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Methylation of Histone H4 Lysine 20 Controls Recruitment of Crb2 to Sites of DNA Damage

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Summary

Histone lysine methylation is a key regulator of gene expression and heterochromatin function, but little is known as to how this modification impinges on other chromatin activities. Here we demonstrate that a previously uncharacterized SET domain protein, Set9, is responsible for H4-K20 methylation in the fission yeast *Schizosaccharomyces pombe*. Surprisingly, H4-K20 methylation does not have any apparent role in the regulation of gene expression or heterochromatin function. Rather, we find the modification has a role in DNA damage response. Loss of Set9 activity or mutation of H4-K20 markedly impairs cell survival after genotoxic challenge and compromises the ability of cells to maintain checkpoint mediated cell cycle arrest. Genetic experiments link Set9 to Crb2, a homolog of the mammalian checkpoint protein 53BP1, and the enzyme is required for Crb2 localization to sites of DNA damage. These results argue that H4-K20 methylation functions as a "histone mark" required for the recruitment of the checkpoint protein Crb2.

Introduction

The nucleosome is central to the faithful packaging and transmission of the genetic information contained within the nucleus of a eukaryotic cell. Composed of a repeating unit of 147 base pairs of DNA wrapped around a core histone octamer, the nucleosome forms the foundation of chromatin (Luger et al., 1997). It has been proposed that distinct histone posttranslational modifications act sequentially or in combination to form a "histone code" within chromatin (Strahl and Allis, 2000). This code is thought to serve as an epigenetic mechanism to extend the information potential stored within DNA.

Methylation of specific histone lysine residues can serve as either a mark of active euchromatin or silent heterochromatin (Lachner et al., 2003; Sims et al., 2003). Methylation of H3-K4, H3-K36, and H3-K79 has been linked with transcription activation and protection of euchromatin. Methylated H3-K9, H3-K27, and H4-K20 are generally associated with gene repression and heterochromatin function. A number of histone lysine methyltransferases (HKMTs) have been identified and characterized as important regulators of chromatin structure and function (Schotta et al., 2004; Sims et al., 2003). Based upon primary sequence homology, many candidate HKMTs are still present within the genomes of a variety of eukaryotes (Schotta et al., 2004). Understanding how these potential enzymes regulate chromatin function is of high interest, since little is known as to how HKMTs might contribute to nuclear processes outside of gene regulation and heterochromatin function. It seems likely that histone lysine methylation will play a key role in other chromatin-templated activities.

After DNA damage, eukaryotic cells orchestrate a complex array of responses (Hoeijmakers, 2001). The coordination of checkpoint-mediated cell cycle arrest and the repair of damaged DNA are essential for cell survival. Histone modification by phosphorylation, acetylation, or deacetylation have all been linked to the repair of damaged DNA (Fernandez-Capetillo and Nussenzweig, 2004). Phosphorylation of histone H2A in fission yeast (referred to as H2AX phosphorylation) or the H2A variant γH2AX in mammals also has a role in checkpoint control (Fernandez-Capetillo et al., 2004; Nakamura et al., 2004). How histone lysine methylation may participate in either the repair process or checkpoint control is unclear.

The fission yeast *Schizosaccharomyces pombe* offers an excellent model organism for the study of chromatin biology. Many aspects of fission yeast chromatin structure and function, such as gene silencing and centromeric function (Allshire, 2003), more closely resemble those of metazoans than of the budding yeast *Saccharomyces cerevisiae*. Because of this, we have sought to investigate the possibility of unknown fission yeast HKMTs. We show that a previously uncharacterized SET domain protein, Set9, is responsible for histone H4-K20 methylation in fission yeast. Surprisingly, H4-K20 methylation has no apparent role in regulating gene expression or heterochromatin function. Rather, we find the modification has a role in DNA damage response. Loss of Set9 function or the H4-K20 residue impairs cell survival after genotoxic challenge and compromises the ability of cells to maintain checkpoint-mediated cell cycle arrest. Set9 is functionally linked to the checkpoint protein Crb2 and is required for its localization to double-strand breaks (DSBs). These results provide a novel connection between histone lysine methylation and checkpoint-mediated genome stability.

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Table 1. Fission Yeast SET Domain Proteins and Related Factors

	Most Related		
S. pombe	*S. cerevisiae*	Human	Substrate
Set1	Set1	Set1 family	H3-K4
Set2	Set2	Set2 family	H3-K36[a]
Clr4	–	SUV family	H3-K9[b]
Set3	Set3/4	–	unknown
Set5	–	–	unknown
Set6	–	HSKM-B	unknown
Set7	–	–	unknown
Set8	–	–	unknown
Set9	–	Suv4-h20 family	H4-K20[b]
SPCC1223.04c	YDR198c	C21orf18	unknown
SPBC16C6.01c	–	FLJ21148	unknown

Blast search analysis was performed using protein sequence from *Schizosaccharomyces pombe* SET domain proteins, as defined by the SMART database (left column). The most related proteins identified (E value cut off of 10^{-5}) from *Saccharomyces cerevisiae* and humans and known substrates are listed across the rows. The naming of SET domain families is defined as previously published (Kouzarides, 2002; Schotta et al., 2004).

[a] Some human SET2 family enzymes also methylate H4-K20.

[b] Denotes residues not methylated in *S. cerevisiae*.

Results

Fission Yeast H4-K20 Methylation Is Set9 Dependent

To assess the function of potential HKMTs, we utilized a candidate approach to identify novel fission yeast enzymes. Except for the Dot1-related enzymes, all known HKMTs contain a catalytic SET domain (Sims et al., 2003). A search of the SMART database (http://smart.embl-heidelberg.de/) revealed eleven SET domain proteins present in *S. pombe* (Table 1). Nine are of uncharacterized function, while three, Set1, Set2, and Clr4, are known to methylate H3-K4, H3-K36, and H3-K9, respectively (Sims et al. [2003] and our unpublished data). Like Clr4, three of these potential enzymes are related to proteins uniquely present in metazoans but not in budding yeast. Immunoprecipitated TAP-tagged Set9 was found to exhibit a methyltransferase activity toward nucleosomal but not free histone H4 (Figure 1A and data not shown). Although reproducible, the Set9-associated activity was relatively modest, and it has not been possible to characterize its specificity in vitro. Regardless, this initial observation identifies a novel fission yeast histone H4 methyltransferase activity.

When we first identified the Set9 methyltransferase activity, the only methylated lysine residue described in histone H4 was methyl-lysine 20. This suggested Set9 to be a H4-K20 HKMT. Because its methylation status was unknown, we first asked whether or not fission yeast H4-K20 was methylated, and Figure 1B indicates the modification is indeed present. Immunoblotting with antibodies specific for the mono-, di-, or trimethylated H4-K20 residue revealed that native but not recombinant fission yeast histone H4 was specifically recognized by each antibody (Figure 1B, left panel). To insure antibody specificity, we asked if detected signals were dependent upon the H4-K20 epitope. Strains were generated in which two of the three histone *h4* genes were deleted and only a single wild-type (wt) (*h4.2*) or mutant *h4.2K20R* gene remained. Loss of the H4-K20 epitope completely abrogated each signal detected by the methyl-specific antibodies (Figure 1B, right panel). Immunostaining also revealed a similar loss of trimethylated H4-K20 in *h4.2K20R* cells (see Supplemental Data at http://www.cell.com/cgi/content/full/119/5/603/DC1/). Peptide competition experiments further verified the specificity of each antibody for the mono-, di-, or trimethylated H4-K20 residue (Figure 1C). Together, these results clearly demonstrate that histone H4 from *Schizosaccharomyces pombe* can be mono-, di-, or trimethylated at lysine 20.

If Set9 is a H4-K20 methyltransferase, we would expect that loss of the enzyme or its catalytic activity should abolish the modification in fission yeast cells. Like other HKMTs, the gene encoding Set9 was found to be nonessential for cell viability (data not shown). Using the *set9*Δ strain, we asked if H4-K20 methylation was dependent upon Set9 protein. Figure 1D shows that the modification is specifically Set9 dependent, as loss of Set9 but not Set1, Set2, Set6, or Clr4 resulted in essentially undetectable mono-, di-, and trimethylated H4-K20. The low amount of monomethyl signal detected in *set9*Δ cells most likely reflects the modest crossreactivity with unmodified H4 observed in Figure 1B. A similar loss of trimethyl H4-K20 was observed after immunostaining *set9*Δ cells (see Supplemental Data on the *Cell* web site). Set9 catalytic activity is also required for H4-K20 methylation. Expression of exogenous wt HA-tagged Set9 but not Set9Y220A was able to rescue H4-K20 methylation in *set9*Δ cells (Figure 1E). Y220 of Set9 is absolutely conserved in all known SET domain HKMTs and corresponds to Y335 of human Set7/9, a residue essential for catalytic activity (Marmorstein, 2003; Xiao et al., 2003). Mutation of the endogenous gene to create a *set9Y220A* allele similarly abolished H4-K20 methylation (Figure 1F). Together, these results clearly indicate that Set9 function is required for methylation of H4-K20 in *Schizosaccharomyces pombe*. Although we have been unable to demonstrate that Set9 directly methylates H4-K20, such seems extremely likely, given the data presented and the recent demonstration that the Set9-related Suv4-20h1/2 proteins do indeed directly methylate H4-K20 (Schotta et al., 2004).

