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The *RAD6/BRE1* Histone Modification Pathway in Saccharomyces Confers Radiation Resistance Through a *RAD51*-Dependent Process That Is Independent of *RAD18*

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ABSTRACT

We examine ionizing radiation (IR) sensitivity and epistasis relationships of several Saccharomyces mutants affecting post-translational modifications of histones H2B and H3. Mutants *bre1*Δ, *lge1*Δ, and *rtf1*Δ, defective in histone H2B lysine 123 ubiquitination, show IR sensitivity equivalent to that of the *dot1*Δ mutant that we reported on earlier, consistent with published findings that Dot1p requires H2B K123 ubiquitination to fully methylate histone H3 K79. This implicates progressive K79 methylation rather than mono-methylation in IR resistance. The *set2*Δ mutant, defective in H3 K36 methylation, shows mild IR sensitivity whereas mutants that abolish H3 K4 methylation resemble wild type. The *dot1*Δ, *bre1*Δ, and *lge1*Δ mutants show epistasis for IR sensitivity. The *paf1*Δ mutant, also reportedly defective in H2B K123 ubiquitination, confers no sensitivity. The *rad6*Δ, *rad51null*, *rad50*Δ, and *rad9*Δ mutations are epistatic to *bre1*Δ and *dot1*Δ, but *rad18*Δ and *rad5*Δ show additivity with *bre1*Δ, *dot1*Δ, and each other. The *bre1*Δ *rad18*Δ double mutant resembles *rad6*Δ in sensitivity; thus the role of Rad6p in ubiquitinating H2B accounts for its extra sensitivity compared to *rad18*Δ. We conclude that IR resistance conferred by *BRE1* and *DOT1* is mediated through homologous recombinational repair, not postreplication repair, and confirm findings of a G_1 checkpoint role for the *RAD6/BRE1/DOT1* pathway.

RECENT research in eukaryotes has demonstrated a much greater role than was initially perceived for histone modifications in basic cellular processes, including transcription, gene silencing, control of carcinogenesis, and responses to DNA damage. As part of this, we reported that Saccharomyces strains deleted for any of several genes involved in histone modifications are substantially more sensitive than wild type to the lethal effects of ionizing radiation (IR) (Game *et al.* 2005). The mutants included strains deleted for the *DOT1* gene, which encodes the methylase that acts on the lysine 79 residue (K79) of the histone H3 protein (Feng *et al.* 2002; van Leeuwen *et al.* 2002), as well as histone H3 mutants in which wild-type Dot1p cannot act because its target lysine is replaced with another amino acid. These findings complemented information from other laboratories that implicates histone H3 lysine 79 methylation in controlling the DNA damage checkpoint induced by ultraviolet radiation and other agents in yeast (Giannattasio *et al.* 2005; Wysocki *et al.* 2005) and in damage recognition by the checkpoint protein 53BP1 in mammalian cells (Huyen *et al.* 2004).

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Substantial information is available indicating that the *DOT1*-mediated methylation of H3 K79 is dependent on the prior modification of histone H2B involving ubiquitination of lysine 123 in Saccharomyces (Briggs *et al.* 2002; Ng *et al.* 2002a) or lysine 120 in mammals (Kim *et al.* 2005). Recently, it was shown that H3 K79 trimethylation and some di-methylation is dependent on H2B K123 ubiquitination, whereas mono-methylation of K79 still occurs fully even in mutants that fail to modify H2B K123 (Shahbazian *et al.* 2005). The Rad6 ubiquitin conjugase and the Bre1 ubiquitin ligase together ubiquitinate H2B K123 (Robzyk *et al.* 2000; Hwang *et al.* 2003; Wood *et al.* 2003a). In addition, the *LGE1* gene product has been found to complex with Bre1 protein and is required for its function (Hwang *et al.* 2003), and mutants involving some members of the RNA polymerase II-associated PAF1 complex, specifically deletions of the *RTF1* and *PAF1* genes, have also been reported to abolish H2B K123 ubiquitination (Ng *et al.* 2003a; Wood *et al.* 2003b). Most recently, the Bur1/Bur2 cyclin-dependent protein kinase has also been implicated in H2B K123 ubiquitination through its role in activating the Rad6 protein by phosphorylation (Wood *et al.* 2005).

