Pds1 phosphorylation

inhibits its degradation by the Anaphase Promoting Complex (APC). During checkpoint

activation, stable Pds1 binds and inhibits Separase, preventing the Cohesin complex from

being degraded; thus keeping sister chromatids together during mitosis. By maintaining

cohesion, the cells become arrested since they cannot proceed with chromosome

separation.

Work on the recruitment and activation of checkpoint proteins has shed light into

an important cell survival mechanism, the DNA damage checkpoint. Yet, the exact

mechanism of activation of the yeast Mec1 kinase has not been explored. I hoped to ask a

simple question, is the DNA break strictly required to activate Mec1? In order to answer

this question, I undertook an artificial co-localization approach that would recruit sensor

proteins to the chromatin in the absence of a DNA break.

CHAPTER 2

Co-localization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage

Running title: Checkpoint activation via co-localization

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Summary

Previous work on the DNA damage checkpoint in *Saccharomyces cerevisiae* has shown that two complexes independently sense DNA lesions: the kinase Mec1-Ddc2 and the PCNA-like 9-1-1 complex. To test whether co-localization of these components is sufficient for checkpoint activation, we fused these checkpoint proteins to the LacI repressor and artificially co-localized these fusions by expressing them in cells harboring Lac operator arrays. We observed Rad53 and Rad9 phosphorylation, Sml1 degradation and metaphase delay, demonstrating that co-localization of these sensors is sufficient to activate the checkpoint in the absence of DNA damage. Our tethering system allowed us to establish that CDK functions in the checkpoint pathway downstream of damage processing and checkpoint protein recruitment. This CDK-dependence is likely, at least in part, through Rad9, since mutation of CDK consensus sites compromised its checkpoint function.

Introduction

Unrepaired DNA damage can lead to the inaccurate propagation of an organism's genome. When eukaryotic cells detect DNA damage, they activate a signal transduction pathway, called a checkpoint, to delay cell division and promote DNA repair. In response to double strand breaks (DSBs), the DNA damage checkpoint in *Saccharomyces cerevisiae* arrests cells at the G2/M phase (Weinert and Hartwell 1988). This response requires the function of at least four classes of checkpoint proteins: a clamp complex, sensor kinases, adaptor proteins and effector kinases.

DSBs are processed by exonucleases that resect the 5' strand, leaving a 3' single stranded DNA (ssDNA) overhang. This structure is thought to be a signal for the recruitment of a damage-specific DNA clamp, referred to as the 9-1-1 complex, that resembles the processivity factor for DNA replication, PCNA (Thelen, Venclovas et al. 1999). The 9-1-1 complex is a heterotrimer composed of three subunits Ddc1, Mec3 and Rad17 (hRad9, hHus1, hRad1). Loading of the PCNA clamp at 3' ssDNA/dsDNA junctions during replication is accomplished by the hetero-pentameric Replication Factor C (RFC) complex (Tsurimoto and Stillman 1991). In contrast, the 9-1-1 complex is thought to be loaded at 5' ssDNA/dsDNA junctions generated at damage sites by a modified form of RFC, in which one subunit, Rfc1, is replaced by a checkpoint-specific subunit called Rad24 (Green, Erdjument-Bromage et al. 2000; Kondo, Wakayama et al.

2001; Melo, Cohen et al. 2001; Bermudez, Lindsey-Boltz et al. 2003; Ellison and Stillman 2003; Majka and Burgers 2003; Zou, Liu et al. 2003).

Mec1 and Tel1 (ATR and ATM in mammals, respectively) are thought of as sensor kinases since they directly recognize DNA damage. These two kinases appear to function somewhat redundantly, Mec1 being the primary checkpoint signaling molecule in yeast. Mec1 associates with damaged chromatin through its partner, Ddc2, which binds RPA-coated ssDNA (Paciotti, Clerici et al. 2000; Rouse and Jackson 2002; Zou and Elledge 2003). One of the functions of the Mec1 kinase is to activate the effector kinase, Rad53 (hCHK2). Rad53 activation is mediated by either of two adaptor proteins, Rad9 or Mrc1. Mrc1 is thought to function as an adaptor during DNA replication, whereas Rad9 can also recognize damage that occurs outside of S phase. Rad9 is phosphorylated in a damage-dependent manner by Mec1 (Aboussekhra, Vialard et al. 1996; Emili 1998; Schwartz, Duong et al. 2002). This promotes its association with Rad53, leading to Rad53 activation by Mec1 and subsequent auto-phosphorylation of Rad53 (Schwartz, Duong et al. 2002; Sweeney, Yang et al. 2005).

The exact mechanism by which Rad9, and its orthologues *S. pombe* Crb2 and mammalian 53BP1, are recruited to break sites remains unclear. Efficient Rad9 and Crb2 recruitment requires at least two histone modifications (Huyen, Zgheib et al. 2004; Nakamura, Du et al. 2004; Sanders, Portoso et al. 2004; Vidanes, Bonilla et al. 2005; Toh, O'Shaughnessy et al. 2006). H2A is phosphorylated by Mec1 or Tel1 at its C-terminus in response to DNA damage (Downs, Lowndes et al. 2000). This

phosphorylation promotes an interaction between Rad9 and H2A. Similarly, vertebrate

53BP1 associates with the related histone H2A variant H2AX after phosphorylation by

ATM or ATR (Celeste, Fernandez-Capetillo et al. 2003; Ward, Minn et al. 2003). In

addition, a constitutive methylation on Lysine 79 of H3 (H3K79) by the Dot1 methyl-

transferase mediates interactions with the Tudor domains in Rad9 (Huyen, Zgheib et al.

2004). Analogously, methylation of Lysine 20 on H4 (H4K20) is required for the

maintenance of Crb2 and 53BP1 (Botuyan, Lee et al. 2006). In summary, adaptor

proteins from all three species use related mechanisms, H2A phosphorylation and

methylations on the histone core, for their enrichment at chromatin adjacent to damage

sites.

The observation that the Ddc2-Mec1 and the 9-1-1 complexes localize

independently to sites of damage (Kondo, Wakayama et al. 2001; Melo, Cohen et al.

2001; Zou, Cortez et al. 2002) suggested a model in which the DNA damage site serves

as a platform to concentrate these molecules. To test this hypothesis, we artificially co-

localized the Ddc2-Mec1 and 9-1-1 complexes by fusing one member of each complex to

LacI and expressing these fusions in a strain with multimerized LacI binding sites (LacO

arrays). Using this system, we show that neither ssDNA nor the 5' ssDNA/dsDNA

junctions are directly required for the activation or function of checkpoint proteins, since

co-localization of Ddc2-Mec1 kinase and the 9-1-1 complex is sufficient to activate

Rad53 and delay cell cycle progression. By altering the exact number of LacO sites, we

were able establish a correlation between the number of checkpoint molecules co-

localized and the degree of Rad53 phosphorylation. Furthermore, we show that the Ddc1

subunit of 9-1-1 is sufficient for Rad53 activation. This activation functions in the context of chromatin, requiring H2A phosphorylation and H3K79 methylation for maximum signaling. Lastly, we show that CDK activity contributes to checkpoint activation through a mechanism independent from its established role in damage processing. Mutating the CDK consensus sites on Rad9 eliminates its cell cycle-regulated electrophoretic shift and generates a checkpoint-deficient allele.

Results

Co-localization of checkpoint proteins activates the Rad53 kinase

We set out to test the requirement for a DNA break in the initial activation step of the DNA damage checkpoint. Recruitment of Ddc2-Mec1 and the 9-1-1 complex to a double stranded break site is essential for activation of the DNA damage checkpoint. The association of Ddc2-Mec1 with ssDNA or RPA could stimulate conformational changes required for direct activation of kinase activity. Alternatively, if ssDNA serves strictly as a scaffold to concentrate Ddc2-Mec1 and the 9-1-1 complex, the requirement for a DSB could be bypassed by artificially co-localizing the two complexes. The prokaryotic repressor protein LacI binds with high affinity to the Lac operator sequence. GFP-LacI fusions have been used to recruit GFP to arrays of LacO repeats in order to visualize chromosome dynamics (Straight, Belmont et al. 1996). We co-opted this approach to recruit Ddc2-Mec1 and the 9-1-1 complex to a region on Chromosome IV containing 256 tandem copies of LacO, encompassing 10.5 Kb, which we will refer to as a LacO array. GFP-LacI fusions of *DDC1* and *DDC2* were placed under a galactose-inducible promoter and introduced into the LacO array strain where a single focus per cell could be seen when Ddc1-GFP-LacI was induced (Figure 2.1 and 2.2). Upon addition of galactose, each fusion was expressed at equivalent levels, as determined by Western blot (Figure 2.3), and could complement deletions of *DDC1* and *DDC2*, respectively (data not shown). To avoid the possibility that these fusions could disrupt DNA replication, we performed all experiments with cells that were first arrested in G2/M with nocodazole. We also

confirmed that co-localization of checkpoint fusions did not create *de novo* DNA breaks by monitoring the formation of Rad52-RFP foci. Rad52 is required for homologous recombination and has been shown to localize to DSB (Lisby, Mortensen et al. 2003). DNA damage created by treatment with 100ug/mL Zeocin for 3 hours resulted in Rad52 focus formation in 42% of cells. In contrast, only 4% of cells showed spontaneous Rad52 foci in the untreated sample, and this was not further increased by the expression of the checkpoint fusions (Figure 2.4). In fact, only 1 out of the 167 fusion-expressing cells examined formed a spontaneous Rad52 focus that co-localized with the checkpoint protein fusions at the LacO array. This suggests that co-localization of Ddc1-GFP-LacI and Ddc2-GFP-LacI at the array does not induce DNA damage.

Expression of both Ddc1-GFP-LacI and Ddc2-GFP-LacI in the presence of a LacO-array induced checkpoint activation (Figure 2.3). In contrast, no Rad53 phosphorylation was seen induced by the localization of either single complex or by expression of both complexes in the absence of LacO arrays (Figure 2.3). The Rad53 that we observed as shifted was activated, as judged by *in situ* kinase assay (data not shown) and was comparable to that induced by a single DSB (Figure 2.3), suggesting that this system mimics the physiological levels of Rad53 activation observed upon DNA damage. Moreover, the Rad53 activation observed was independent of Mre11 (Figure 2.4) arguing against ssDNA formation by Mre11's exonuclease activity (Nakada, Hirano et al. 2004) leading to Rad53 phosphorylation during co-localization.