Loss of H4-K20 Methylation Does Not Impair Gene Expression or Heterochromatin Function

Histone lysine methylation is known to be involved in transcriptional regulation and heterochromatin formation. We next turned our attention to understanding how Set9 and H4-K20 methylation might regulate these processes. To determine the influence of H4-K20 methylation on transcriptional regulation, DNA microarrays were employed to characterize alterations in mRNA gene expression in *set9*Δ versus wt cells. Surprisingly, of 4641 genes scored in two of two experiments, 99% displayed a change (either up or down) of $<30\%$ (see Supplemental Data). The expression of only one gene was altered >2-fold, a change considered significant. We further found that Set9 was not required for the induction of the glucose-repressed *fbp1*$^+$ and *inv1*$^+$ genes (data not shown). Thus it seems Set9 and H4-K20 methylation either do not significantly contribute to the regulation

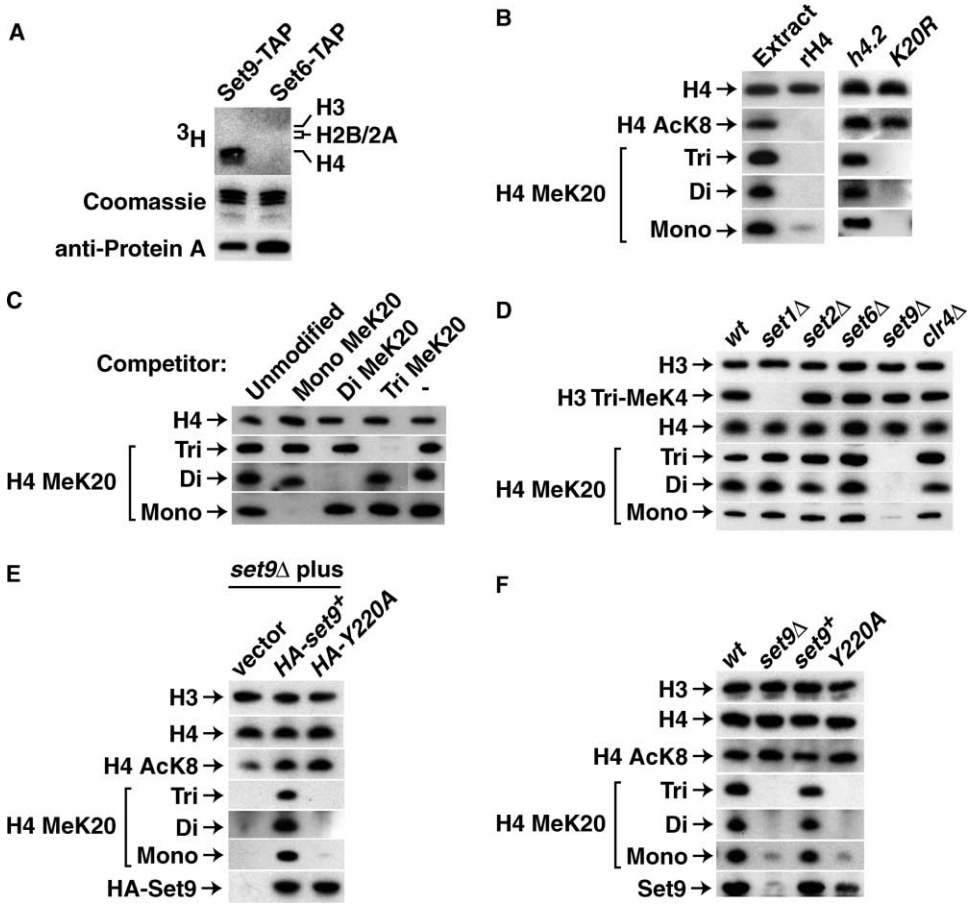

Figure 1. Methylation of Fission Yeast H4-K20 Is Set9 Dependent

(A) Set9 is associated with histone H4 methyltransferase activity. Protein A purifications were performed with extracts from strains in which the native allele for the indicated protein (top) were TAP tagged. Bound proteins were used for histone methyltransferase assays with chicken polynucleosomes. A portion of each reaction was subjected to SDS-PAGE and either coomassie staining to visualize histones (labeled right), autoradiography to detect ^{3}H histones, or immunoblotting to detect tagged proteins (as indicated left).

(B) Detection of methylated H4-K20. (Left panel) Whole-cell extracts derived from wt fission yeast cells (Extract) or *E. coli* cells expressing recombinant (r) fission yeast histone H4 or (right panel) extracts from fission yeast strains containing only a single *h4.2* or *h4.2K20R* (*K20R*) allele were subjected to immunoblotting with methyl (Me)-specific or control antibodies indicated left.

(C) Peptide competition of methyl-specific H4-K20 antibodies. A fission yeast whole-cell extract was subject to immunoblotting with each of the antibodies indicated at the left that had been preincubated with either no peptide (−) or a peptide encompassing fission yeast H4 amino acids 17–24 with K20 unmodified, mono-, di-, or trimethylated as indicated across top. For each antibody presented, peptides are in approximate 200-fold molar excess.

(D) Set9 is required for H4-K20 methylation. Immunoblotting was performed with antibodies indicated using cell extracts prepared from labeled strains.

(E and F) A SET domain catalytic mutation abolishes H4-K20 methylation. Cell extracts were prepared from (E) *set9*Δ cells transformed with empty vector or plasmids expressing HA-tagged *set9*$^+$ or *set9Y220A* (*Y220A*) or from (F) strains with the indicated *set9* locus. Immunoblotting was then performed with antibodies labeled. Anti-HA (E) and a polyclonal anti-Set9 antibody (F) were used to detect Set9 protein. All results presented are representative of at least two independent experiments.

of gene expression, or their roles are redundant with other factors under the conditions tested.

A recent report has demonstrated trimethylated H4-K20 is focally enriched at mammalian pericentric heterochromatin, and a Set9-related HKMT regulates position effect variegation in *Drosophila* (Schotta et al., 2004). Localization of the modification was also dependent upon the Suv39h1/2 H3-K9 methyltransferases and the H3-K9 methyl binding protein HP1. From these data, a silencing pathway involving H3-K9 methylation followed by HP1 binding and subsequent recruitment of H4-K20 trimethylation was proposed (Schotta et al., 2004). Like metazoans, fission yeast centromeres are composed of a complex array of repeated sequences (see illustration, Figure 2B), and centromeric function is dependent upon heterochromatin factors (Allshire, 2003). It therefore seemed highly likely that H4-K20 methylation would be required for fission yeast centromere structure and function. Unexpectedly, the series of experiments presented in Figure 2 demonstrates that H4-K20 methylation does not play a central role in fission yeast centromeric function.

Immunostaining revealed trimethylated H4-K20 is present through much of the genome but does not ap-