Given this information, and to better understand the role of the *RAD6* gene in different DNA repair pathways, we chose to study the X-ray sensitivity of additional

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Saccharomyces histone modification mutants, including those with reported defects in H2B K123 ubiquitination and H3 K79 methylation and those involved in methylation elsewhere on histone H3. In addition, we constructed double-, triple-, and multiple-mutant strains involving H2B K123 ubiquitination and H3 K79 methylation mutations combined with each other and with key mutations in previously known DNA repair pathways. We assessed IR sensitivity in these strains to determine epistasis relationships for this phenotype both within the proposed *BRE1/DOT1*-mediated histone modification pathway and between this pathway and others to identify the probable IR repair processes involved.

With the exception of *paf1* deletion strains, we found increased sensitivity to X-rays in all the mutants that we tested that are reported to affect histone H3 K79 methylation. We also found that *set2* mutants, which fail to methylate histone H3 lysine 36, show mild X-ray sensitivity, whereas mutants that abolish histone H3 lysine 4 methylation retain wild-type resistance to X-rays. We obtained evidence that genes required for histone H3 K79 methylation predominantly fall into a single *RAD6*-dependent IR epistasis group that falls outside the well-known family of recovery processes mediated by *RAD6/RAD18*-dependent postreplication/translesion synthesis mechanisms. Instead, these histone modification genes appear to function in a process that facilitates *RAD51*-dependent homologous recombinational repair (HRR), although they are not completely required for such repair since significant *RAD51*-dependent IR resistance remains in *dot1*Δ, *bre1*Δ and related mutants. We show, in agreement with evidence from others, that some aspects of the DNA damage response cell-cycle checkpoints are abrogated in mutants unable to methylate histone H3 K79, and discuss this as a possible cause of their IR sensitivity.

MATERIALS AND METHODS

Yeast strains: As described earlier (Game *et al.* 2005), our starting strains were from the library of ~4700 individual haploid deletion strains in the α mating type (background strain BY4742) created by an International Consortium and obtained from Research Genetics, Huntsville, Alabama (now Invitrogen Life Technologies). The genotype of strain BY4742 and the construction of the deletion strains have been described (Brachmann *et al.* 1998; Winzeler *et al.* 1999). Information is also available at the Saccharomyces Genome Deletion Project website at http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html. We also used our background-isogenic strains MW5067-1C and g1201-4C, described earlier (Game *et al.* 2005), as a wild type for survival curves and a wild-type *MAT***a** parent for initial crosses with *MAT*α mutants from the deletion strain library, respectively. For crosses involving *rad51*, we primarily used a *rad51::URA3* disruption *null* allele (originally obtained from Vladimir Larionov) that we had backcrossed eight times into the library background to give expected unlinked nonisogenicity <1%. This enabled us to use the *URA3* marker in place of *KanMX4* to quickly distinguish *rad51* from other mutants in crosses. In the text, we refer to the *rad51::URA3* allele as *rad51null* and the *rad51::KanMX4* replacement allele from the library as *rad51*Δ. Strains containing either of these *rad51* alleles show equivalent survival curves, as shown in Figure 7.

Genetic methods and media: Genetic methods including tetrad dissection were as described (Sherman *et al.* 1982). Cultures were routinely incubated at 30°. Rich media (YPD) and supplemented minimal media were prepared as described (Sherman *et al.* 1982). To induce meiosis, we incubated cultures for 4 or more days, usually at 30°, on solid Fogel's sporulation medium. This contains 9.65 g potassium acetate, 1 g glucose, 2.5 g yeast extract (Difco), and 2% agar per liter. To score geneticin (GEN) resistance, hygromycin B (HYG) resistance, or nourseothricin (NAT) resistance, we used YPD plates separately supplemented with geneticin (Sigma, St. Louis), hygromycin B (Research Products International), or nourseothricin (Werner BioAgents) added from filter-sterilized solution shortly before pouring plates to give a final concentration of 150 μg/ml (GEN), 300 μg/ml (HYG), or 100 μg/ml (NAT), respectively.

Transformations: To facilitate scoring multiple deletion mutations in crosses, for several relevant genes we replaced the *KanMX4* cassette that was used to create the original deletion library with cassettes containing *LEU2* (obtained from James A. Brown, Stanford University) or a hygromycin B (*HYG*) or nourseothricin resistance (*NAT*) gene (obtained from Beth Rockmill, Yale University), using described cassettes (Goldstein *et al.* 1999) and a standard transformation procedure (Ito *et al.* 1983). To restore *BRE1* to *bre1*Δ mutant strains, we transformed with a *CEN URA3* plasmid containing *BRE1*, obtained from James A. Brown, using a lithium acetate procedure (Gietz *et al.* 1995).