Mec1 phosphorylation of the adaptor protein Rad9 is required for its association with, and activation of, Rad53. Rad9 exhibits a damage-independent electrophoretic shift in G2/M, that is super-shifted upon DNA damage by Mec1/Tel1 phosphorylation (Emili 1998; Sun, Hsiao et al. 1998; Vialard, Gilbert et al. 1998). Rad9 was super-shifted in a LacO array-dependent manner when both Ddc2-Mec1 and 9-1-1 were co-localized (Figure 2.3). The observation that the localization of neither Ddc2-Mec1 nor 9-1-1 alone was sufficient to promote Rad9 or Rad53 phosphorylation further demonstrates that we have recapitulated the physiological DNA damage response.

In order to estimate the number of checkpoint complexes required for signaling, we tested the minimum number of LacO repeats required for Rad53 activation. To this end, we integrated LacO arrays of different sizes, ranging from 8-256 repeats in length, into a strain co-expressing Ddc1-GFP-LacI and Ddc2-GFP-LacI. As shown in figure 1F, Rad53 phosphorylation is observed in strains with as few as 40 LacO sites. The shifted form of Rad53 continued to increase with an increasing number of LacO sites, whether they were continuous (Figure 2.5) or integrated in small groups separated by 3 Kbs (Figure 2.5). This suggests that the total number of co-recruited molecules determined the extent of Rad53 phosphorylation.

Rad53 properly targets downstream substrates following artificial sensor localization

Having shown that Rad9 and Rad53 are phosphorylated after Ddc1 and Ddc2 co-localization, we wanted to test whether Rad53 kinase was active and led to downstream signaling. One direct target of Rad53 is the kinase Dun1, which is activated by Rad53 phosphorylation (Zhou and Elledge 1993). Dun1 phosphorylates the ribonucleotide reductase inhibitor Sml1, inducing its degradation (Zhao and Rothstein 2002). We were unable to observe Dun1 phosphorylation directly in our system or after a single DSB. Therefore, we used Sml1 protein levels as a read out of Dun1 activation. Induction of multiple DSBs with the bleomycin derivative Zeocin promoted Sml1 degradation (Figure 2A, lanes 2-5). Similarly, when both checkpoint fusions were induced in the presence of the LacO array, Sml1 levels consistently decreased (Figure 2.6, lanes 11-15). Sml1 protein level decreased in an array dependent manner on induction of the fusions, suggesting that the Rad53 phosphorylation seen in figure 2.6 represents *in vivo* activation of the Rad53 kinase.

The DNA damage checkpoint acts primarily at G2/M to arrest the cell cycle prior to chromosome segregation. We asked whether co-localization was sufficient to signal arrest at this cell cycle stage upon release from nocodazole into alpha factor, which subsequently arrests cells in G1. Cell cycle progression was monitored by Flow Cytometry (FACS). Cells without LacO arrays began to enter G1 90 minutes after release from a nocodazole arrest (150' after galactose induction) (Figure 2.6). The strain co-

expressing Ddc2 and Ddc1 fusions in the presence of a LacO array maintained a 2N peak

after nocodazole release for the duration of the experiment (most obvious at 150'),

indicating maintenance of the G2/M arrest (Figure 2.6). To ensure that this represented a

checkpoint-mediated arrest, we examined an isogenic strain deleted for *RAD9* and found

that the cell cycle delay was relieved (Figure 2.6). Rad53 phosphorylation levels

correlated with the observed delay in G2/M (Figure 2.6).

The Rad24 requirement is bypassed by co-localization of Ddc2-Mec1 and 9-1-1

To further characterize the genetic requirements for checkpoint activation in our

system, we examined which other checkpoint genes were required for Rad53 activation

after Ddc2-Mec1 and 9-1-1 co-localization. We deleted *MEC1, RAD9* and *RAD24* in

strains co-expressing both checkpoint fusions and carrying LacO arrays. Unlike wild type

strains, strains deleted for *RAD9* and *MEC1* did not display Rad53 phosphorylation upon

co-expression, suggesting that both Rad9 and Mec1 are still needed to transduce the

signal to Rad53 (Figure 2.7). Importantly, this demonstrates that the Ddc2-GFP-LacI

fusion was activating Rad53 through its association with Mec1.

Rad24-RFC has been shown to function as the clamp loader for the 9-1-1

complex. We observed that Rad24 was dispensable for Rad53 phosphorylation in the co-

localization strain, suggesting that the role of Rad24 is restricted to localizing 9-1-1 to

damage (Figure 2.7). These data also suggest that the 9-1-1-LacI-clamp did not need to

be loaded (i.e. it does not need to encircle DNA) at the array. Rad24 was still required for checkpoint activation when a Ddc1-GFP-LacI, Ddc2-GFP-LacI strain lacking a LacO array was treated with Zeocin (Figure 2.7). This result verifies that the Ddc1 fusion is capable of interacting with Rad24 and can be loaded onto DNA damage sites. These data also confirm that expression of LacI fusions does not activate Rad53 by producing damage, since damage-induced activation requires Rad24 function.

Ddc1 can act independently of the 9-1-1 complex

The Ddc1 subunit of the 9-1-1 complex was sufficient to activate Rad53 when co-localized with Ddc2-Mec1. We tested whether the other 9-1-1 subunits, Mec3 and Rad17, were required for checkpoint activation. Deletion of *MEC3* and *RAD17* in the strain co-expressing Ddc1-GFP-LacI and Ddc2-GFP-LacI containing a LacO array did not abolish Rad53 phosphorylation (Figure 2.8). The Rad53 phosphorylation observed corresponded to a metaphase delay in the *rad17Δ* and *mec3Δ* strains as assessed by FACS (Figure 2.8). Deletion of *MEC3* and *RAD17* did not prevent cells from delaying with a 2N peak like wild type strains by three hours of galactose induction. This result suggested that Ddc1 is capable of activating Mec1 without the other two 9-1-1 subunits. If Ddc1 is the subunit that activates Mec1, it should be indispensable when the 9-1-1 complex is recruited through either of the other subunits, Mec3 or Rad17. To test this hypothesis, we fused Mec3 to GFP-LacI and recruited it to the array along with Ddc2-Mec1. Unlike the Ddc1-GFP-LacI fusion, the Mec3-GFP-LacI fusion was hypomorphic for DNA damaged-induced checkpoint activation (Figure 2.9). Still the co-localization of Mec3-GFP-LacI

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and Ddc2-GFP-LacI at the array also resulted in Rad53 activation, although not to the

degree seen for Ddc1-GFP-LacI. Mec3-GFP-LacI required the Ddc1 subunit to activate

Mec1, as seen by the lack of Rad53 phosphorylation in the *ddc1Δ* strain, consistent with

the hypothesis that Ddc1 mediates checkpoint activation (Figure 2.9)

Chromatin is required for checkpoint activation

In recent years, histone modifications have been implicated in the DNA damage

checkpoint. Phosphorylation of the H2A variant H2AX by ATM and ATR on S139 is a

hallmark of DNA damage. While yeast does not possess the H2AX variant, the C-

terminal tail of yeast H2A is phosphorylated by Mec1 and Tel1 on the analogous residue.

This phosphorylation is thought to help recruit Rad9 to break sites by the physical

interaction between Rad9 and phosphorylated H2A (Ogiwara, Ui et al. 2006).

Methylation of histone H3 on lysine 79 (H3K79) by the Dot1 methyltransferase is also

required for full Rad9 phosphorylation and localization (Giannattasio, Lazzaro et al.

2005; Toh, O'Shaughnessy et al. 2006). Since our co-localization system brings Ddc2-

Mec1 and 9-1-1 to DNA, we examined the role of neighboring chromatin in this

checkpoint activation. Mutation of the phosphorylation sites on H2A (Figure 2.10,

bottom panel) or deletion of *DOT1* resulted in a slight decrease in Rad53 phosphorylation

upon co-localization. When both H2AS129 phosphorylation and H3K79 methylation

were abolished, Rad53 phosphorylation decreased markedly (Figure 2.10). At later time

points (4 hours), the double mutant strain displayed some Rad53 phosphorylation, but not

to the extent seen in the wild type strain (Figure 2.10). Thus, while we have bypassed the

requirement for DNA damage in checkpoint activation, chromatin is still required for efficient signaling.

CDK activity promotes Rad53 activation

Several studies have suggested that checkpoint activation is less efficient in G1. In part, this effect can be attributed to a reported reduction in damage processing (Pellicioli, Lee et al. 2001; Clerici, Baldo et al. 2004; Ira, Pellicioli et al. 2004). Efficient 5'-3' resection is thought to require the cyclin-dependent kinase (CDK), Cdc28. Thus ssDNA, which is thought to be the intermediate recognized by the checkpoint machinery, is less abundant in G1 when CDK is inactive. We used our recruitment system to ask whether a reduction in damage processing, leading to reduced Ddc2-Mec1 and 9-1-1 recruitment, was the sole reason for low checkpoint activity in G1. Strains were arrested in G1 with alpha factor or in G2/M with nocodazole and then both checkpoint fusions were induced with galactose. As expected from our previous results, co-localizing Ddc2-Mec1 and 9-1-1 resulted in Rad53 phosphorylation and Rad9 hyper-phosphorylation in G2/M (Figure 2.11). When cells were arrested in G1, induction of checkpoint fusions did not result in Rad53 or Rad9 phosphorylation (Figure 2.11), suggesting that CDK activity is required for efficient checkpoint activation independent of its described role in damage processing.

In order to address directly whether our inability to activate the checkpoint in G1 was due to low CDK activity, we used an analog-sensitive allele of *CDC28 (cdc28-as)* that renders the kinase inactive upon addition of the inhibitor 1NM-PP1 (Ubersax,

Woodbury et al. 2003). Isogenic *CDC28* and *cdc28-as* strains were arrested in G2/M with nocodazole. 1NM-PP1 was added at the initiation of galactose induction to inhibit Cdc28-as, and Rad53 phosphorylation was measured. When cells carrying the *cdc28-as* allele were treated with inhibitor, they failed to activate Rad53 (Figure 2.11), suggesting that CDK activity was required even when both sensors were co-localized (Figure 2.11). These data support the idea that CDK activity controls checkpoint activation downstream of damage processing.