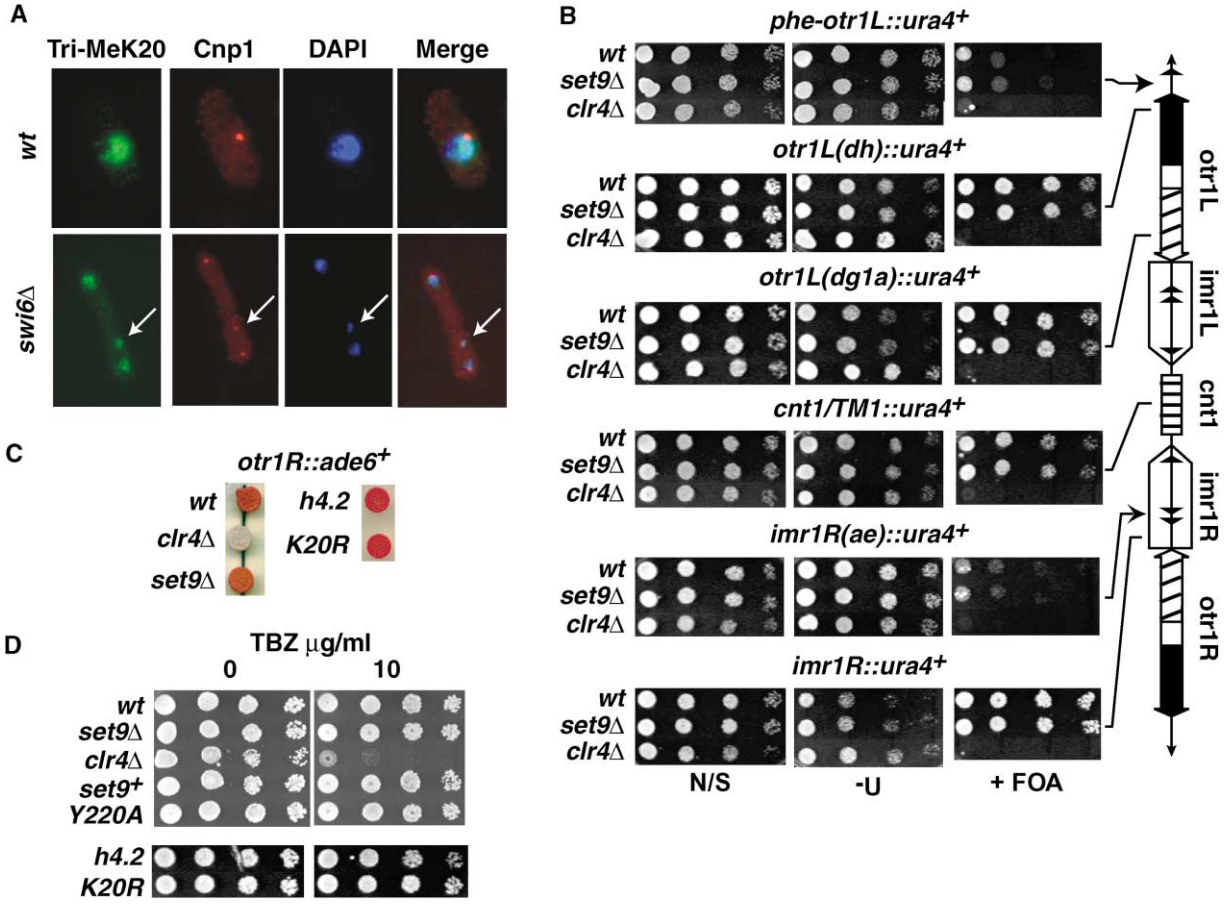

Figure 2. Centromere Function Is Not H4-K20 Methylation Dependent

(A) Trimethylated H4-K20 is not a marker of fission yeast centromeres. wt (top row) or *swi6*Δ (bottom row) cells were fixed, and localization was performed with anti-TriMe-H4-K20, anti-Cnp1, or DAPI (labeled top). Merge represents an overlay of all three images. Note the presence of the lagging chromosome in *swi6*Δ cells marked by the arrow.

(B and C) Centromeric gene silencing is not dependent upon Set9 function or H4-K20. (B) Right, diagram of *cen1* structure and sites of *ura4*$^+$ insertions, not drawn to scale. Silenced *ura4*$^+$ alleles are marked by lines, arrowheads mark expressed *ura4*$^+$ alleles, and filled triangles indicate tRNA genes. See Allshire [2003] for further detail. (B) Left, strains harboring diagramed *ura4*$^+$ insertions were plated on nonselective (N/S), minus uracil (−U), or FOA-containing media. (C) Strains with an *otr1R*-inserted *ade6*$^+$ allele were spotted on low adenine media.

(D) Loss of Set9 function or H4-K20 does not render cells sensitive to TBZ. Strains were spotted onto rich media containing the indicated amounts of TBZ and incubated at 30°C. Note that *clr*Δ cells act as a control for impaired centromeric function in shown assays. All results presented are representative of at least two independent experiments.

pear concentrated at any single area (top left panel, Figure 2A). Even a lagging chromosome present during mitosis appears to be completely decorated by the modification (see *swi6*Δ, bottom panels, Figure 2A). Trimethyl H4-K20 does not appear to mark centromeres, as no significant overlap with the centromere-specific histone H3 variant Cnp1$^{CENP\text{-}A}$ was observed (see Merge panels, Figure 2A). Chromatin immunoprecipitation also did not reveal enrichment of trimethyl H4-K20 at centromeric repeats (Supplemental Data), nor was Cnp1$^{CENP\text{-}A}$ localization Set9 dependent (data not shown). Further, localization of trimethyl H4-K20 was not dependent upon the Hp1-related Swi6 protein (bottom panels, Figure 2A) or the H3-K9 Clr4 methyltransferase (data not shown). Our attempts to perform immunostaining with the mono- and dimethyl antibodies have so far been unsuccessful.

Fission yeast centromeric gene silencing was also found not to be dependent upon H4-K20 methylation (Figures 2B and 2C). Reporter genes (either *ura4*$^+$ or *ade6*$^+$) located within centromeric repeats but not distal to outer repeats or between tRNA genes are silenced in a heterochromatin-dependent manner (see illustration, Figure 2B, and Allshire et al. [1995]). Loss of Set9 did not alter *ura4*$^+$ expression or silencing, as judged by growth on media lacking uracil or containing 5-fluoro-orotic acid (FOA) (Figure 2B). *ade6*$^+$ gene silencing was also not dependent upon Set9 protein or the H4-K20 residue (Figure 2C). Gene silencing at the mating type, ribosomal DNA repeats, and telomeric loci were also found not to require Set9 protein (Supplemental Data). Cells with impaired centromere function are frequently hypersensitive to the microtubule poison thiabendazole (TBZ). Neither loss of Set9 protein, its catalytic activity, nor its H4-K20 substrate rendered cells hypersensitive to TBZ over a range of concentrations (10–30 μg/ml) or temperatures (18°C–36°C) (Figure 2D and data not shown). The rate of loss for a circular minichromosome (CM3112) was also unaffected by *set9*Δ in the presence

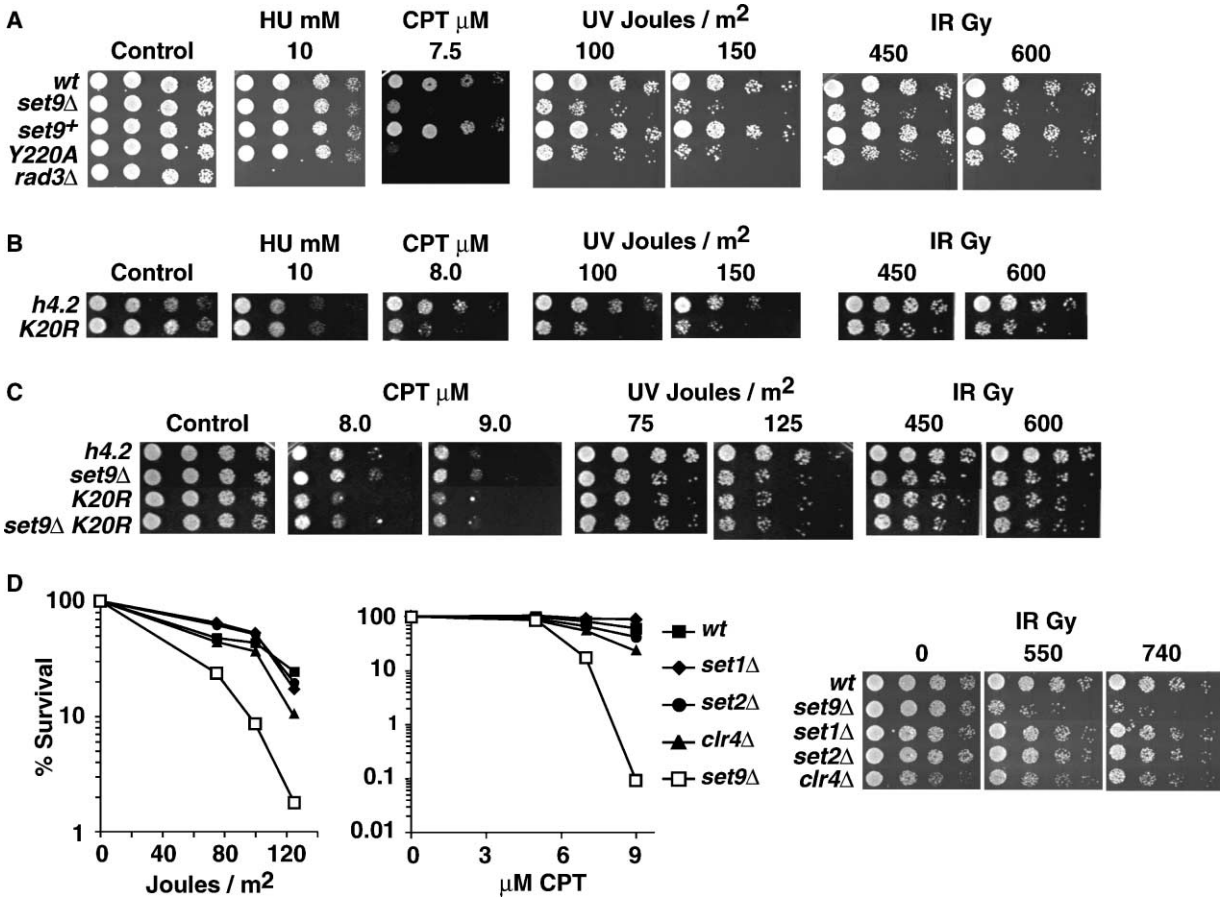

Figure 3. Set9 and H4-K20 Methylation Have a Role in Fission Yeast Genome Stability

(A) Set9 function is required for cell survival after DNA damage. Labeled strains (left) were spotted in a 1:5 serial dilution series (initial density 1×10^4 cells) onto rich media either lacking (control) or containing the indicated (top) amounts of each compound and incubated at 30°C. For UV and IR, cells were spotted onto rich media, then irradiated as indicated.