Determining X-ray sensitivity: As described earlier (Game *et al.* 2005), for X-ray exposures we used a Machlett OEG 60 X-ray tube with a beryllium window and a Spellman power supply operated at 30 kV and 15 mA to deliver a dose rate of 1.3 Gy (130 rad)/sec of "soft" X-rays. To determine initially whether a mutant strain was likely to exhibit IR sensitivity, we essentially followed the spot-testing procedure described previously (Game *et al.* 2005). To quantify the degree of sensitivity, we obtained X-ray survival curves using log-phase cells from overnight liquid YPD cultures, freshly sonicated to reduce any clumpiness, as described in the same article. Colonies were counted after incubation for 4–6 days at 30°. We obtained survival curves for at least two separate strains for most of the single-, double-, or multiple-mutant genotypes that we present here, and in many cases additional survival assays (not shown) over part or all of the dose range served to confirm our findings. For the most part, we find good agreement in X-ray sensitivity between different spore clones with the same genotype in the same genetic background. We prefer to present individual survival curves instead of averaging measurements at each dose from separate curves, both because dose points within a curve are related by serial dilutions and because their statistical robustness will vary from curve to curve on the basis of colony counts as well as on the accuracy of the unirradiated control. This means that error bars calculated for a mean value based on separate curves can be misleading (see Game *et al.* 2005). In addition, despite our isogenic genetic background, we prefer to obtain confirmatory survival curves using separate spore clones rather than repeating the same strain, as a better control for modifier mutations that might arise. Clearly, taking average values for separate strains would obscure any variability that we hope to expose.

Ultraviolet radiation treatments: Log-phase cells were prepared for UV survival as for X-ray curves. They were irradiated

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on YPD plates using a shielded apparatus containing five General Electric G8T5 tubes giving most of their radiation at 254 nm. Plates were incubated in the dark and colonies counted as for X-ray curves.

Cell cycle checkpoint studies: Standard methods (Day *et al.* 2004) were adapted as follows: To study the IR-induced G_1 checkpoint, cells were arrested at G_1 using α-factor (Zymo Research). One microliter of 10 mm α-factor was added to 2 ml of log-phase cells shaken in liquid YPD at OD ~0.2 at 30°. After 1.5 hr, a second microliter was added and synchrony was assessed microscopically after another 1.5 hr. The cultures were split: one-half was irradiated using a ^{137}Cs source (Mark 1 model 3 from J. L. Shepherd, San Francisco; dose rate 28.4 Gy/min) and one-half was mock treated. After irradiation, cells were released from the block and 0.25-ml aliquots were fixed in 70% ethanol at 15-min intervals. Fixed cells were spun down and washed with 1 ml 0.05 m sodium citrate. Cell pellets were mixed with 0.5 ml 0.05 m sodium citrate containing 0.25 mg/ml RNase A and incubated at 50° for 1 hr. After addition of propidium iodide (16 μg/ml final concentration), samples were incubated at room temperature for 30 min, briefly resonicated, and analyzed by flow cytometry (Nash *et al.* 1988) with a FACSCalibur machine.

To study the IR-induced G_2 checkpoint, nocodazole (Sigma) was added (15 μg/ml final concentration) to midlog-phase cultures (OD ~0.2) shaking in liquid YPD and cells were incubated for 2.5 hr at 30° to achieve >90% large buds. Cultures were split and then mock treated or irradiated with a ^{137}Cs source (see above), nocodazole was removed by resuspending in sterile water, and cells were then resuspended in fresh YPD and shaken at 30°. Aliquots of 250 μl were fixed in 70% ethanol at 30-min intervals, spun down, resuspended in PBS (120 mm sodium chloride; 2.7 mm potassium chloride; pH 7.3 with 10 mm phosphate buffer), and incubated with DAPI (1 μg/ml final concentration) (Williamson and Fennell 1979) at room temperature for 20 min. Percentages of uninucleate and binucleate cells were assessed by fluorescence microscopy.