CDK activity could be required for either the initiation or the maintenance of the checkpoint. We tested the requirement for CDK activity in checkpoint maintenance by inhibiting CDK after the checkpoint signal had been established. Checkpoint fusions were induced with galactose after a nocodazole arrest in the *cdc28-as* strain. After three hours of galactose induction (a time when the checkpoint signal is robust), the inhibitor 1NM-PP1 was added to half the culture (Figure 2.11). Checkpoint inhibition was visible within 30 minutes and by 2 hours the checkpoint signal was almost eliminated. Therefore, CDK activity is required to maintain Rad53 activation.

Discussion

The DNA damage checkpoint employs two related protein kinases, ATR/Mec1 and ATM/Tel1, which must act on their substrates in a regulated manner: they do not become activated until a DNA lesion occurs. We set out to understand the mechanism by which the yeast checkpoint kinase Mec1 is activated and have shown that its co-localization with the 9-1-1 complex is sufficient to activate the checkpoint *in vivo*. By recruiting Ddc2-Mec1 and the 9-1-1 complex to LacO arrays, we were able to bypass the requirement for DNA damage, arguing against a strict damage-dependent activation step for Mec1/ATR. Having shown that co-localization activates the DNA damage checkpoint *in vivo*, we sought to understand the relationship between the number of sensors recruited and the extent of Rad53 phosphorylation. We consistently observed an array size-dependent increase in Rad53 phosphorylation and propose that checkpoint signaling correlates with the number of sensors co-recruited. This result could explain the kinetics of Rad53 activation when a DSB is being processed: more checkpoint molecules on ssDNA result in more Rad53 phosphorylation.

Rad24-RFC is an essential component of the DNA damage checkpoint, presumably because it is required to recruit the damage-specific clamp (9-1-1) to a double strand break (Kondo, Wakayama et al. 2001; Melo, Cohen et al. 2001). *rad24* mutants fail to form damage-induced Ddc1-GFP foci or fully activate Rad53. We found that the requirement for Rad24-RFC could be bypassed if we co-localized 9-1-1 with Ddc2-Mec1 through LacO arrays. Our result argues against the existence of any

additional role of Rad24 in Rad53 activation. It also suggests that 9-1-1 activity does not require that it encircle DNA.

The Ddc1 subunit of 9-1-1 can activate Mec1

Despite the similarities between the ATM and ATR kinases, the mechanisms used by cells to activate them appear quite different. Mammalian ATM has been shown to be activated by DNA *in vitro*. Dimeric ATM is directly activated by the MRN complex in the presence of DNA (Lee and Paull 2005; Dupre, Boyer-Chatenet et al. 2006). This activation leads to the formation of active monomers that act on their substrates (Bakkenist and Kastan 2003; Lee and Paull 2005). Mec1/ATR kinase is activated through an alternate mechanism that does not require a DNA break, as we have shown. Activation of Mec1 could take place directly through an intimate Mec1-Ddc1 interaction or Ddc1 could promote the activation of Mec1 by recruiting a second factor. Mec1 activation by DNA damage outside of S phase requires the 9-1-1 complex, whereas its activation during DNA replication is less dependent of the 9-1-1 complex. Recently, *in vitro* data showed that the replication protein TopBP1 is able to activate ATR through an <u>A</u>TR <u>A</u>ctivating <u>D</u>omain (AAD) (Kumagai, Lee et al. 2006). BRCT domains I and II of TopBP1 interact with the C terminus of the vertebrate Ddc1 homologue Rad9, suggesting that the 9-1-1 complex could recruit TopBP1 to ATR (Kumagai, Lee et al. 2006; Delacroix, Wagner et al. 2007; Lee, Kumagai et al. 2007). Both *S. cerevisiae* and *S. pombe* TopBP1 homologues, scDpb11 and spCut5, interact with scDdc1 and spRad9 respectively, suggesting a similar mechanism where Dbp11 could be recruited to Mec1 *in*

vivo through the 9-1-1 complex in response to replication damage in S phase (Wang and Elledge 2002; Furuya, Poitelea et al. 2004).

Alternatively, Ddc1 could activate Mec1 directly. It has recently been shown *in vitro* by Majka *et al.* 2006 that 9-1-1 loaded onto a DNA template mimicking a processed damage site could promote Mec1 activation (Majka, Niedziela-Majka et al. 2006). Our system furthers the understanding of this mechanism by showing that *in vivo* activation of Mec1 by the 9-1-1 complex does not require an association of either complex with DNA damage and that this co-localization is sufficient for checkpoint activation. The authors also reported that low salt conditions allowed the Ddc1 subunit to activate Mec1 *in vitro*. We show that Ddc1 is able to activate Mec1 and recapitulate the whole checkpoint signaling pathway *in vivo* in the absence of other 9-1-1 members. The Mec3 and Rad17 subunits could serve to recognize the Rad24/RFC complex and allow loading of the 9-1-1 complex, which is important for maintaining Ddc1 in proximity to Mec1 at the DNA break. In support of this hypothesis, recruitment of Mec3 to Ddc2-Mec1 also activated the checkpoint, but it strictly required Ddc1, arguing that Ddc1 is the activating subunit.

Co-localization requires chromatin for full activation of Rad53

The role of chromatin in checkpoint signaling could be explained by its ability to maintain a pool of adaptor proteins close to the sensor kinases. This hypothesis is supported by the interaction between the BRCT domains of Rad9 and phosphorylated H2AX. Rad9 Tudor domains and methylated histone residues also provide a binding interface that contributes to adaptor protein localization. Elimination of both H2AX phosphorylation and H3K79 methylation decreases Rad9 phosphorylation upon IR

treatment (Ogiwara, Ui et al. 2006). Consistent with these data, removing both, H2A phosphorylation and H3K79 methylation decreased Rad53 phosphorylation, but did not completely eliminate it, in our system. Therefore, recruitment of Ddc2-Mec1 and the 9-1-1 complex alone is insufficient to fully activate the checkpoint. Thus, DNA is important not only as a scaffold for co-recruitment of sensors, but also as neighboring chromatin where it may serve to amplify the signal by retaining other checkpoint components.

Activation and maintenance of checkpoint signal requires CDK

Efficient checkpoint signaling requires CDK activity, and recent experiments have suggested that this is due to a requirement for CDK in DSB processing (Pellicioli, Lee et al. 2001; Clerici, Baldo et al. 2004; Ira, Pellicioli et al. 2004; Jazayeri, Falck et al. 2006). We found that CDK activity is required for full checkpoint signaling even when co-localization was achieved by artificially concentrating Ddc2-Mec1 and the 9-1-1 complex. While resection does play an important role in signal amplification through damage processing, we have now shown that CDK is also required during additional steps downstream of sensor recruitment.

Experimental Procedures

Plasmids and Strains

All strains are derivatives of CBY36 W303, mat a, *ddc1Δ, ade2, leu2, trp1, his3, ura3*. Plasmid pJAM150 was used to integrate the checkpoint fusion Gal-Ddc2-GFP-LacI at the *his3* locus. Plasmid pCB5 was used to integrate GalS-Ddc1-GFP-LacI at the *ura3* locus. Plasmid pCB10 was used to integrate GalS-Mec3-GFP-LacI at the *ura3* locus. Integrations were checked by PCR and Western blotting using antibodies against GFP. Endogenous *RAD53* and *RAD9* were C' terminally tagged with HA::*LEU2*. Plasmid pAFS52 was digested with EcoRV to integrate 265 LacO arrays at the *trp1* locus. Integration of correct number of arrays was verified by Southern Blot by BglII digest, probing against a LacO array specific sequence. Smaller array plasmids were integrated and verified similarly. *MEC3* and *RAD17* were deleted by gene replacement using the Kan-G418 cassette. *DDC1*, *RAD24, RAD9* and *SML1* were deleted by gene replacement using the Hygromycin resistance cassette. *MEC1* was deleted in the *sml1*::Hygromycin strain by gene replacement with the Kan-G418 cassette. Serine 129 in *HTA1* and *HTA2* were deleted and replaced with the Hygromycin resistance cassette and the KanMX-G418 cassette respectively. *DOT1* was deleted by gene replacement with the Nat resistance cassette. The *cdc28-as* allele was obtained from Dave Morgan and crossed into the required strain. The Rad52-mRFP allele was obtained from Rodney Rothstein and used to tag the endogenous Rad52.

Galactose Induction and Zeocin Treatment

Galactose induction experiments were conducted with nocodazole arrested cells. Log cycling cells were arrested with nocodazole for two hours in rich media + raffinose. Galactose was added for one hour, at the end of which dextrose was added to prevent continued induction. Checkpoint fusions were stable for up to 6 hours after induction as determined by Western blot. In the nocodazole release experiment, cells were arrested with nocodazole for two hours, induced with galactose for one hour in the presence of nocodazole, and released into rich media with dextrose and 8 ug/ml alpha factor. For, experiments performed in G1, cells were first arrested in alpha factor for two hours, and then galactose was added for two hours in the presence of alpha factor. Experiments using the *cdc28-as* allele containing strain were done in nocodazole arrested cells, and 5 uM 1-NM-PP1 was used as final concentration. For experiments performed with Zeocin, 10 ug/mL were used commonly, unless otherwise stated.

Protein Detection

Cell pellets were collected and lysed in boiling SDS Buffer for 3 minutes and loaded onto 10% SDS-PAGE for Rad53-HA detection and 8% SDS-PAGE for Rad9-HA detection and 6% for Rad9-Flag. Proteins were transferred to nitrocellulose and incubated with 16B12 anti-HA antibody or M2 anti-Flag antibody. For Sml1 detection, samples were loaded onto a 15% SDS-PAGE, and incubated with an anti-Sml1 antibody obtained from Rodney Rothstein. Cdc28 was visualized with Santa Cruz antibody cY-20. H2A

phosphorylation was assayed using a yeast phosphor-H2A antibody obtained from William Bonner.

Figure Legends

Figure 2.1. Inducible checkpoint fusions experimental design.

Figure 2.2 Ddc1-GFP-LacI protein fusion localizes to DSBs. Fluorescence microscopy of Gal-Ddc1-GFP-LacI fusion after galactose induction. Ddc1-GFP-LacI was nuclear and formed a single GFP focus when a LacO array was introduced as well as formed damaged induced foci. Ddc2-GFP-LacI was more difficult to localize unambiguously, as cytoplasmic vacuolar aggregates appeared in 23% of the cells (data not shown).