(B) H4-K20 is required for cell survival after DNA damage. As (A).

(C) Loss of Set9 does not further enhance the DNA damage sensitivity of the *h4.2K20R* mutant. As (A), except that all strains are in the *h4.2* background.

(D) Set9 has a unique role in DNA damage response. (Left) Survival curves for the indicated strains after exposure to UV light or when plated on CPT containing rich media. (Right) Comparison of IR sensitivity of indicated strains. All results presented are representative of at least two independent experiments.

or absence of TBZ (data not shown). Taken together, these data indicate that fission yeast centromeric structure and function do not require methylation of histone H4-K20.

Set9 and H4-K20 Methylation Have a Role in Fission Yeast DNA Damage Response

Our results have not revealed a requirement for H4-K20 methylation in any process normally associated with histone lysine methylation. In an attempt to expose the elusive function, we performed a variety of phenotypic tests. When challenged with a number of stresses including osmotic, heavy metal, or oxidative stress, *set9*$^-$ cells displayed viability comparable to that of wt (data not shown). Phenotypic analysis did reveal, however, that H4-K20 methylation does function in fission yeast DNA damage response (Figure 3). Deletion of *set9*$^+$ or inactivation of catalytic activity resulted in cells hypersensitive to DNA damage induced by ultraviolent (UV) light, ionizing radiation (IR), and the topoisomerase I poison camptothecin (CPT) (Figure 3A). Loss of Set9 function did not render cells hypersensitive to stalled replication complexes induced by hydroxyurea (HU). H4-K20 itself is also required for cell viability after DNA damage (Figure 3B). Relative to the control *h4.2*, *h4.2K20R* cells displayed a similar pattern of hypersensitivity as *set9*$^-$ cells, though the sensitivity to CPT was less pronounced. This most likely reflects the slower growth rate of the *h4.2* background strain, as we have found that fission yeast cells are generally more resistant to CPT when growing at a reduced rate (data not shown). To allow for a direct comparison between the *set9*Δ and *h4.2K20R* mutants, *set9*$^+$ was deleted in the *h4.2* strain. When in the same background, *h4.2K20R* and *h4.2set9*Δ cells are equally sensitive to UV light- and IR-induced damage but loss of Set9 did not result in detectable sensitivity to CPT in the *h4.2* background (Figure 3C). Again, the lack of CPT sensitivity is most likely related

to the slow growth rate of *h4.2* cells. Nonetheless, the similar patterns of sensitivity observed in Figures 3A–3C argue that Set9-dependent methylation of H4-K20 is required for cell survival after DNA damage. We next asked if H4-K20 was the only Set9 substrate required for DNA damage response. If methylation of other unknown substrates were required, we would expect loss of the Set9 enzyme to further increase the sensitivity of the substrate H4-K20 mutant. This is clearly not the case, as the double *h4.2set9Δ-K20R* mutant is no more sensitive to any damaging agent than either single mutant (Figure 3C). This strongly argues that Set9-dependent methylation of histone H4-K20 and not other substrates is required to maintain fission yeast cell survival after DNA damage. We next asked if the role of Set9 in DNA damage response was unique when compared to other known SET domain HKMTs. Figure 3D shows that only loss of Set9 and not loss of Set1, Set2, or Clr4 proteins results in significant sensitivity to IR, CPT, or UV light. Together, these data indicate that Set9 and H4-K20 methylation play a role in maintaining fission yeast genome stability after DNA damage.

H4-K20 Methylation Functions in the DNA Damage Checkpoint

We next sought to determine how Set9 and H4-K20 methylation contribute to DNA damage response. One possibility was that H4-K20 methylation functions in the repair of damaged DNA, but several initial observations suggested this not to be the case. In fission yeast, IR- and CPT-induced DSBs are repaired primarily by homologous recombination (HR). UV light-induced damage is repaired by both HR and excision repair. Genetic studies indicated that loss of Set9 protein further increased the UV sensitivity of excision repair mutants (*rad13Δ*, *uvdeΔ*, and *rad13Δ-uvdeΔ*, data not shown), arguing that Set9 does not function in excision repair. An initial analysis did not expose any significant defects in HR in mitotically dividing *set9Δ* cells, nor did deletion of *set9*$^{+}$ result in any phenotypes (such as low spore viability or mating type switching defects) normally associated with impaired HR function (data not shown). Though we cannot rule out a possible connection, H4-K20 methylation does not seem to be generally required for HR. It was also possible that H4-K20 methylation is required to regulate the expression of DNA damage response genes. In fission yeast, the majority (approximately 70%) of genes whose expression is induced after DNA damage are involved in core stress response (Chen et al., 2003; Watson et al., 2004). Of the approximate 50 noncore stress response genes induced by IR, only four are known to be directly involved in DNA repair, and none are known to function in checkpoint control (Watson et al., 2004). As *set9*$^{-}$ cells are not hypersensitive to any stress tested other than DNA damage, H4-K20 methylation is not generally required for the regulation of core stress response genes. Analysis of our DNA microarray data did not reveal the expression of any known DNA damage repair or checkpoint gene to be significantly altered in *set9Δ* cells (data not shown). Thus it seems highly unlikely that the role of H4-K20 methylation in DNA damage response is due to the regulation of gene expression.

We next asked whether or not H4-K20 methylation functions in checkpoint control after DNA damage. Two major evolutionarily conserved checkpoint pathways, the DNA damage and replication checkpoint, have been defined in fission yeast (Carr and Casprari, 2003). Activation of the Rad3 ATM/ATR-related kinase and other checkpoint Rad proteins is essential to both pathways and leads to a cascade of signaling events. The S phase-specific replication checkpoint responds to replication stress and is defined by checkpoint Rad protein and Mrc1-dependent activation of the Cds1 kinase. Independent association of the BRCT domain protein Crb2 and checkpoint Rad proteins at sites of damage is thought to initiate the G2-specific DNA damage checkpoint. Subsequent activation of the Chk1 effector kinase triggers downstream events leading to delay of the G2-M transition. High sensitivity to S phase perturbation after acute or chronic exposure to HU is a defining phenotype of proteins involved in the replication checkpoint. In contrast, proteins specifically involved in the DNA damage checkpoint are not sensitive to acute HU exposure and only moderately sensitive to chronic HU exposure. H4-K20 methylation-deficient cells are not sensitive to HU, even after chronic exposure, but are sensitive to other damaging agents during asynchronous growth where >70% of the cells are in G2 (Figure 3). From this, we can predict that, if involved in checkpoint control, H4-K20 methylation would function in the DNA damage checkpoint. The data obtained and presented in Figure 4 indicate H4-K20 methylation does function in the DNA damage checkpoint.

To examine the integrity of the DNA damage checkpoint G2 synchronized cells were irradiated with IR and mitotic progression was monitored (Figure 4A). With no or low IR (150 Greys [Gy], data not shown), wt and *set9Δ* cells progressed through mitosis at a similar rate. At higher doses (450 Gy, a point where *set9Δ* cells are approximately 10 times more sensitive to IR than wt cells), *set9Δ* cells arrested similar to wt but reentered mitosis markedly faster. Like wt, *set9Δ* cells displayed an elongated G2 morphology (data not shown), but, unlike wt, 20%–30% of *set9Δ* cells exhibited an abberant or "cut-like" mitotic phenotype (Figure 4B). The cut phenotype is a clear indication that *set9Δ* cells did prematurely leak through the G2-M checkpoint entering mitosis with damaged DNA. Similar results were seen with the catalytically dead *set9Y220A* or when the target lysine residue for Set9 was mutated in *h4.2K20R* cells (Figure 4A and data not shown). Together, these data indicate that Set9 function and H4-K20 are required to maintain DNA damage checkpoint-dependent cell cycle arrest in fission yeast.

We next examined the DNA damage checkpoint at the molecular level by asking if checkpoint signaling to the Chk1 effecter kinase requires the Set9 methylase. Although the functional consequences are unknown, checkpoint Rad protein and Crb2-dependent phosphorylation of Chk1 serves as a specific marker of DNA damage checkpoint activation (Carr and Casprari, 2003). Consistent with the checkpoint defect described above, Figure 4C shows that phosphorylation of Chk1 (Chk1-Pi) is compromised at high doses of IR by deletion of *set9*$^{+}$. Together, these data indicate that Set9 and H4-K20 methylation are important elements of the fission yeast DNA damage checkpoint.