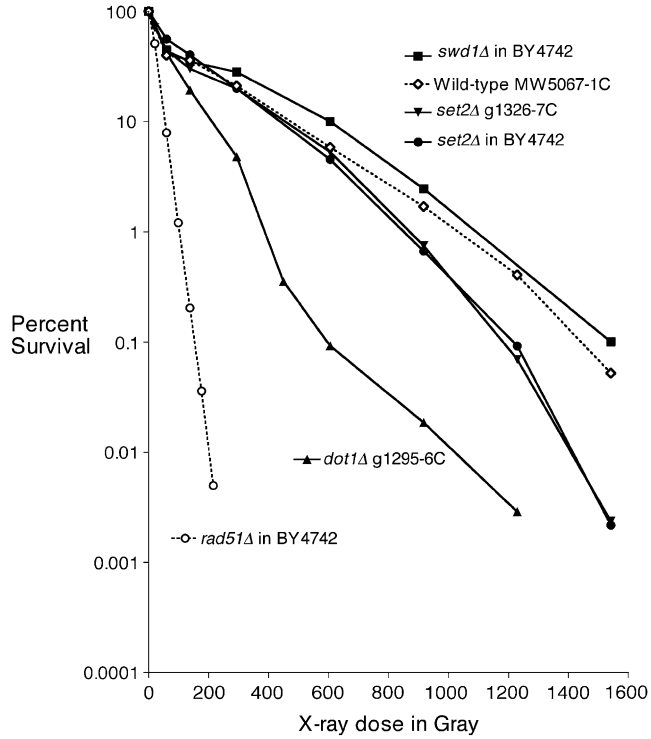

Figure 1.—Survival *vs.* X-ray dose for haploid mutant strains separately affected in methylation of three sites on histone H3. Two *set2*Δ strains, blocked in H3 K36 methylation, are compared to *swd1*Δ and *dot1*Δ strains, blocked in H3 K4 and H3 K79 methylation, respectively. A wild-type and a *rad51*Δ haploid strain are included for comparison. The strains share the same genetic background, and the *dot1*Δ mutant shows X-ray sensitivity equivalent to that of previously published *dot1*Δ strains in this background (Game *et al.* 2005).

RESULTS

IR survival of mutants separately blocked in histone H3 K4, K36, and K79 methylation: In addition to histone H3 K79, two other histone H3 lysine residues, K4 and K36, are known to be methylated in both Saccharomyces and higher eukaryotes (Roguev *et al.* 2001; Strahl *et al.* 2002; Lee and Skalnik 2005). We studied mutants blocked in each of these methylations to determine if they too played a role in IR resistance, as is the case for H3 K79 methylation. H3 K4 methylation resembles that of H3 K79 in being dependent on prior ubiquitination of histone H2B K123 for di- and trimethylation of the lysine residue (Dover *et al.* 2002; Sun and Allis 2002; Shahbazian *et al.* 2005). Methylation of H3 K4 is carried out by the SET1 protein complex, also known as COMPASS, which is thought to include at least eight component proteins (Miller *et al.* 2001; Roguev *et al.* 2001; Krogan *et al.* 2002; Schneider *et al.* 2005). Information is available concerning the IR sensitivity ranking for homozygous diploid deletion mutants involving six of the COMPASS-encoding genes relative to the rest of the mutants in a pooled deletion library after a single dose (200 Gy) of IR (Brown *et al.* 2006). These mutants are deleted for *BRE2*, *SDC1*, *SHG1*, *SPP1*, *SWD1*, and *SWD3*, respectively. This assay involves microarray hybridization to assess the relative prevalence of molecular markers for each mutant relative to the whole pool (Birrell *et al.* 2001; Game *et al.* 2003; Brown *et al.* 2006). While the assay is less rigorous than survival curves, none of the six COMPASS-component mutants tested in this way came within the top 20% of mutants ranked in the pool for IR sensitivity (Brown *et al.* 2006), collectively arguing strongly against a significant role for the COMPASS complex in ensuring diploid survival after IR. Additional observations based on qualitative assays of replica plates with patches of haploid cultures of the same mutants also showed no evidence of sensitivity. To confirm lack of sensitivity, we assayed survival *vs.* dose for one of these mutants, the *MAT*α haploid deleted for the *SWD1* gene, and found sensitivity equivalent to that of wild type (Figure 1).

To test for a role for H3 K36 methylation in IR resistance, we studied the *set2*Δ mutant, since Set2p is responsible for methylating this residue (Strahl *et al.* 2002). We observed that the *set2*Δ *MAT*α haploid strain showed mild X-ray sensitivity in survival curves, which was reproducible in *set2*Δ spore clones derived from a

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TABLE 1

Meiotic spore viability and cosegregation data for four deletion-mutant heterozygous diploids

Gene name	Systematic name	% spore viability	No. tetrads obtained with four live spore clones	No. of tetrads showing 2+:2− cosegregation for geneticin resistance and IR sensitivity
BRE1	YDL074C	95	22	22
LGE1	YPL055C	94.1	11	11
RTF1	YGL244W	98.1	25[a]	24[b]
PAF1	YBR279W	96.3	60	No IR sensitivity. 57 showed 2+:2− segregation for geneticin resistance and small colony size.[c]