Figure 2.3 Rad9 and Rad53 are phosphorylated when Mec1 and 9-1-1 are co-localized. Strains were arrested with nocodazole and maintained arrested while galactose was added to induce fusions or the HO endonuclease as a positive control. Rad53-HA and Rad9-HA were visualized with anti-HA antibody. Checkpoint fusions were visualized with anti-GFP antibodies.

Figure 2.4 Co-localization of Ddc1 and Ddc2 at a LacO array does not cause Rad52 foci formation. Cells containing Ddc1-GFP-LacI, Ddc2-GFP-LacI, LacO array and Rad52-RFP were arrested with nocodazole and treated as follows: 100 ug/mL Zeocin for 3 hours in rich media plus dextrose, rich media plus dextrose, and rich media plus a one hour galactose pulse. Digital microscopy was used to measure the frequency of Rad52-RFP foci formation in all samples. Mre11 is not required for checkpoint activation through co-localization. Isogenic wild type and *mre11* deleted strains containing Ddc2-GFP-LacI

and Ddc1-GFP-LacI +array were arrested with nocodazole and galactose was added to induce expression.

Figure 2.5 Rad53 phosphorylation correlates with the amount of sensors co-localized. Arrays of tandem LacO repeats were introduced in the strain co-expressing the checkpoint fusions. Strains were nocodazole arrested (top panel) and galactose was added to induce fusion expression for 2.5 hours (bottom panel).

Figure 2.6. Co-Localization activates multiple checkpoint readouts. (A) Sml1 is degraded upon co-localization. A wild type strain was left untreated or was treated 100 ug/ml Zeocin for four hours. Strains expressing checkpoint fusions –array and +array were arrested with nocodazole and galactose was added to induce fusions. The Westerns were blotted against endogenous Sml1 and Rad53-HA. A s*ml1* delete strain served as control for specificity (last lane). (B, C, D) Strains containing both fusions –array (B), +array (C), and +array, *rad9* (D) were arrested in nocodazole, induced with galactose for one hour while arrested, and then released into media with alpha factor. A time course of FACS analysis is shown starting at two hours (120') into galactose induction. (E) Analysis of Rad53-HA by Western blot of samples taken from B, C, D.

Figure 2.7. 9-1-1 and Ddc2-Mec1 co-localization bypasses the requirement for Rad24. (A) Both checkpoint fusions were induced in nocodazole arrested isogenic wild type, *rad24*, *rad9* and *mec1* strains containing a LacO array. (B) Checkpoint fusions were

induced in nocodazole arrested wild type and *rad24* strains. Galactose and Zeocin were added simultaneously in the presence of nocodazole.

Figure 2.8. Ddc1 is the Mec1 activating subunit. (A) Ddc1-GFP-LacI and Ddc2-GFP-LacI checkpoint fusions were induced in nocodazole arrested isogenic wild type, *mec3, rad17* strains containing a LacO array. Strains containing Ddc1-GFP-LacI and Ddc2-GFP-LacI fusions + array that were wild type(A), *rad17Δ* (B), *mec3Δ* (C) and *rad9Δ* (D) were treated as explained in figure 2.6.

Figure 2.9 Mec3-GFP-LacI requires Ddc1 in order to activate Mec1. Mec3-GFP-LacI and Ddc2-GFP-LacI checkpoint fusions were induced in nocodazole arrested isogenic wild type and *ddc1* strains containing a LacO array. Mec3-GFP-LacI fusion is partially checkpoint proficient. Wild type and *mec3* deleted, Mec3-GFP-LacI strains were treated with galactose and Zeocin as to test for Rad53 phosphorylation.

Figure 2.10. Rad53 activation requires H2AX-P and H3K79Me. (A) Ddc1-GFP-LacI and Ddc2-GFP-LacI checkpoint fusions were induced in nocodazole arrested isogenic wild type; *htaS129; dot1* and *htaS129, dot1* strains containing a LacO array. (B) Same as in A for longer time.

Figure 2.11. CDK promotes Rad53 activation. (A, B) Strains were arrested in G1 with alpha factor and G2/M with nocodazole, galactose was added to induce checkpoint fusions. (C) Checkpoint fusions were induced in nocodazole arrested isogenic wild type and analogue (1NM-PP1) sensitive, *cdc28-as*, strains containing a LacO array. Both

strains were treated as follows: Inhibitor alone (5uM), galactose plus inhibitor, and galactose alone. (D) The strain carrying the *cdc28-as* allele, checkpoint fusions and array was arrested with nocodazole and induced with galactose. After three hours, the culture was split and inhibitor was added.
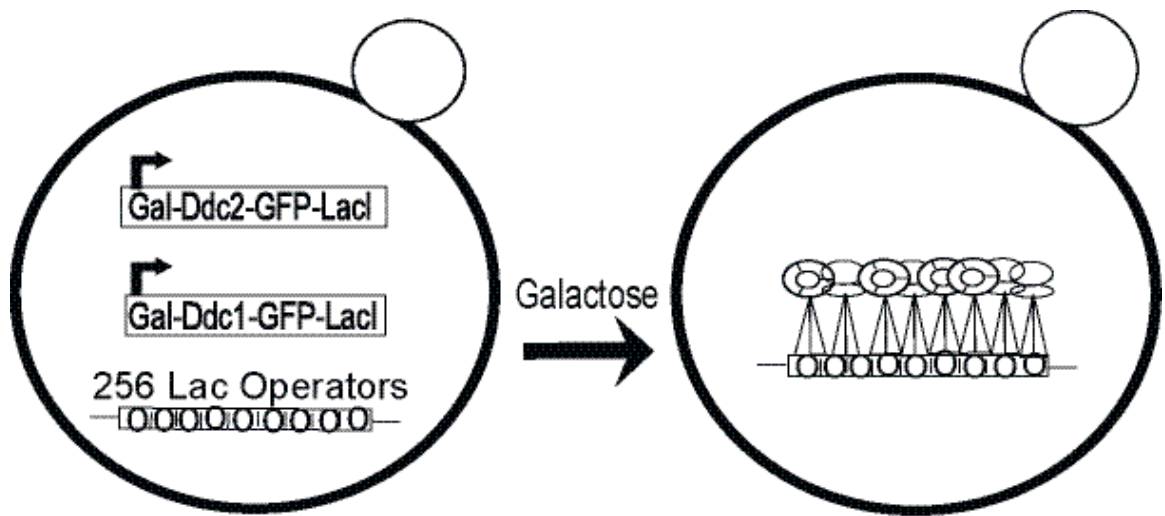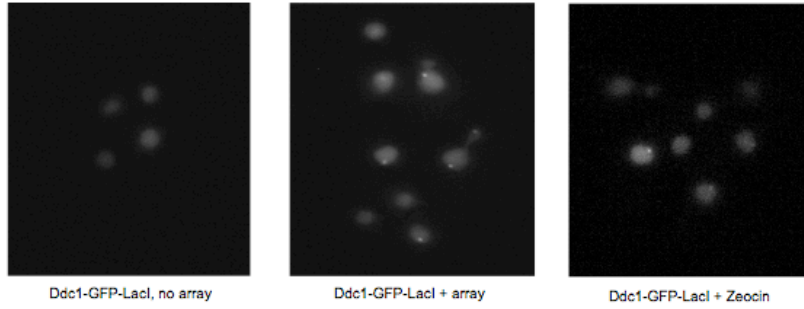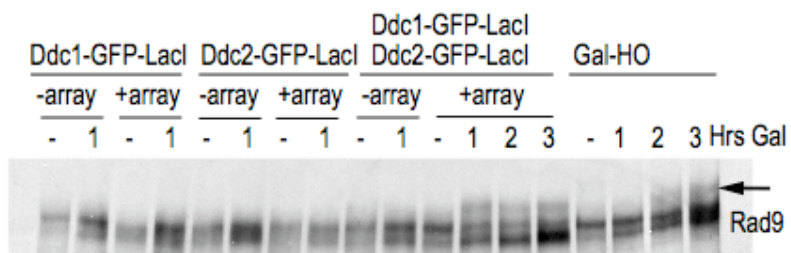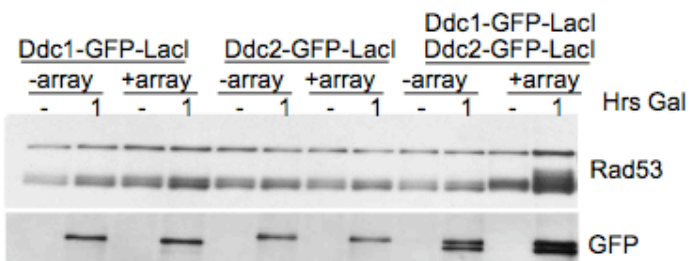
Gal-Ddc2-GFP-LacI

Gal-Ddc1-GFP-LacI

256 Lac Operators

Galactose

Figure 2.1

Figure 2.2

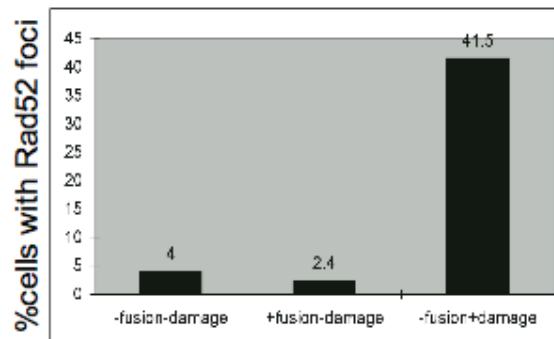Ddc1-GFP-LacI, no array   Ddc1-GFP-LacI + array   Ddc1-GFP-LacI + Zeocin
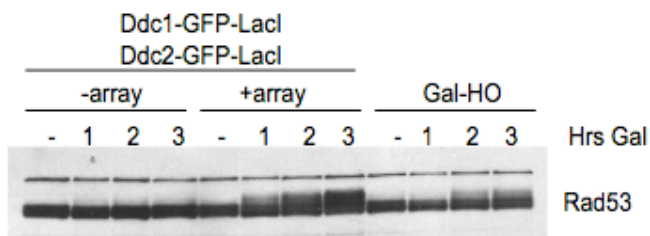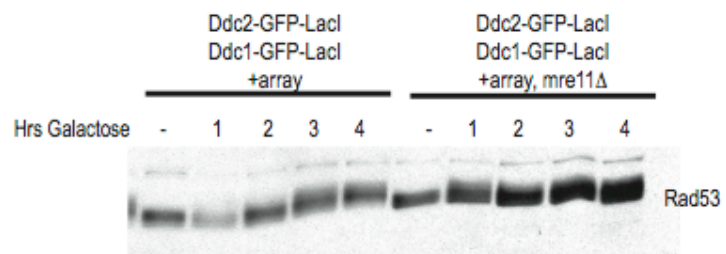
Figure 2.3