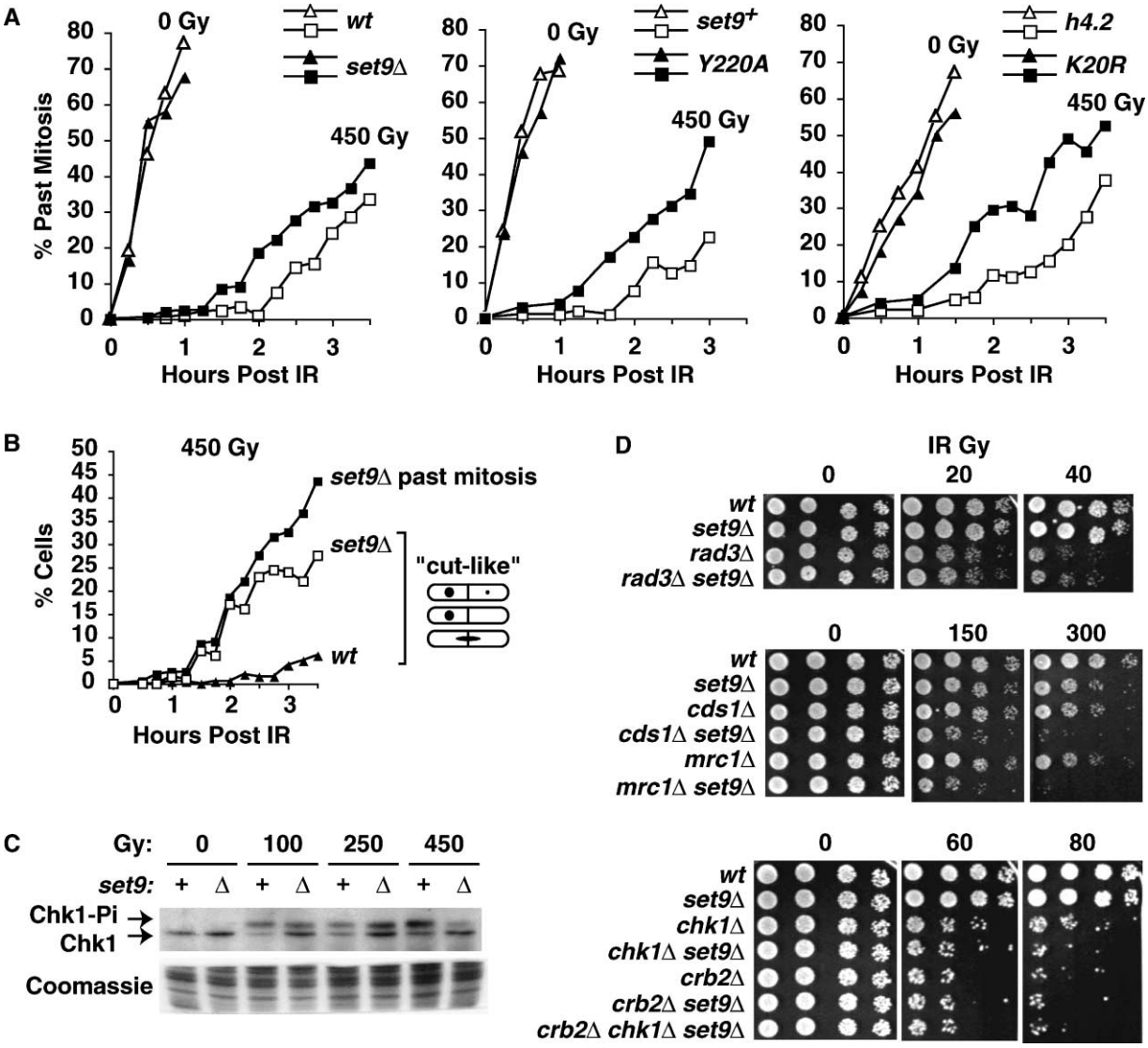

Figure 4. Set9 and H4-K20 Methylation Function in the DNA Damage Checkpoint

(A) Set9 function and H4-K20 are required to maintain DNA damage checkpoint cell cycle arrest. The indicated strains (labeled on each graph) were synchronized in G2 by lactose gradients, irradiated with IR (dose indicated), and grown at 30°C. Mitotic progression was determined by DAPI and calcofluor staining and counting binucleated/septated cells (at least 200 cells for each point).

(B) "Cut-like" mitotic phenotypes after IR in *set9*Δ cells. The percent wt or *set9*Δ cells with cut-like phenotype (see illustration) from the experiment presented in (A), left graph, were plotted. The *set9*Δ past mitosis data represents the same data from (A), left graph replotted for comparison.

(C) Loss of Set9 impairs Chk1 phosphorylation. Asynchronously growing *chk1*$^+$*-HA* cells with a *set9*$^+$ (+) or *set9*Δ (Δ) locus where IR irradiated (dose indicated top) and immediately processed for immunoblotting. A section of the gel was Coomassie stained as a loading control.

(D) Set9 functions in a pathway dependent upon checkpoint Rad proteins and Crb2. As Figure 3A. All results presented are representative of at least two independent experiments.

We next sought to delineate the pathway via which H4-K20 methylation functions in the DNA damage checkpoint. To this end, the genetic relationship between Set9 and checkpoint proteins was examined by asking if deletion of *set9*$^+$ altered the IR sensitivity of checkpoint mutants. The checkpoint Rad mutants *rad3*Δ, *rad26*Δ, *rad17*Δ, *rad9*Δ, or *hus1*Δ did not exhibit increased sensitivity when combined with *set9*Δ (Figure 4D, top panels, and data not shown). In contrast, double *mrc1*Δ-*set9*Δ and *cds1*Δ-*set9*Δ mutants were markedly more IR sensitive than any single mutant alone (Figure 4D, middle panels). This indicates that Set9 functions in a checkpoint Rad protein-dependent pathway but not in the replication checkpoint defined by Mrc1 and Cds1. This is also consistent with the lack of HU sensitivity for methyl H4-K20-deficient cells (Figure 3).

Because loss of Set9 or H4-K20 methylation impairs the DNA damage checkpoint that is defined by Crb2-dependent activation of the Chk1 kinase, in some fashion, Set9 should genetically function within this pathway. Deletion of *crb2*$^+$ results in cells moderately more sensitive to IR than *chk1*Δ mutants (Willson et al., 1997).

However, double *crb2*Δ-*chk1*Δ mutants are no more sensitive to IR than *crb2*Δ cells, and Chk1 phosphorylation after DNA damage is strictly Crb2 dependent (Saka et al., 1997; Willson et al., 1997). Based upon these results, it has been argued that Crb2 function is required prior to Chk1 activation within the DNA damage checkpoint. The IR sensitivity of *crb2*Δ was unaffected by deletion of *set9*$^+$ either alone or in combination with deletion of *chk1*$^+$ (Figure 4D, lower panels). This indicates that Set9 functions in a Crb2-dependent pathway. Interestingly, loss of Set9 did increase the sensitivity of *chk1*Δ to a level similar to that of the *crb2*Δ mutant (Figure 4D, lower panels). Because *set9*Δ is epistatic with *crb2*Δ but not *chk1*Δ, these data argue that the role of Set9 in the DNA damage checkpoint is to contribute to Crb2 function prior to Chk1 activation.

Set9 Regulates Crb2's Recruitment to Sites of DSBs and Subsequent Phosphorylation

The epistatic relationship between Set9 and Crb2 (Figure 4D) prompted us to further investigate the functional relationship between the two proteins. Similar to its mammalian counterpart 53BP1, Crb2 rapidly localizes to distinct nuclear foci representing sites of DSBs after IR (Du et al., 2003). DNA damage also induces hyperphosphorylation of a number of Crb2 residues (Esashi and Yanagida, 1999). Interestingly, mutation of a single threonine, T215, to alanine severally compromises the of ability of Crb2 to become phosphorylated but not its ability to initiate cell cycle arrest after DNA damage (Esashi and Yanagida, 1999). Rather, Crb2T215A cannot function in the repair of DSBs by regulating Rqh1/Top3-dependent helicase/topoisomerase function (Caspari et al., 2002). To understand how H4-K20 methylation contributes to Crb2 function, we examined the role of Set9 in regulating Crb2 localization and phosphorylation in the series of experiments presented in Figure 5.