[a] Data combined from the initial cross and a follow-up cross using a spore clone from the first cross.
[b] A single tetrad appeared to show three X-ray-sensitive spore clones; see text.
[c] Two 1:3 and one 4:0 tetrads were observed, with each spore clone consistent with cosegregation for colony size and geneticin resistance.

backcross of this strain to wild type (Figure 1). A clear segregation for sensitivity in this cross was difficult to observe on replica plates, although segregation for a borderline X-ray-sensitive phenotype was apparent after 1540 Gy of X-rays. A homozygous *set2*Δ/*set2*Δ diploid strain constructed from our spore segregants also showed a small increase in sensitivity compared to a wild-type diploid (not shown). In addition, the *set2* diploid from the deletion library pool showed a ranking of 105 for relative growth after IR treatment (Brown *et al.* 2006), consistent with mild sensitivity. We conclude that methylation of histone H3 K36 plays at least a minor role in resistance to radiation.

Mutants defective in histone H2 K123 ubiquitination are X-ray sensitive: We reported earlier (Game *et al.* 2005) that either yeast strains deleted for the *DOT1* gene, whose product methylates the histone H3 K79 residue (Ng *et al.* 2002b), or yeast strains in which the H3 K79 residue is altered to another amino acid, showed sharply increased X-ray sensitivity compared to wild type. At the same time, work from other laboratories showed that methylation of histone H3 K79 as well as H3 K4 is dependent on prior ubiquitination of histone H2B at residue K123 (Briggs *et al.* 2002; Ng *et al.* 2002a). The H2B K123 ubiquitination reaction has been shown to result from the combined action of the *RAD6*-encoded ubiquitin conjugase and the *BRE1*-encoded ubiquitin ligase (Robzyk *et al.* 2000; Hwang *et al.* 2003). IR sensitivity in *rad6* mutants was first reported in 1968 (Cox and Parry 1968) and is well known (Game and Mortimer 1974; Lawrence 1994) but has been thought previously to result from the interaction of Rad6p with Rad18p and their joint role in the ubiquitination of proliferating cell nuclear antigen (PCNA) (Hoege *et al.* 2002). The *dot1*Δ mutant's IR sensitivity implied that the role of *RAD6* in H2B K123 ubiquitination might also contribute to the sensitivity conferred by *rad6*Δ, and we anticipated that *bre1*Δ itself should confer X-ray sensitivity comparable to that conferred by *dot1*Δ but less than that conferred by *rad6*Δ. In addition, deletion mutations involving the *LGE1*, *RTF1*, and *PAF1* genes have also each been reported to abolish H2B K123 ubiquitination (Hwang *et al.* 2003; Ng *et al.* 2003a; Wood *et al.* 2003b) and hence might also be expected to confer IR sensitivity. While Lge1p directly interacts with Bre1p, both Rtf1p and Paf1p are members of the separate Paf1/RNA polymerase II complex and may have additional effects on other histone modifications (Mueller and Jaehning 2002; Krogan *et al.* 2003). We therefore tested X-ray sensitivity in *bre1*Δ, *lge1*Δ, *rtf1*Δ, and *paf1*Δ deletion strains.

Initial plate tests indicated that *MAT*α haploid strains carrying any of *bre1*Δ, *lge1*Δ, or *rtf1*Δ showed clearly increased X-ray sensitivity compared to wild type, whereas the *paf1*Δ mutant showed at most marginal sensitivity. The *bre1*Δ, *lge1*Δ, and *rtf1*Δ diploids from the deletion library pool show rankings of 36, 51, and 32, respectively, for relative growth after IR treatment (Brown *et al.* 2006), consistent with sensitivity and in the same range as the *dot1*Δ mutant (rank 42). The *paf1*Δ mutant, which has a slow-growth phenotype (Shi *et al.* 1996), is not ranked in the pool assay. As done with other mutants from the library (Game *et al.* 2005), we backcrossed each of the mutant *MAT*α haploid strains to a wild-type strain (g1201-4C) carrying the same genetic background as the deletion library to confirm haploidy and to test whether the X-ray-sensitive phenotype cosegregates with the geneticin-resistance phenotype marking the known deletion mutation. Results are shown in Table 1, where it can be seen that a cosegregation for both phenotypes occurs in crosses of *bre1*Δ, *lge1*Δ, and *rtf1*Δ, confirming that the deletion itself is responsible for conferring the X-ray sensitivity. For *paf1*Δ, no segregation for X-ray sensitivity was apparent, but a convincing cosegregation was observed for the geneticin-resistance marker and a slow-growth phenotype conferred by the original mutant (see Shi *et al.* 1996).