Figure 2.4

Figure 2.5

Figure 2.6

Figure 2.7

A

B

44

Figure 2.8

Ddc1-GFP-Lacl, Ddc2-GFP-Lacl +array

WT			rad17 Δ			mec3 Δ			
-	1	2	-	1	2	-	1	2	Hrs Gal

Rad53

Figure 2.9

Mec3-GFP-Lacl, Ddc2-GFP-Lacl +array

WT				ddc1Δ				
-	2	3	4	-	2	3	4	Hrs Gal

Rad53

-	-	+	-	-	+	Galactose
-	+	+	-	+	+	Zeocin

Rad53

wild type   mec3Δ
Gal-Mec3-GFP-Lacl

Figure 2.10

A WT   H2AS129   dot1 Δ   H2AS129
                            dot1Δ
   - 1 2 - 1 2 - 1 2 - 1 2   Hrs Gal

   Rad53

   P-H2A

B   WT   H2AS129, dot1Δ
   - 1 2 3 4 - 1 2 3 4   Hrs Gal

   Rad53

Figure 2.11

A

G1 | G2/M
-array +array | -array +array
Gal
− + − + | − + − +
← Rad53

B

G1 | G2/M
-array +array | -array +array
Gal
− + − + | − + − +
← Rad9

C

WT   cdc28-as

Rad53

+ + − + + −   Inhibitor
− + + − + +   Gal

D

− 1 2 3 3.5 4 4.5 5   Hrs Gal
Rad53

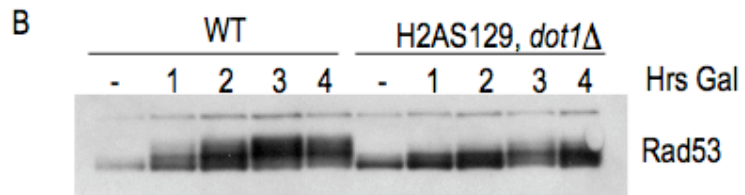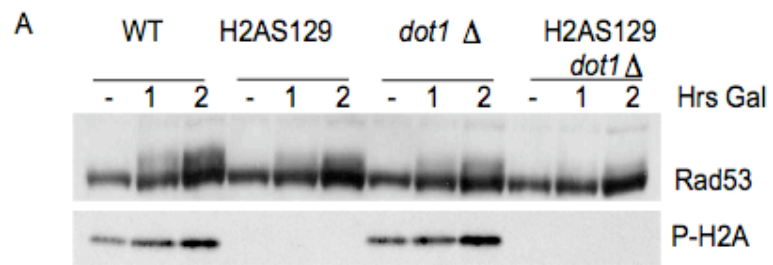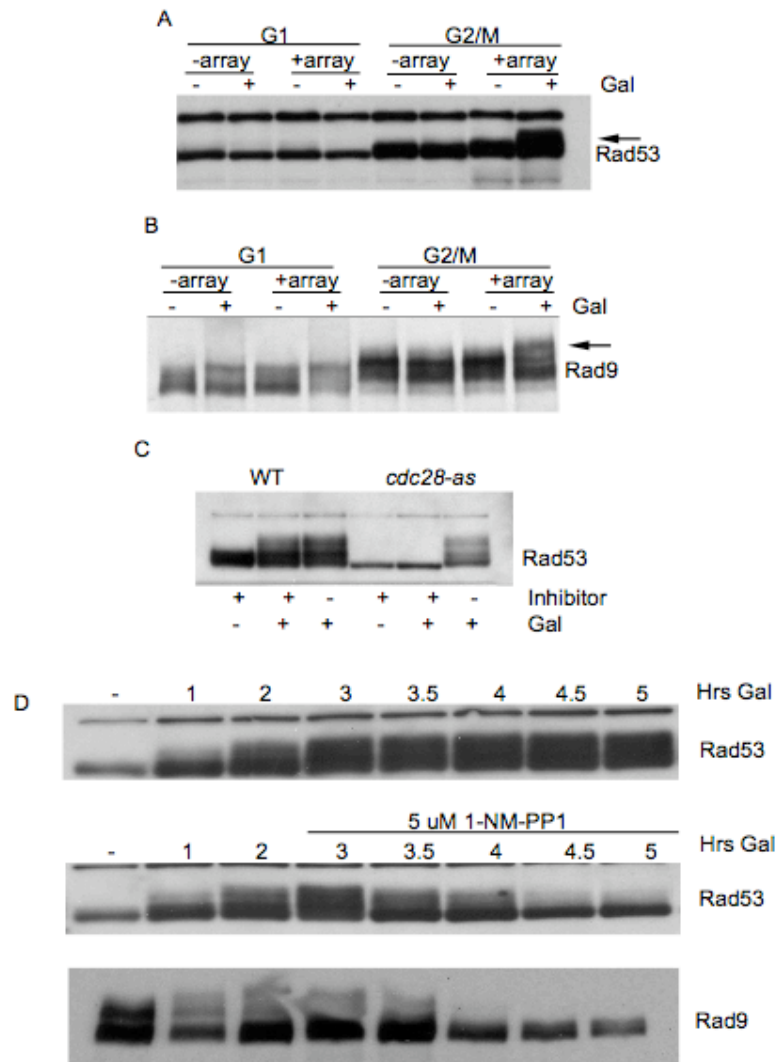5 uM 1-NM-PP1
− 1 2 3 3.5 4 4.5 5   Hrs Gal
Rad53

Rad9

CHAPTER 3

CDK regulation of the checkpoint protein Rad9

The DNA damage checkpoint impinges on CDK for full activity by requiring DNA break resection. It has been shown that the 5-3 resection that creates the ssDNA signal depends on CDK for full activity (Ira, Pellicioli et al. 2004). By limiting the amount of ssDNA that can recruit Mec1/Ddc2 and the 9-1-1 clamp, CDK can affect the strength of the signal created. This has been thought to be the reason for the lack of cell cycle arrest in cells that encounter damage in G1. With our colocalization system, we showed that there is an additional role for CDK in checkpoint activation. The stable recruitment of Mec1 and the 9-1-1 clamp to the LacO array did not yield activation of the checkpoint in cells lacking CDK, via alpha factor arrest or with direct inhibition of Cdc28. We went on to show that neither Rad53 or Rad9 could undergo the damage dependent phosphorylation in cells with low CDK despite having Mec1 and 9-1-1 co-localization.

Furthermore, Rad9 underwent rapid dephosphorylation upon CDK inactivation even when the checkpoint was strongly activated via co-localization (Figure 2.11). Given that Rad9 lays upstream of Rad53 in the checkpoint signaling cascade and that it is required for Rad53 activation, it seemed reasonable to hypothesize that Rad9 might be a target of CDK leading to inactivation of Rad53. Rad9 has been shown to be a CDK target in vivo and in vitro by multiple groups. The CDK consensus site is well characterized, therefore it was possible to identify putative CDK sites on Rad9. Sequence scanning of the Rad9 protein resulted in 20 complete and incomplete CDK sites that we went on to test for their role in the checkpoint.

Results

CDK sites are important for checkpoint function

Having shown that CDK activity was important for the DNA damage checkpoint, we next wanted to identify the CDK target responsible for this requirement. The adaptor protein Rad9 has 9 full (S/T-P-x-K/R) and 11 partial (S/T-P) CDK consensus sites and shows a cell cycle-dependent mobility shift indicative of phosphorylation (Figure 3.1). We made alanine substitutions of the N-terminal 18 sites, naming this allele *rad9-18A*. Upon initial examination, it was clear that Rad9-18A did not undergo cell cycle dependent phosphorylation compared to the wild type Rad9 when cells were nocodazole arrested or allowed to cycle (Figure 3.2). This observation suggests that we have eliminated the CDK -dependent phosphorylations on Rad9. The allele was then tested for its ability to activate the checkpoint in response to co-localization of sensors and induction of DSBs with Zeocin. As we have previously shown, co-localization of Ddc2-Mec1 and the 9-1-1 clamp at an array induced Rad53 and Rad9 phosphorylation. The Rad9-18A allele failed to undergo DNA damage-dependent phosphorylation upon co-localization of Ddc1-GFP-LacI and Ddc2-GFP-LacI (Figure 3.2). Moreover, the Rad9-18A allele did not support Rad53 phosphorylation. Similarly, when the Rad9-18A mutant was challenged with Zeocin, the *rad9-18A* strain did not show Rad53 phosphorylation (Figure 3.2). Therefore, phosphorylation of CDK consensus sites on Rad9 is important for transducing the checkpoint signal from the sensors (Ddc2-Mec1 & 9-1-1) to the effector kinase Rad53.

Mutating multiple serine and threonine residues on Rad9 could render the protein unstable, resulting in its checkpoint deficiency. However, we noticed that at long time points, slight Rad53 phosphorylation could be seen in the *rad9-18A* strain. We determined whether the CDK site requirement could be over-ridden with very high levels of damage. To test this, a range of Zeocin concentrations were used to damage wild type and *rad9-18A* strains and Rad9 and Rad53 phosphorylation was monitored (Figure 3.3). At low Zeocin concentrations, Rad9-18A and Rad53 were not phosphorylated. At higher Zeocin concentrations, most of the Rad9-18A exhibited a damage -dependent mobility shift and Rad53 qualitatively activated. Thus, the *rad9-18A* allele is activated at very high damage doses suggesting it is not misfolded.