We first tested the requirement of Set9 for Crb2 phosphorylation immediately after exposure to a range of IR doses. Crb2 phosphorylation is markedly compromised in the absence of Set9, even at low IR doses (Figure 5A). We next asked if Set9 function was required only to regulate Crb2 phosphorylation. If this were the case, we would expect deletion of *set9*$^+$ not to further enhance the IR sensitivity of the Crb2T215A phosphorylation mutant. Clearly, this is not the case, as the sensitivity of the double *crb2T215A-set9*Δ mutant is much greater than either single mutant alone and equal to deletion of *crb2*$^+$ (Figure 5B). Further, the double mutant displays a checkpoint defect equivalent to that of the *crb2*Δ mutant (Figure 5C). These results argue that Set9 function is required prior to Crb2 phosphorylation. To examine the requirement for Set9 in Crb2 foci formation, live imaging of strains expressing GFP-tagged Crb2 and harboring either a *set9*$^+$ or *set9*Δ allele was performed. Whereas Crb2 foci were readily formed immediately after IR in the presence of Set9, in its absence, Crb2 foci formation was severally compromised (Figures 5D and 5E). Together, these results argue that the role of Set9 in the DNA damage checkpoint is to regulate the initial recruitment of Crb2 to sites of DSBs, and this recruitment is required for subsequent phosphorylation of Crb2.

Our results argue that loss of H4-K20 methylation compromises the localization of Crb2 to DSBs, and this impairs the ability of cells to maintain cell cycle arrest after IR. Interestingly, histone H2AX phosphorylation has also been linked to Crb2 recruitment and checkpoint control in fission yeast (Nakamura et al., 2004). Most strikingly, cells harboring mutations of the phosphorylated residues (S129 and S128 of histones H2A.1 and H2A.2, respectively) display a checkpoint maintenance defect similar to that of H4-K20 methylation-deficient cells, and Crb2 foci are almost completely lacking after IR. This led us to ask whether or not a defect in H2AX phosphorylation could explain the loss of Crb2 recruitment to DSBs in *set9*Δ cells. Figure 5A shows that IR-induced H2A phosphorylation is not significantly altered by the absence of Set9 in cells where Crb2 phosphorylation is clearly compromised. This result argues that the pathway via which Set9 facilitates Crb2 localization is independent of H2AX phosphorylation.

Discussion

Here we provide evidence that a HKMT and its methylated target function in DNA damage checkpoint control. A previously uncharacterized SET domain protein, Set9, is responsible for methylation of fission yeast histone H4-K20. Surprisingly, this modification does not have any apparent role in the regulation of gene expression or heterochromatin function. We have found the Set9 enzyme, its catalytic activity, and its substrate have a role in maintaining cell survival after DNA damage and loss of H4-K20 methylation impairs the DNA damage checkpoint. Set9 is functionally linked to the checkpoint protein Crb2 and regulates its localization to DSBs and subsequent phosphorylation. These results provide a novel connection between histone lysine methylation and checkpoint surveillance of genome integrity.

In metazoans, methylation of histone H4-K20 can be associated with either transcriptional activation or repression (Sims et al., 2003). Trimethylation of H4-K20 by enzymes related to Set9 has also been linked to heterochromatin formation in mammals and *Drosophila* (Schotta et al., 2004). In contrast, we have yet to identify a role for the modification in regulating either of these processes in fission yeast. Most surprising is the lack of involvement in heterochromatin function, given the similar centromere structure and gene silencing mechanisms in fission yeast and metazoans. How might we resolve this apparent functional distinction? Perhaps the role of H4-K20 methylation in genome stability described here represents an evolutionarily early function that has simply yet to be described in higher eukaryotes. It may be that the heterochromatin role of H4-K20 methylation arose only later in evolution in response to the increasing genome complexity of higher eukaryotes. It is also interesting that metazoans have at least two phylogenetically distinct H4-K20 methyltransferases, the Suv4-h20 and PR-Set7/Set8 enzymes (Fang et al., 2002; Nishioka et al., 2002; Schotta et al., 2004), whereas fission yeast appears to have only a single enzyme. Presumably, the Set9 enzyme can catalyze mono-, di-, or trimethylation of the H4-K20 residue, as all three modifications are strictly Set9 dependent in fission yeast cells. The Set9-related Suv4-h20 enzymes show a strong

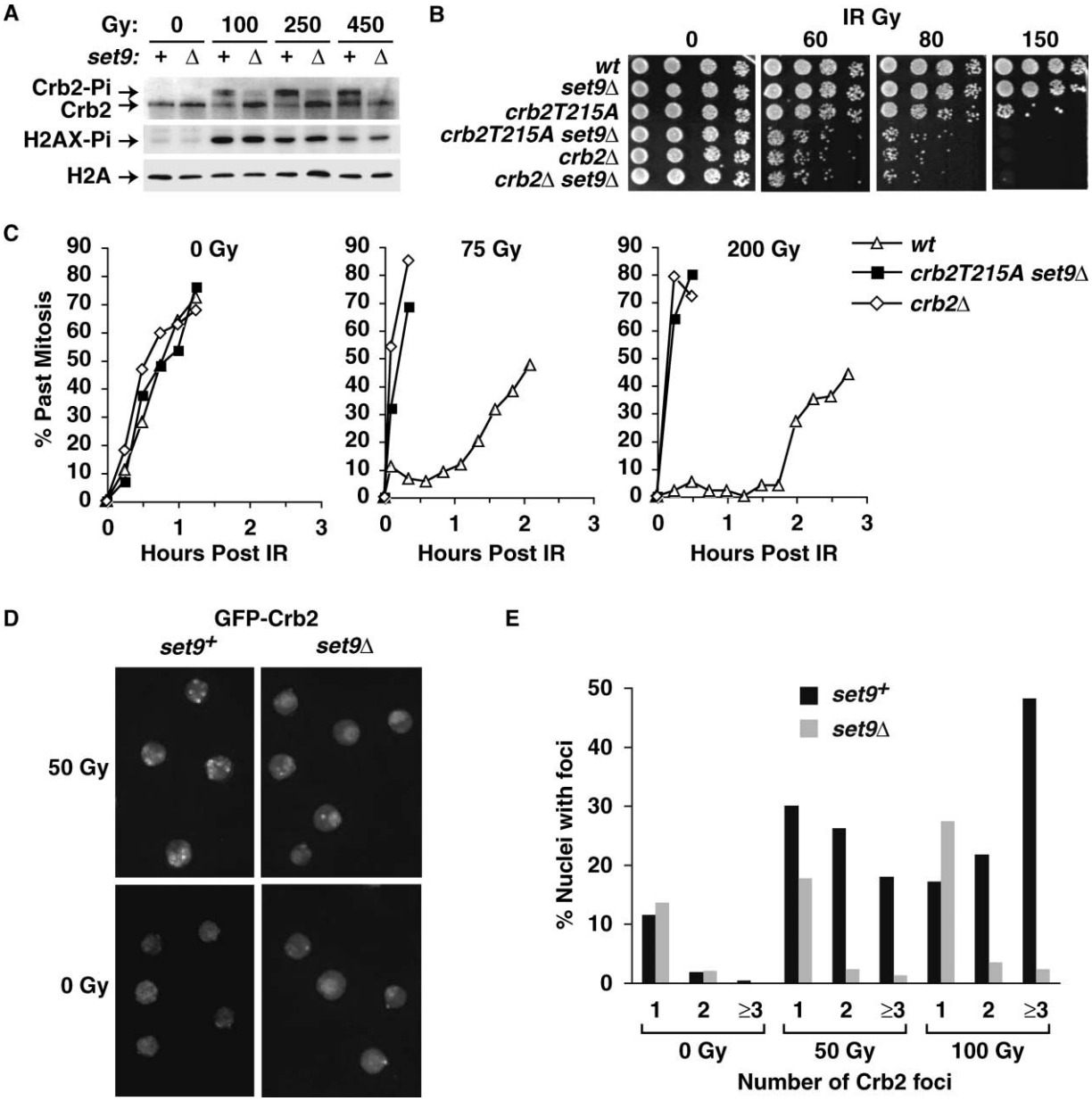

Figure 5. Set9 Regulates Crb2's Recruitment to Sites of DSBs and Subsequent Phosphorylation

(A) Set9 regulates Crb2 phoshporylation but not H2A phosphorylation. Asynchronously growing *crb2*$^{+}$*-MYC* cells with a *set9*$^{+}$ (+) or *set9*Δ (Δ) locus were IR irradiated and immediately processed for immunoblotting. Note that the decrease in H2A phosphorylation at higher IR doses is most likely reflective of the extended time required for irradiation.

(B) *set9*Δ and *crb2T215A* are not epistatic. As Figure 3A.

(C) A *set9*Δ*-crb2T215A* double mutant is checkpoint defective. As Figure 4A. Note that, due to the time required for irradiation, a large fraction of *set9*Δ*-crb2T215A* and *crb2*Δ cells have already progressed through mitosis by the first time point.

(D and E) Set9 is required for Crb2 recruitment to DSBs. Live imaging of *set9*$^{+}$ or *set9*Δ strains expressing GFP-Crb2 as the sole source of Crb2 was performed before or immediately after indicated IR dose. Representative images and quantitative data (100–200 nuclei counted for each point) from a single experiment are shown in (D) and (E). All results presented are representative of at least two independent experiments.

preference for the trimethyl reaction (Schotta et al., 2004), but the unrelated PR-Set7/Set8 enzymes seem to catalyze mono- and/or dimethylation (Julien and Herr, 2004; Nishioka et al., 2002). Perhaps this distribution of catalytic activity in higher organisms may provide a mechanism to accomplish two distinct functions, one of which is heterochromatin function and a second being DNA damage response.