Next, to quantify sensitivity, we performed X-ray survival curves for at least two haploid strains carrying each of the deletion mutations, using the original strains

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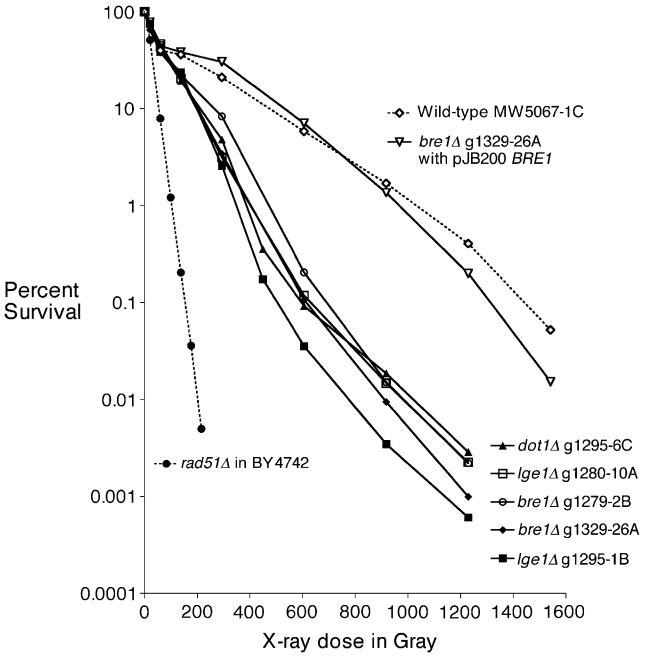

FIGURE 2.—Survival *vs.* X-ray dose for two *bre1*Δ and two *lge1*Δ haploid deletion strains. Wild-type, *rad51*Δ, and *dot1*Δ haploids are included for comparison. In addition, a curve for the *bre1*Δ strain g1329-26A transformed with a plasmid containing the *BRE1* gene and its native promoter (pJB200 from James Brown at Stanford University) is shown.

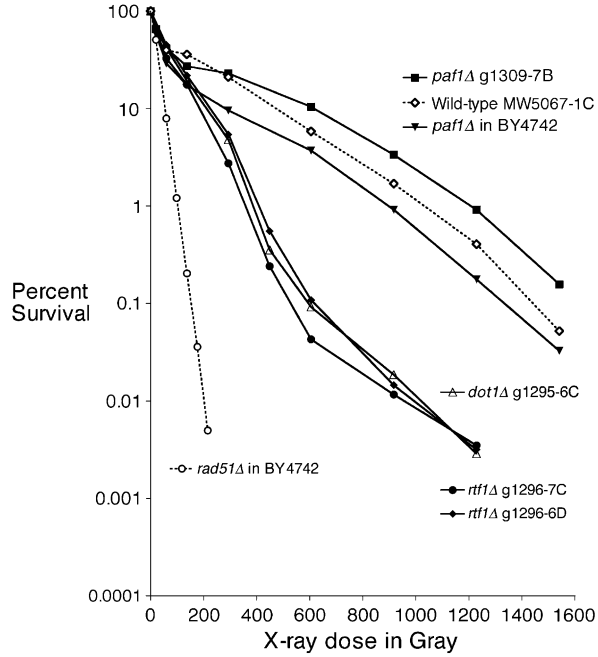

FIGURE 3.—Survival *vs.* X-ray dose for two *rtf1*Δ and two *paf1*Δ haploid deletion strains. Wild-type, *rad51*Δ, and *dot1*Δ haploids are included for comparison.

from the deletion library and one or more spore clones derived from the crosses to wild type. Results are shown in Figures 2 and 3, where an additional *dot1*Δ mutant survival curve is shown for comparison. It can be seen that the *bre1*Δ, *lge1*Δ, and *rtf1*Δ mutations confer sensitivity comparable to that seen in *dot1*Δ strains, consistent with a repair defect that in each case arises from abolition of the Dot1p-mediated histone H3 K79 methylation. In the case of *BRE1*, we further confirmed that the deletion itself conferred the IR sensitivity by transforming a *bre1*Δ strain with a plasmid containing the *BRE1* gene and finding that this restored wild-type resistance (Figure 2). Surprisingly, however, the *paf1* deletion mutant shows a wild-type response to IR, in contrast to the other three mutants (Figure 3). We considered that the lack of sensitivity of *paf1*Δ might arise from a secondary mutation in the mutant strain acting as a suppressor or modifier, but rejected this as unlikely when we found a uniform lack of sensitivity in *paf1*Δ spore clones segregating from a cross with wild type, as judged from irradiated replica plates. A survival curve of one of these *paf1*Δ spore clones shown with the original mutant in Figure 3 resembles wild type, and a curve from another spore clone (not shown) was equivalent. We note that Paf1p has multiple functions in addition to facilitating H2 K123 ubiquitination (CHANG *et al.* 1999; KROGAN *et al.* 2003; MUELLER *et al.* 2004; SHELDON *et al.* 2005), and it seems possible that the slow-growth phenotype of *paf1*Δ counteracts the expected IR sensitivity, as discussed later.