Rad918A is sensitive to DNA damaging agents

The initial screen for checkpoint mutants was performed in conditions of DNA damage with the assumption that cells defective in DNA damage signaling or processing would not survive. A *RAD9* delete strain is sensitive to damaging agents because the checkpoint cannot be activated. We tested whether the impaired checkpoint observed in the Rad9-18A allele made the cells sensitive to DNA damaging agents. We used the genotoxic agents MMS and 4NQO to test for damage sensitivity. Ten fold serial dilutions of wild type untagged Rad9, rad9 delete, Rad9-Flag and Rad9-18A-Flag cultures were spotted on MMS and 4NQO plates. As evident by the lack of growth in the rad9 delete and the Rad918A-Flag strains in MMS plates, mutating CDK sites on Rad9 makes cells sensitive to DNA damage. The phenotype of Rad918A is intermediate, similar to the Rad53 activation observed.

Rad9 is phosphorylated in G1

Upon closer inspection of the phosphorylation state of wild type Rad9 in G1 cells, it became evident that not all the shift accredited to phosphorylation was gone, especially when compared to the Rad918A allele, which shows no shift. Rad9 isolated from cultures grown to stationary phase for 24 or 72 hours were still shifted as compared to Rad918A isolated from similarly grown cultures (Figure 3.5). I wanted to verify that the remaining shift on wild type Rad9 was due to phosphorylation; therefore, I performed a phosphatase assay on Flag-immunoprecipitated (IP) Rad9 from asynchronous and G1 arrested cells. IPed Rad9 was treated with λ phosphatase alone or with inhibitors. In both cases, asynchronous and G1, the Rad9-Flag band collapsed to a tighter band when phosphatase was added. This result confirms that even in G1 cells the Rad9 protein contains some phosphorylated residues. Rad9 contains many CDK phosphorylation sites and they may not all be dephosphorylated upon CDK inactivation in G1, perhaps allowing the cell to maintain some sensitivity to damage.

Rad918A is not phosphorylated by Mec1

We have shown that Rad53 is not phosphorylated when the cells contain a Rad9 protein mutated for its CDK phosphorylation sites; and Rad9 must be phosphorylated by Mec1/Tel1 in order to serve as an adaptor to Rad53. From immunoblotting , it seemed as if Rad918A was unable to undergo the DNA damage dependent shift usually created by Mec1/Tel1. An antibody that recognizes the specific phosphorylated S/T-Q residues

phosphorylated by Mec1/Tel1 can allow us to specifically look at the damage dependent phosphorylation of Rad9-18A. Wild type Rad9 and Rad9-18A were immunoprecipitated from Zeocin treated and untreated cells and blotted with the phospho S/T-Q antibody. As figure 3.6 shows Rad9-18A was not phosphorylated by Mec1/Tel1 as no signal is detected in those samples. Wild type Rad9 shows a strong signal when Zeocin was added.

Phosphorylation of Rad9 by Mec1 is a prerequisite for the interaction between Rad9 and Rad53 upon damage. Since the Rad9-18A allele did not show Mec1 phosphorylation in the presence of Zeocin, we hypothesize that the Rad9-Rad53 interaction would also be abolished in this mutant, explaining the lack of Rad53 activation. I used differentially tagged versions of Rad9 and Rad53 to probe at their interaction. Samples were treated with damaging agent and wild type Rad9-Flag and Rad9-18A-flag were immunoprecipitated with the Flag antibody. The presence of Rad53-HA co-immunoprecipitated with Rad9 was seen by probing against Rad53 in the IP samples (Figure 3.6). As shown in the HA blot, Rad53 levels were lower in the Rad9-18A samples as compared to wild type Rad9, even though similar amounts of Rad9 were brought down. This suggests that in the presence of damage, Rad9-18A is unable to bind Rad53. This may be due to the inability of Mec1 to phosphorylate Rad9, preventing Rad9 from performing its adaptor role.

Rad9 Oligomer functions in the Rad918A allele

As an initial read out of checkpoint function, we look at Rad53 phosphorylation in response to DNA damage. Yet, we want to understand where the CDK phosphorylation of Rad9 may be regulating the checkpoint. We showed that Mec1 is unable to phosphorylate Rad9 when the CDK sites are mutated (figure 3.6). This inability to phosphorylate Rad9 could be explained by a number of ways: first Rad9 may be unfolded and unable to be recognized by Mec1; second, Rad9 may not be recruited to break sites efficiently; third, CDK phosphorylation may aid Mec1 in recognizing Rad9.

I aimed to test the first scenario, improper folding, by probing the un-damage state of Rad9 as a proxy for its folded state. Rad9 is thought to exist in a higher order structure bound to its chaperons Ssa1 and Ssa2 (Gilbert, Green et al. 2001). I was unable to definitively answer whether the Rad918A allele was stably bound to its chaperones. Therefore, I decided to test the ability of the Rad918A allele to bind other wild type Rad9 proteins. I created a strain that contained two differently tagged versions of Rad9; a Flag tagged version and a Myc tagged version. In a strain containing wild type versions of both tags, Rad9-flag and Rad9-myc, I was able to co-immunoprecipitate (Co-IP) Rad9-flag with Rad9-Myc in the absence of damage (Figure 3.7). This result shows that Rad9 exists in a structure containing at least two Rad9 proteins, perhaps more. When the Rad918A-flag allele was tested for its ability to Co-IP with wild type Rad9, it showed that it was capable of binding wild type Rad9-Myc with equal affinity as its wild type counter part (Figure 3.7). Since the known functions of Rad9 are in the DNA damage

checkpoint and the 18A allele is impaired for checkpoint functions, we sought a DNA damage-independent Rad9 interaction to test the allele of functionality. The oligomer state of Rad9 proved to be a way to show that Rad918A was properly folded to form Rad9 oligomers. It still remains to be shown that Rad918A can oligomerize with other Rad18A alleles.

Discussion

CDK is an important regulator of cell cycle progression. Therefore, identifying a role for CDK in regulating the DNA damage checkpoint was no surprise. We have shown that CDK plays an important role in the direct regulation of a checkpoint adaptor protein. Rad9 contains 9 full and 11 partial consensus CDK sites, more than any other protein in the yeast genome, and is phosphorylated by CDK in vitro (Ubersax, Woodbury et al. 2003). Rad9 is phosphorylated in the absence of damage in G2/M, when CDK activity is high. If Rad9 phosphorylation by CDK promotes Rad9's activity as an adaptor, eliminating the CDK phosphorylation could impair its checkpoint function. By mutating 18 putative CDK sites on Rad9, we eliminated its cell cycle-dependent electrophoretic shift and Rad9's ability to activate Rad53, suggesting that CDK also plays a role in controlling Rad9 as an adaptor of the DNA damage checkpoint. Importantly, a residual electrophoretic shift in Rad9 was often observed in G1 arrested. This, and the fact that the Rad9-18A defect can be over-ridden by high levels of damage, may explain the variation seen in the literature for the role of CDK in checkpoint signaling. The residual Rad9 damage-independent phosphorylation could be due to these sites being shielded from phosphatases, or to their phosphorylation being mediated by an alternative proline-directed kinase.

Rad9 activation involves multiple steps that include recruitment of Rad9 to a damage site, change in its oligomerization state, phosphorylation by Mec1/Tel1, and binding of Rad53 to allow for Rad53's activation (Emili 1998; Gilbert, Green et al. 2001; Schwartz, Duong et al. 2002). Phosphorylation by CDK could be important for one or

more of these steps. The Rad9-18A allele described in this manuscript will allow us to probe into the role of CDK in the checkpoint signaling cascade through a specific protein and avoid additional CDK effects. Preliminary work showed that the Rad918A allele was not phosphorylated by Mec1 and bound Rad53 inefficiently. This may be the result of a failure in Rad9 recruitment to the site of damage that would never bring Rad9 to the kinase Mec1. Alternatively, Rad9 may be recruited properly, yet it is not recognized by Mec1 and therefore not phosphorylated.

Cells that encounter damage in G1 delay progression into S-phase for only 20-30 minutes. This delay is significantly shorter than the checkpoint mediated delay in G2/M, which lasts up to 8 hours. The differences between the two checkpoint responses in *S. cerevisiae* is consistent with the fact that cells do not require a strong checkpoint response in G1. The difference may reflect the fact that a wider range of repair options are available in G2/M, such as sister chromatid exchange. Previous experiments have suggested that the mechanisms of checkpoint activation are different in G1. The requirement for both H2AX phosphorylation and H3K79 methylation for checkpoint activation is made stronger in G1 (Wysocki, Javaheri et al. 2005; Javaheri, Wysocki et al. 2006; Hammet, Magill et al. 2007). CDK phosphorylations on Rad9 may help promote Rad9 localization such that H2AX phosphorylation and H3K79 methylation are more important for the activation of Rad9 in G1, when Rad9 would have low levels of CDK phosphorylation. Similarly, Crb2, a *S. pombe* BRCT containing checkpoint protein similar to Rad9, has also been shown to undergo CDK phosphorylation (Esashi and Yanagida 1999; Caspari, Murray et al. 2002; Du, Nakamura et al. 2006). Initial reports

suggested a role for this phorphorylation in checkpoint adaptation and repair (Esashi and

Yanagida 1999; Caspari, Murray et al. 2002). More recently, it has been suggested that

this phosphorylation has a redundant role in Crb2 localization (Du, Nakamura et al.

2006).

Experimental Procedures

Plasmids and Strains

All strains are derivatives of strain PGY1834 W303, *mat a, rad9::Hygro, ade2, leu2, trp1, his3, ura3, Rad53-HA::Trp1.* Plasmids pCB18 and pCB19 were used to integrate Rad9-flag and Rad9-18A-flag respectively at the *RAD9* locus under its endogenous promoter with the KanMX marker.

Zeocin treatment and Cell Cycle Experiments. Experiments with Zeocin and were performed as stated in Chapter 2 experimental procedures. Cell cycle position experiments were performed as previously stated in Chapter 2.

Phosphatase assay

Colleted 50 ODs of cells, resuspend in Hepes Buffer (100mM Hepes, pH7.5, 100 mM NaCl, 0.2% Triton X, PMSF, phosphatase inhibitors, protease inhibitors. Beadbeat pellet in 250 ul buffer, remove 10% for input. Add 25 ul washed beads+Flag antibody and incubate for 2 hours at 4 degrees. Wash 4X, and wash 2X with phosphates buffer to remove inhibitors. Resuspend IPs in 100 ul Phosphatase buffer (50 mM Tris-HCl pH7.8, 5mM DDT, 1mg BSA/mL, 1ug Leupeptin/mL, 1ug pepstatin A/mL, 0.10 TIU aprotinin/mL, 1mM PMSF). Split IP in three tubes and add 2 mM MnCl2 or 2 mM MnCl2 plus 100 U Lambda phosphatase, or phosphatase plus 2 mM ZnCl2, 50 mM NaF, 1mM Sodium Vanadate. Incubate for 1 hr at 30 degrees, wash three times with Hepes buffer and run on gel.