Following DNA damage, phosphorylation of histone H2AX (in fission and budding yeast) or γH2AX (in mammals), carried out by the ATM/ATR-related checkpoint kinases, functions specifically at sites of DNA repair (Fernandez-Capetillo et al., 2004; Nakamura et al., 2004). In both fission yeast and mammals, this phosphorylation is thought to function in part by regulating the localization of the Crb2 (fission yeast) and 53BP1 (mammals)

proteins to sites of DSBs (Fernandez-Capetillo et al., 2004; Nakamura et al., 2004). These and our results suggest that H2AX phosphorylation and H4-K20 methylation both function in the DNA damage checkpoint by regulating the binding of Crb2 to sites of DSBs in some fashion. Despite the similarities, several observations argue that H4-K20 methylation contributes to checkpoint control and Crb2 function in a manner that is mechanistically distinct from H2AX phosphorylation. First, the mono-, di-, and trimethylated H4-K20 residue is readily detectable during unperturbed cell growth (Figure 1). Second, bulk levels of the modification do not show any significant increase after DNA damage or any apparent dependence upon checkpoint proteins (our unpublished data). Third, immunostaining indicates that trimethylated H4-K20 is distributed through much of the genome (Figure 2A), and we have been unable to detect any significant changes in localization of the modification or the Set9 enzyme after DNA damage (our unpublished data). H2AX phosphorylation is also not significantly dependent upon methylation of H4-K20 (Figure 5A). Thus, unlike H2AX phosphorylation, H4-K20 methylation does not seem to function in a manner that requires specific relocalization to sites of DNA repair. This is consistent with the static nature of histone lysine methylation (Bannister et al., 2002) and suggests a novel mechanism by which histone posttranslational modification can regulate a chromatin binding factor.

Though present in the N-terminal tail region of H4, evidence argues that lysine 20 is not exposed but "buried" in the context of stacked nucleosomes and that the H4 tail has a critical role in higher order nucleosome packing (Dorigo et al., 2003; White et al., 2001). Based upon this and the apparent presence of the modification throughout much of the genome, we suggest the model of H4-K20 methylation function illustrated in Figure 6. During unperturbed cell growth, the methylated H4-K20 residue is hidden or buried in the context of packed chromatin. Introduction of a DSB would then generate a region of unstacked or open chromatin exposing a preexisting methylated H4-K20 residue. Recognition of the modification by Crb2 then leads to recruitment of Crb2 to the DSB. Though not illustrated, we further suggest that the independent association of Rad3 (Du et al., 2003) and subsequent phosphorylation of H2AX is further required for the stable association of Crb2 at DSBs. In the absence of H4-K20 methylation or H2AX phosphorylation, the ability of Crb2 to recognize a DSB is compromised, leading to a lack of focal enrichment and an attenuated checkpoint signal insufficient to maintain an extended cell cycle arrest after IR.

Whether or not the proposed recognition of methyl H4-K20 by Crb2 is through direct binding is currently unknown, but, for the reasons below, we predict that the binding will be direct. Outside of its tandem BRCT domains, which may bind phosphorylated H2AX (Nakamura et al., 2004), Crb2 contains no other obvious functional domains. However, our unpublished data suggest that Crb2 does contain a noncanonical tudor fold similar to that of 53BP1 (Charier et al., 2004). We have previously postulated that tudor domains may bind methylysines, since they are part of the Royal Family of chromo-like domains (Hughes-Davies et al., 2003; Maurer-Stroh et al., 2003). This suggests that Crb2 may recognize chromatin through its tudor domain by directly binding to methyl H4-K20. Interestingly, 53BP1 contains two adjacent tudor domains (Charier et al., 2004) and has the ability to recognize methylated H3-K79 (T. Halazonetis, personal communication). This raises the intriguing possibility that Crb2's mammalian counterpart may recognize chromatin via interaction with other modifications in addition to methyl H4-K20.

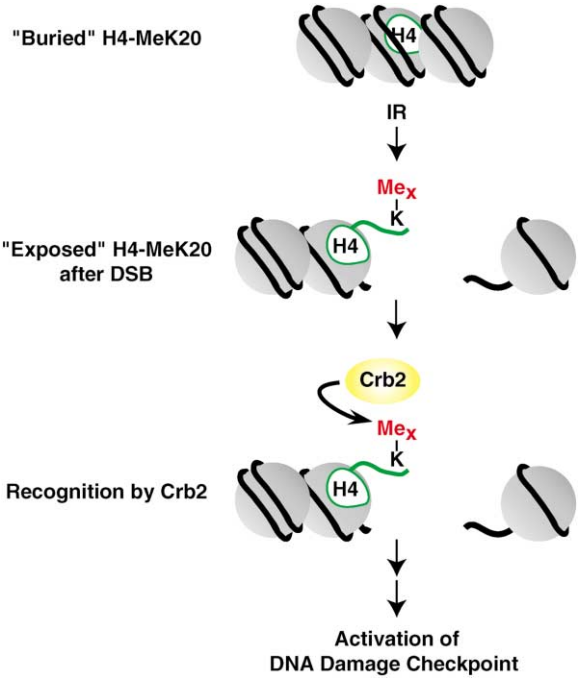

Figure 6. A Model of How H4-K20 Methylation May Function in DNA Damage Checkpoint Control

Introduction of a DSB generates a region of unstacked or open chromatin exposing a preexisting methyl H4-K20 residue that can then be recognized by Crb2. Though not shown, Rad3-dependent H2AX phosphorylation would further stabilize the association of Crb2. See Discussion for specific details. Me$_X$ represents the possibility that mono-, di-, or trimethylated H4-K20 (or some combination) could be the required modification. Whether or not the proposed recognition of methyl H4-K20 by Crb2 is through direct binding is currently unknown.

Experimental Procedures

Strains and Plasmids

Standard laboratory methods and techniques for fission yeast manipulations were used (Moreno et al., 1991). Relevant strains are listed in Supplemental Data. Silencing assays and strains have been described (Allshire et al., 1995; Bjerling et al., 2002; Nimmo et al., 1998). Checkpoint mutant strains were obtained from T. Carr. Because we have found that auxotrophic strains, which are slower growing than wt cells on rich media, display a less pronounced *set9*Δ DNA damage sensitivity, all except required markers were crossed out. Double deletion strains were generated by random spore analysis. PCR-mediated TAP tagging and generation of *KAN*-marked deletion strains were performed as described (Bahler et al., 1998; Tasto et al., 2001), utilizing strain *972h−*. Markers were crossed in as required. The *h4.2K20R* strain was generated as described (Mellone et al., 2003).

The Set9 ORF was PCR amplified from genomic DNA and cloned into pREP81-HA. PCR-mediated mutagenesis was then performed to generate pREP81-HA-Y220A. To generate the *set9Y220A* strain,

a DNA fragment composed of the Set9Y220A ORF, the transcription termination and *KAN* marker sequence from pFA6a-3HA-kanMx6 (Bahler et al., 1998), followed by 500 base pairs of Set9 ORF 3′ flanking sequence was transformed into 972h$^-$. *KAN*$^+$ colonies were screened by immunoblotting to identify cells harboring *set9Y220* (*set9*$^+$) or *set9Y220A* alleles. The entire ORF from *set9*$^+$ and *set9Y220A* strains was PCR amplified and verified by automated DNA sequencing. For GFP-Crb2 strains, a plasmid expressing the GFP-Crb2 ORF under the control of the attenuated REP81 promoter was integrated at the *leu1* locus of the appropriate strain. The ORF for Histone H4 was PCR amplified from genomic DNA and cloned into pET15b (Novagen) such that the His6 tagging sequence was deleted, yielding an untagged H4.

DNA Damage Assays

Damage and checkpoint assays were performed essentially as described (Edwards and Carr, 1997). Data presented for survival curves is the average of three to five experiments performed in duplicate. A 254 nm UV-C lamp (dose rate of 2–3.7 J/m^2/s) and a Faxitron 43855D X-ray source (dose rate of 3.15 Gy/min) were utilized. For checkpoint assays, cells were G2 synchronized using lactose gradients, plated onto YES plates, irradiated, washed off into conditioned YES media, and incubated at 30°C using a water bath shaker. GFP-Crb2 foci experiments were performed as described (Du et al., 2003).

Protein Methods

Whole-cell extract preparation from logarithmically growing cells in rich media and protein A purifications have been described (Logie and Peterson, 1999; Rigaut et al., 1999). Histone methyltransferase assays using protein A-purified proteins bound to beads were performed as described (Nishioka et al., 2002). For recombinant H4 production, Rosetta(DE3) cells (Novagen) were utilized. Induced cell cultures were harvested, boiled in SDS-PAGE sample buffer, subjected to a brief centrifugation, and used directly for immunoblotting. Extracts for immunoblotting were prepared as described (Caspari et al., 2000).