Mutants defective in histone H2 K123 ubiquitination or H3 K79 methylation interact epistatically for IR sensitivity: If IR sensitivity in mutants defective in H2B K123 ubiquitination arises from their downstream effects on H3 K79 methylation, then combining *bre1*Δ and *lge1*Δ with each other or with *dot1*Δ should add no additional sensitivity. We constructed strains with each of the double-mutant genotypes involving these three genes, as well as triple-mutant strains. Figures 4–6 show that all three genes interact epistatically. The data are compelling for *bre1*Δ *lge1*Δ (Figure 4), *bre1*Δ *dot1*Δ (Figure 5), and the *bre1*Δ *dot1*Δ *lge1*Δ triple-mutant strain (Figure 6). For *dot1*Δ *lge1*Δ strains, we observed some scatter among strains of equivalent genotype, with two strains showing possibly increased sensitivity and a third falling closer to the single mutants (Figure 6).

Both *rad6*Δ and *rad51*Δ are epistatic to *dot1*Δ: Most previously characterized mutants that show substantial X-ray sensitivity in Saccharomyces either are defective in HRR, mediated by *RAD51, RAD52*, and related genes, or are defective in one or more aspects of postreplication repair/translesion synthesis that are dependent on the *RAD6* and *RAD18* genes.

Mutants in the latter group, including *rad6*Δ and *rad18*Δ, confer additional sensitivity in double-mutant combinations with *rad51*Δ (MCKEE and LAWRENCE 1980; GAME 2000; see Figure 7), supporting the view that these repair processes are essentially separate. However, *rad6*Δ mutants show substantially greater X-ray sensitivity than *rad18*Δ mutants (see Figure 8), although *rad6*Δ and *rad18*Δ mutants are equally defective in ubiquitination of PCNA, which is a prerequisite for the subsequent

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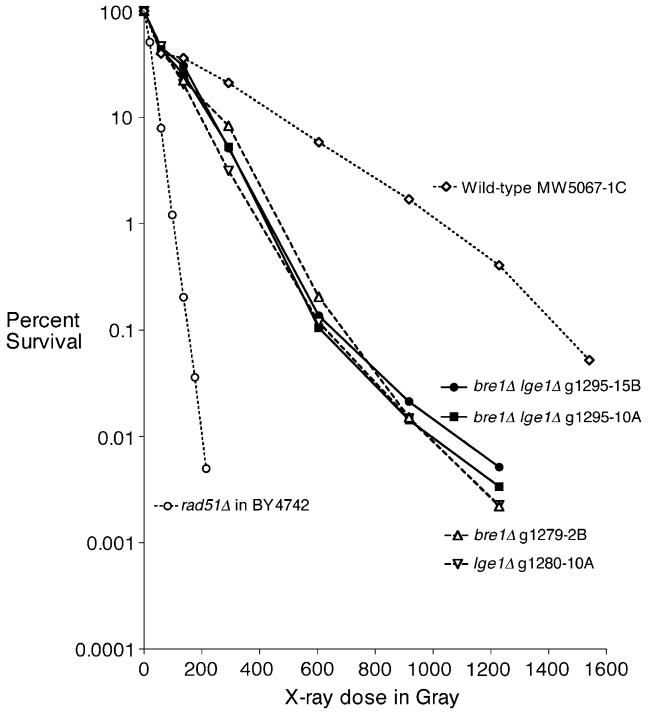

FIGURE 4.—Survival *vs.* X-ray dose for two *bre1*Δ *lge1*Δ double-mutant haploid deletion strains, shown with representative *bre1*Δ and *lge1*Δ single mutants (see Figure 2). Wild-type and *rad51*Δ haploids are included for comparison.