Plate sensitivity assay. Poured plates containing 0.006%MMS and 4NQO at 1:50,000

dilution of 4mg/mL. Made ten fold serial dilutions of overnight cultures from 1:10 to

1:100000 and used pinning tool to plate dilutions on MMS, 4NQO, and Dextrose plates.

Incubate at 30 degrees for two days.

Figure Legends

Figure 3.1 Rad9 CDK site mutant is checkpoint deficient. Isogenic strains carrying wild type Rad9-Flag or Rad9-18A-Flag were alpha factor arrested, nodocazole arrested, allowed to cycle or treated with *cdc28-as* inhibitor 1NM-PP1.

Figure 3.2 Rad9 CDK mutant is defective in the DNA damage checkpoint. Ddc1-GFP-LacI and Ddc2-GFP-LacI checkpoint fusions were induced in nocodazole arrested Rad9-Flag and Rad9-18A-Flag strains. Zeocin was added to Rad9-Flag, Rad9-18A-Flag, and *rad9Δ* strains.

Figure 3.3 Rad918A has some checkpoint activity. Rad9-Flag, Rad9-18A-Flag, and *rad9Δ* cycling strains were left untreated or treated with Zeocin at 10ug/mL, 20ug/mL and 200 ug/mL final concentrations for two hours.

Figure 3.4 Rad918A makes cells sensitive to DNA damaging agents. Ten fold serial dilution of Rad9, rad9 delete, Rad9-Flag and Rad918A-Flag strains were plated on .006%MMS plates and 1:50,000 dilution of 4NQO plates and grown at 30 degrees for two days.

Figure 3.5 Rad9 remains phosphorylated when CDK levels are low. Wild type Rad9 and Rad918A cultures were grown to saturation for 24 and 72 hours at 30 degrees in rich media. Samples were collected at those timepoints along with an asynchronous sample and immunoblotted for Rad9-Flag. Wild type Rad9 was immunoprecipitated from

cycling and G1 arrested cultures using the Flag antibodies conjugated to beads and treated with λ phosphatase +/- inhibitor.

Figure 3.6 Rad918A is not phosphorylated by Mec1. Wild type Rad9 and Rad918A were immunoprecipitated and blotted against the Mec1 phosphorylated using the S/TQ antibody. The presence of Rad53-HA co-immunoprecipitaed with Rad9-Flag was tested in the absence and presence of damage with an anti-Flag IP.

Figure 3.7 Rad18A can bind wild type Rad9. Strains containing Rad9-Myc, Rad9-Flag and Rad9-Myc, Rad9-18A-Flag were grown asynchronously. Beads containing anti-Flag antibodies were used to IP Rad9-Flag and Rad9-18A-Flag. Samples were blotted against Flag and Myc, run on a 6% SDS-PAGE.
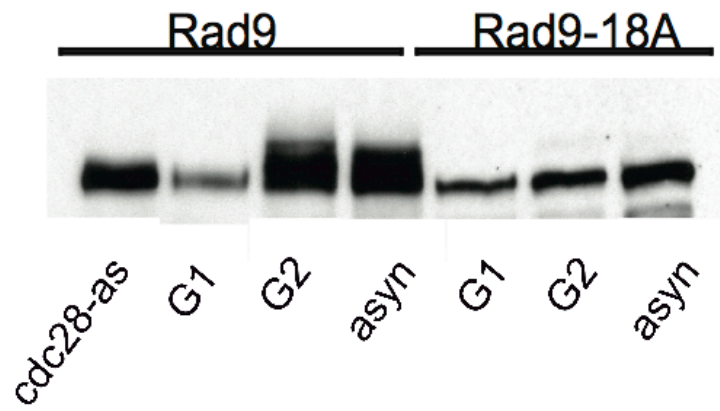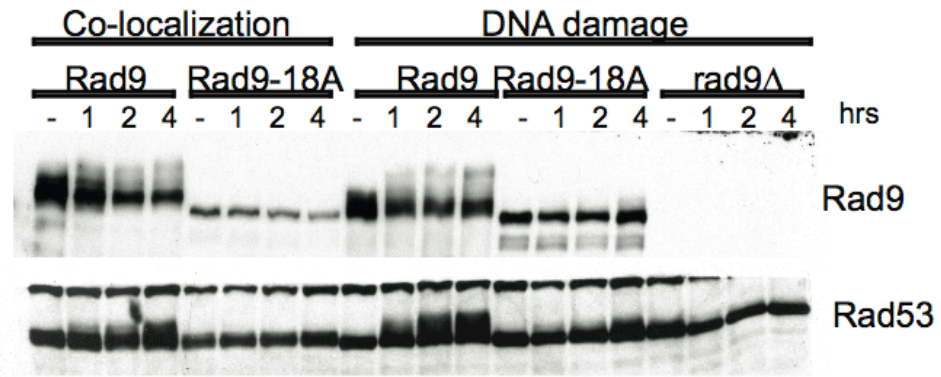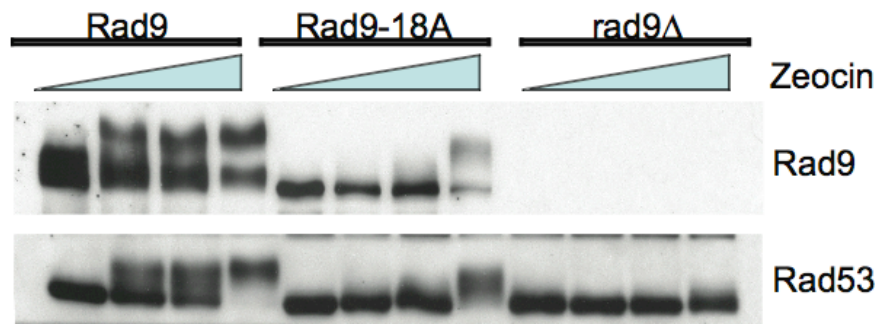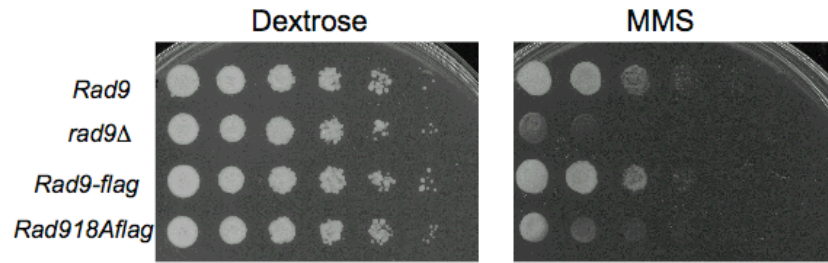
Figure 3.1

Figure 3.2

Figure 3.3

Dextrose　　　　　MMS

Rad9
rad9Δ
Rad9-flag
Rad918Aflag

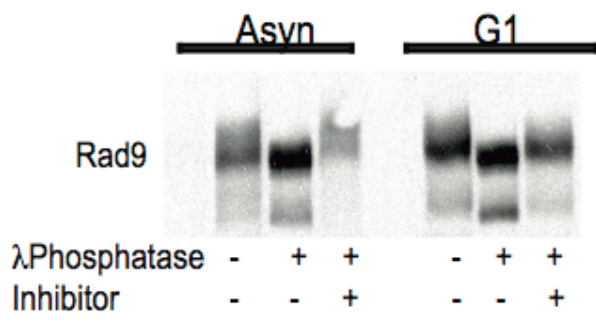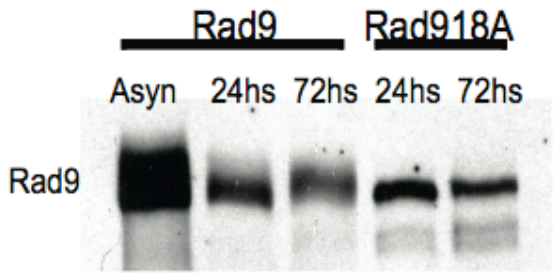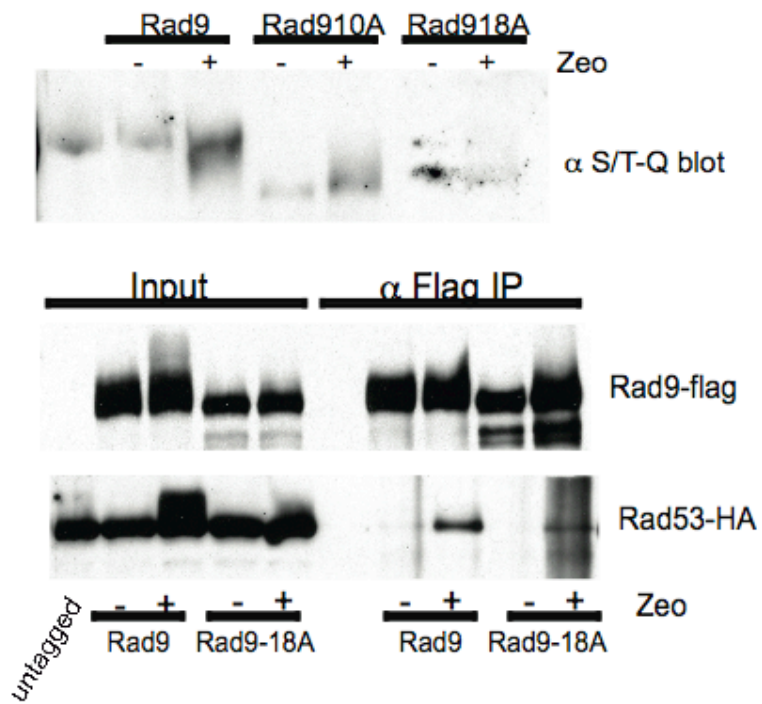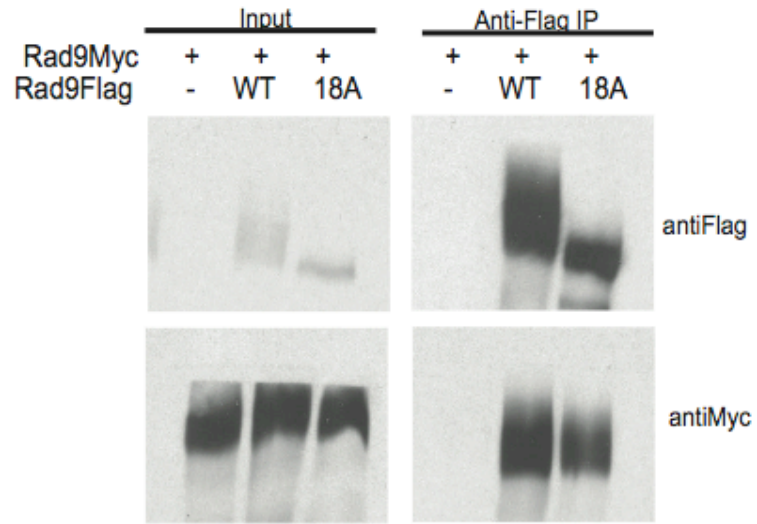Similar result with 4NQO