Antibodies

Mono-, di-, and trimethyl H4-K20 antibodies from Abcam (ab9051, ab9052, and ab9053) and Upstate (07-440, 07-367, and 07-463) were utilized as well as polyclonal anti-histone H3 and H2A (Abcam ab1791 and ab13923). Polyclonal anti-histone H4, anti-acetyl H4-K8, and anti-Pi scH2A-S129 antibodies were kindly provided by A. Verreault, B. Turner, and C. Redon, respectively. Polyclonal anti-Set9 antibody was generated by Abcam. Immunostaining was performed using cells fixed with 3.7% paraformaldehyde for 20 min and Alexafluor 488 anti-rabbit secondary antibody (Molecular Probes).

DNA Microarray Analysis

DNA microarray analysis was performed as described (Mata et al., 2002) using strains 972h− and YSLS252 grown to mid-log in YES media at 30°C. For genes scored in two of two experiments with less than 10% error, the top 20 most up- or downregulated are listed in Supplemental Data. The complete data set is available upon request. Similarly few changes in gene expression were also seen in a single experiment using cells grown in minimal media.

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References

Allshire, R. (2003). Centromere and kinetochore structure and function. In The Molecular Biology of *Schizosaccharomyces pombe*, R. Egel, ed. (Berlin: Springer), pp. 149–166.

Allshire, R.C., Nimmo, E.R., Ekwall, K., Javerzat, J.P., and Cranston, G. (1995). Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev. *9*, 218–233.

Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast *14*, 943–951.

Bannister, A.J., Schneider, R., and Kouzarides, T. (2002). Histone methylation: dynamic or static? Cell *109*, 801–806.

Bjerling, P., Silverstein, R.A., Thon, G., Caudy, A., Grewal, S., and Ekwall, K. (2002). Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and in vivo specificity. Mol. Cell. Biol. *22*, 2170–2181.

Carr, A.M., and Casprari, T. (2003). Checkpoint controls halting the cell cycle. In The Molecular Biology of *Schizosaccharomyces pombe*, R. Egel, ed. (Berlin: Springer), pp. 41–56.

Caspari, T., Dahlen, M., Kanter-Smoler, G., Lindsay, H.D., Hofmann, K., Papadimitriou, K., Sunnerhagen, P., and Carr, A.M. (2000). Characterization of *Schizosaccharomyces pombe* Hus1: a PCNA-related protein that associates with Rad1 and Rad9. Mol. Cell. Biol. *20*, 1254–1262.

Caspari, T., Murray, J.M., and Carr, A.M. (2002). Cdc2-cyclin B kinase activity links Crb2 and Rqh1-topoisomerase III. Genes Dev. *16*, 1195–1208.

Charier, G., Couprie, J., Alpha-Bazin, B., Meyer, V., Quemeneur, E., Guerois, R., Callebaut, I., Gilquin, B., and Zinn-Justin, S. (2004). The Tudor tandem of 53BP1: a new structural motif involved in DNA and RG-rich peptide binding. Structure (Camb.) *12*, 1551–1562.

Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., and Bahler, J. (2003). Global transcriptional responses of fission yeast to environmental stress. Mol. Biol. Cell *14*, 214–229.

Dorigo, B., Schalch, T., Bystricky, K., and Richmond, T.J. (2003). Chromatin fiber folding: requirement for the histone H4 N-terminal tail. J. Mol. Biol. *327*, 85–96.

Du, L.L., Nakamura, T.M., Moser, B.A., and Russell, P. (2003). Retention but not recruitment of Crb2 at double-strand breaks requires Rad1 and Rad3 complexes. Mol. Cell. Biol. *23*, 6150–6158.

Edwards, R.J., and Carr, A.M. (1997). Analysis of radiation-sensitive mutants of fission yeast. Methods Enzymol. *283*, 471–494.

Esashi, F., and Yanagida, M. (1999). Cdc2 phosphorylation of Crb2 is required for reestablishing cell cycle progression after the damage checkpoint. Mol. Cell *4*, 167–174.

Fang, J., Feng, Q., Ketel, C.S., Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Tempst, P., Simon, J.A., and Zhang, Y. (2002). Purification and functional characterization of SET8, a nucleosomal histone H4-lysine 20-specific methyltransferase. Curr. Biol. *12*, 1086–1099.

Fernandez-Capetillo, O., and Nussenzweig, A. (2004). Linking histone deacetylation with the repair of DNA breaks. Proc. Natl. Acad. Sci. USA *101*, 1427–1428.

Fernandez-Capetillo, O., Lee, A., Nussenzweig, M., and Nussenzweig, A. (2004). H2AX: the histone guardian of the genome. DNA Repair (Amst.) *3*, 959–967.

Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. Nature *411*, 366–374.

Hughes-Davies, L., Huntsman, D., Ruas, M., Fuks, F., Bye, J., Chin, S.F., Milner, J., Brown, L.A., Hsu, F., Gilks, B., et al. (2003). EMSY

links the BRCA2 pathway to sporadic breast and ovarian cancer. Cell *115*, 523–535.

Julien, E., and Herr, W. (2004). A switch in mitotic histone H4 lysine 20 methylation status is linked to M phase defects upon loss of HCF-1. Mol. Cell *14*, 713–725.

Kouzarides, T. (2002). Histone methylation in transcriptional control. Curr. Opin. Genet. Dev. *12*, 198–209.

Lachner, M., O'Sullivan, R.J., and Jenuwein, T. (2003). An epigenetic road map for histone lysine methylation. J. Cell Sci. *116*, 2117–2124.

Logie, C., and Peterson, C.L. (1999). Purification and biochemical properties of yeast SWI/SNF complex. Methods Enzymol. *304*, 726–741.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. Nature *389*, 251–260.

Marmorstein, R. (2003). Structure of SET domain proteins: a new twist on histone methylation. Trends Biochem. Sci. *28*, 59–62.

Mata, J., Lyne, R., Burns, G., and Bahler, J. (2002). The transcriptional program of meiosis and sporulation in fission yeast. Nat. Genet. *32*, 143–147.

Maurer-Stroh, S., Dickens, N.J., Hughes-Davies, L., Kouzarides, T., Eisenhaber, F., and Ponting, C.P. (2003). The Tudor domain 'Royal Family': Tudor, plant Agenet, Chromo, PWWP and MBT domains. Trends Biochem. Sci. *28*, 69–74.

Mellone, B.G., Ball, L., Suka, N., Grunstein, M.R., Partridge, J.F., and Allshire, R.C. (2003). Centromere silencing and function in fission yeast is governed by the amino terminus of histone H3. Curr. Biol. *13*, 1748–1757.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. *194*, 795–823.

Nakamura, T.M., Du, L.L., Redon, C., and Russell, P. (2004). Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast. Mol. Cell. Biol. *24*, 6215–6230.

Nimmo, E.R., Pidoux, A.L., Perry, P.E., and Allshire, R.C. (1998). Defective meiosis in telomere-silencing mutants of *Schizosaccharomyces pombe*. Nature *392*, 825–828.

Nishioka, K., Rice, J.C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuikov, S., Valenzuela, P., Tempst, P., Steward, R., et al. (2002). PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. Mol. Cell *9*, 1201–1213.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. *17*, 1030–1032.

Saka, Y., Esashi, F., Matsusaka, T., Mochida, S., and Yanagida, M. (1997). Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. Genes Dev. *11*, 3387–3400.

Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupt, R., Reuter, G., Reinberg, D., and Jenuwein, T. (2004). A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. *18*, 1251–1262.

Sims, R.J., 3rd, Nishioka, K., and Reinberg, D. (2003). Histone lysine methylation: a signature for chromatin function. Trends Genet. *19*, 629–639.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41–45.

Tasto, J.J., Carnahan, R.H., McDonald, W.H., and Gould, K.L. (2001). Vectors and gene targeting modules for tandem affinity purification in *Schizosaccharomyces pombe*. Yeast *18*, 657–662.

Watson, A., Mata, J., Bahler, J., Carr, A., and Humphrey, T. (2004). Global gene expression responses of fission yeast to ionizing radiation. Mol. Biol. Cell *15*, 851–860.

White, C.L., Suto, R.K., and Luger, K. (2001). Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions. EMBO J. *20*, 5207–5218.

Willson, J., Wilson, S., Warr, N., and Watts, F.Z. (1997). Isolation and characterization of the *Schizosaccharomyces pombe* rhp9 gene: a gene required for the DNA damage checkpoint but not the replication checkpoint. Nucleic Acids Res. *25*, 2138–2146.

Xiao, B., Jing, C., Wilson, J.R., Walker, P.A., Vasisht, N., Kelly, G., Howell, S., Taylor, I.A., Blackburn, G.M., and Gamblin, S.J. (2003). Structure and catalytic mechanism of the human histone methyltransferase SET7/9. Nature *421*, 652–656.