Figure 3.4

Figure 3.5

Figure 3.6

Figure 3.7

Conclusion and Future Directions

Progression through the cell cycle is a highly regulated process and any mechanism that aims to regulate that progression, such as the DNA damage checkpoint, should be tightly regulated itself. The proper activation of the checkpoint pathway can be regulated in different ways, temporal recruitment of proteins, allosteric changes in proteins imposed by other regulatory proteins, or the DNA break itself can be an activator. How the cell activates the checkpoint to halt the cell cycle has been the subject of research and we know that protein complexes are specifically recruited to break sites. In this work, we showed that the DNA break is not required to initiate the checkpoint when the ATR homologue, Mec1 and the 9-1-1 clamp are recruited to chromatin. Recent work by Misteli's group showed that ATM is also capable of being activated in the absence of DNA damage (Soutoglou and Misteli 2008). These results suggest that the DNA damage checkpoint is subject to regulation through the simultaneous increased concentration of signaling molecules.

CDK and the DNA damage checkpoint

Circumventing the DNA break allowed us to test different functions of checkpoint components, i.e. the need for CDK in the absence of resection, and identified a previously uncharacterized regulation of the checkpoint adaptor, Rad9. More work needs to be done to understand how and why CDK might regulate this checkpoint protein. Rad9's role in Rad53 activation is crucial and composed of many steps. Rad9 is phosphorylated by the

sensor kinases, it undergoes an oligomerization state change, it is localized to the break and lastly binds Rad53. Phosphorylation by CDK may affect one of more of these steps. I began to look at this and showed that phosphorylation by Mec1 was affected by mutating the CDK phosphorylation sites on Rad9. Looking upstream of Mec1 phosphorylation will help in identifying the exact steps CDK may control. The phosphomimic version of a protein has been a useful tool to look at when misregulation of a protein happens. For example, constitutive CDK phosphorylation of Rad9 might have an effect on the checkpoint if expressed during times when the phosphorylation does not happen. I made a version of Rad9 that contains phosphomimic residues at 18 of the 20 postulated CDK sites. This allele is slightly hypomorphic. The checkpoint defective phenotype observed in the Rad9-18A allele might also be due to misfolding due to multiple mutations. Yet, I showed that it is folded sufficiently well to form dimers. The mutations to create the phosphomimic Rad9 allele may disturb the structure more than Ala substitutions making the protein even more unstable. Therefore it'll be useful to reduce the number of mutations before testing its ability to imitate a fully phosphorylated Rad9.

We consistently observed residual phosphorylation on Rad9 in G1, stationary and CDK-inhibited cells. Yet, the Rad9-18A allele seems devoid of any phosphorylation. It is puzzling that Rad9 may retain some phosphorylation even when CDK activity is low and therefore Rad9 from G1 cells may maintain some checkpoint function as compared to the Rad9-18A allele. Those phosphorylations may be due to kinases active in G1. I tested the role of one such kinase, Pho85. I looked at the phosphorylation state of Rad9 in G1 arrested cells deleted for Pho85 and saw no change in its phosphorylation (data not

shown) suggesting Pho85 is not the responsible kinase. A more likely scenario is that some CDK phosphorylations persist on Rad9. To test this hypothesis, I overexpressed one known CDK phosphatase, Cdc14. Overexpression of Cdc14 from the Gal promoter yielded a marked dephosphorylation of Rad9 (data not shown). It would be interesting to test whether Cdc14 can mimic a situation when Rad9 is devoid of phosphorylations.

Activation of the checkpoint and repair

Work on DNA repair has taught us that the cell has many ways to deal with the different kinds of exogenous and endogenous damage it can receive. There are pathways to deal with gaps on the double stranded DNA and perform blunt end ligations. One of the most complex ways to repair damage is by homologous recombination, which requires strand invasion of an intact double strand. It is thought that the processing by repair proteins may aid in signaling to the DNA damage checkpoint. Additionally, repair proteins are recruited through checkpoint proteins, such as the 9-1-1 clamp (Parrilla-Castellar, Arlander et al. 2004). This co-localization system can be used to ask whether repair proteins are recruited to damage by the mere presence of the sensors; or is the DNA structure the signal that recruits them. I have shown that the homologous recombination protein Rad52 is not recruited to the site of Mec1 and 9-1-1 clamp co-recruitment in the absence of a DNA break. Yet, Rad52 recruitment is not the initial step in homologous recombination, therefore there may be other earlier proteins that could be recruited. Tagging a repair protein with a different fluorescent tag, such as mRFP, can tell us whether it co-localizes to the array when checkpoint fusions are expressed.

The checkpoint activation system we developed allowed us to separate ssDNA recognition functions from protein recruitment functions. We hypothesize that some checkpoint or repair proteins may be recruited to the site of sensor localization by H2A phosphorylation. One such case could be the MRX complex, which could be recruited through Mec1 and not ssDNA as the current models suggest. I have attempted to tag the repair protein Mre11 with the mCherry fluorescent tag, but was unable to visualize it under the microscope. Mre11 belongs to the complex that binds dsDNA breaks before they are recessed. The recruitment of Mre11 at the array would suggest a feedback mechanism where Mec1 could recruit Mre11 perhaps through phospho-H2A. As an indirect read out of Mre11 recruitment one could look at the phosphorylation state of Mre11 since it is phosphorylated upon checkpoint activation. The Mre11 phosphorylation shift is well documented and I have shown that Mre11 is phosphorylated in the presence of damage using an endogenous antibody to Mre11 (data not shown). Immunoblotting for Mre11 in the co-localization strain might shed light into this question.

Dpb11 and the DNA damage checkpoint

As explained in the introduction, the replication protein TopBP1 has been shown to have ATR activating functions. The yeast orthologue of this protein, Dpb11, may also function to activate Mec1, yet there is not much evidence to prove it. One recent paper by Puddu showed that Dpb11 may act in the checkpoint by recruiting Rad9. This may be a different function as the one showed in the Xenopus and mammalian systems with

TopBP1, where it can directly activate ATR. We can test whether Dpb11 can activate Mec1 by co-localizing these proteins at the array. We know that the 9-1-1 clamp must be localized with Mec1 in order to get checkpoint activation. The reason for this activation might be the recruitment of Dpb11 by the 9-1-1 clamp as shown in vertebrates. By making a Dpb11-GFP-LacI fusion we may be able to bypass the 9-1-1 clamp and show that in at least some cases Dpb11 can activate Mec1 in vivo.

There may be a differential requirement for Mec1 activation, that is to say depending on the conditions Dpb11 and Ddc1 could activate Mec1 direclty. If such differences are due to the DNA substrate, we could not differentiate between them with our system. However, we could show that in the absence of the other, either Ddc1 of Dpb11 can activate Mec1 at an array.

Mec1-GFP-LacI and Tel1-GFP-LacI

Mec1/Ddc2 is a constitutive complex that makes the recruitment of Mec1 to ssDNA immediate through Ddc2. Recently, Ddc2 and its homologue ATRIP have been implicated in the recruitment of Dpb11 and TopBP1, aiding in Mec1 and ATR activation respectively (Mordes, Glick et al. 2008). The authors suggest a model in which the formation of such tertiary complex is required for activation of the Mec1 kinase activity. We can directly test if such complex is required for Mec1 activation or if Ddc2 simply serves to stabilize the Dpb11-Mec1 interaction. One can make a fusion by tagging Mec1

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at the N terminus with GFP-LacI. Expressing this fusion in an array containing strain should bypass the requirement for Ddc2, if recruitment is Ddc2's only role. We could test whether co-localization of Mec1-GFP-LacI and Ddc1-GFP-LacI leads to Rad53 phosphorylation in a wild type and *ddc2* deleted strain.

Similar to the experiments outlined in the previous section, we could co-localize Mec1-GFP-LacI with Dpb11-GFP-LacI and ask whether they are sufficient to get Rad9 and Rad53 phosphorylation. These experiments could be done in the absence of Ddc2 and the 9-1-1 clamp. Another advantage of using a Mec1-GFP-LacI fusion that does not require Ddc2 for function would be the ability to test activation in S phase. Currently, Ddc2-GFP-LacI overexpression in S phase cells leads to checkpoint activation due to its binding to the ssDNA formed during the process of replication. The Mec1-GFP-LacI fusion should not be recruited to this substrate and therefore more thorough cell cycle experiments could be performed. It would also allow us to ask questions about cross talk between the replication checkpoint and the DNA damage checkpoint.

The sensor kinase Tel1 has separate roles from Mec1. Its recruitment and activation is thought to happen through the MRX complex to the blunt end of double strand breaks. We could use this fusion system to test whether an MRX component can recruit Tel1 to non-break sites and whether Tel1 is activated. The Xrs2 protein would be the best candidate for a GFP-LacI fusion since its homologue, Nbs1, is responsible for activating ATM. Misteli's work in mammalian cells would argue that recruitment of Xrs2 should be sufficient to recruit Tel1 and activate the checkpoint. While the initial

result would be proof of principal, once both sensors can be activated in the absence of damage, we could ask whether co-recruitment and activation of them leads to a stronger checkpoint. Is one kinase more active than the other, do they act synergistically or do they antagonize each other's function?

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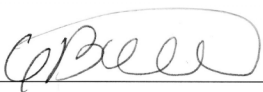
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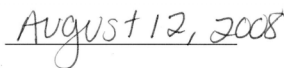
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