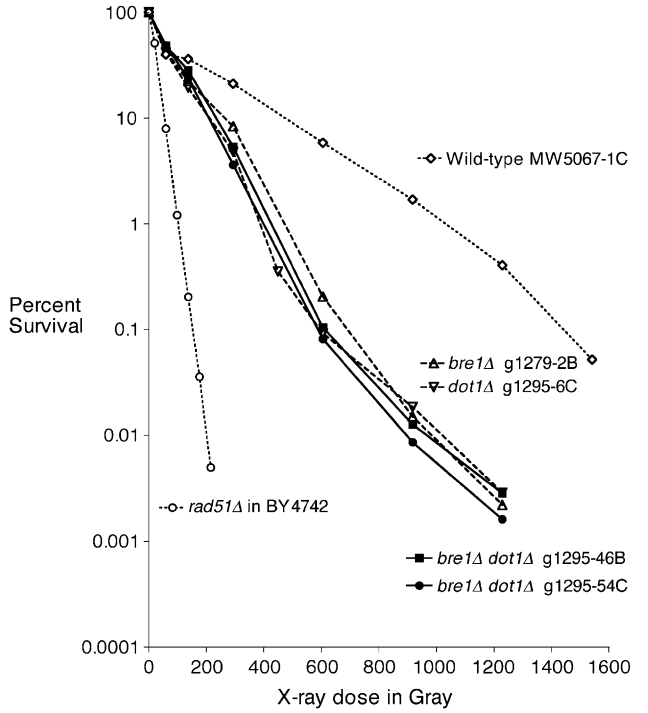

FIGURE 5.—Survival *vs.* X-ray dose for two *bre1*Δ *dot1*Δ double-mutant haploid deletion strains, shown with representative *bre1*Δ and *dot1*Δ single mutants (see Figure 2). Wild-type and *rad51*Δ haploids are included for comparison.

steps of postreplication repair/translesion synthesis (HOEGE *et al.* 2002; STELTER and ULRICH 2003; HARACSKA *et al.* 2004). This suggests an additional role for *RAD6* in mediating IR resistance outside the PCNA ubiquitination pathway. Further support for a separate role for *RAD6* in DNA transactions may come from the fact that *rad6* mutants are completely defective in meiotic division and fail to commit to meiotic recombination (GAME *et al.* 1980), whereas *rad18* mutants show little if any meiotic phenotype (GAME and MORTIMER 1974; DOWLING *et al.* 1985).

Given the X-ray sensitivity of *bre1*Δ, we anticipated that this additional role for *RAD6* could be mediated by its involvement in H3 K79 methylation through its function with *BRE1* in H2B K123 ubiquitination, and this in turn could involve the *RAD51*-dependent HRR pathway. We therefore constructed double mutants involving *dot1*Δ with *rad6*Δ and with *rad51*Δ. Figure 7 shows that *dot1*Δ confers no additional X-ray sensitivity when combined with either *rad6*Δ or *rad51*Δ. However, we and others have observed that *rad6*Δ single mutants tend to vary in radiation sensitivity and to quickly pick up modifier mutations, especially in the *SRS2* gene (SCHIESTL *et al.* 1990). To address possible variation here, we performed seven survival assays involving six *rad6*Δ strains. In comparing double mutants with r*ad6*Δ, we show either the curve with the median IR sensitivity (Figures 8, 9, 12, and 17) or a *rad6*Δ strain from the same cross as the double mutant to which we compare it (Figure 7). Also, we show both the most sensitive and the least sensitive of the six strains in Figure 10. The *dot1*Δ *rad6*Δ double mutant (g1238-2B, Figure 7) has a sensitivity equivalent to the most related *rad6*Δ single mutant (g1238-7B, Figure 7) and very similar to that of the median *rad6*Δ strain (MW5094-8A, shown on the same scale in Figure 17). Hence, we conclude that *DOT1* mediates a pathway of radiation resistance that requires the *RAD6* gene but also facilitates HRR, thus demonstrating a role for *RAD6* in enabling effective HRR.

The *bre1*Δ, *lge1*Δ, and *dot1*Δ mutations add extra IR sensitivity when combined with the *rad18*Δ mutation: Given that the HRR mutation *rad51*Δ is epistatic to *dot1*Δ, we expected that the latter mutation would confer increased sensitivity in double-mutant combinations with *rad18*Δ, since *RAD18* is known to act in postreplication repair (PRR) and itself interacts additively with mutants in HRR (MCKEE and LAWRENCE 1980; GAME 2000). Figures 8 and 9 show that there is a strong, rather similar increase in sensitivity in each of the double mutants that we constructed involving *rad18*Δ with *bre1*Δ, *lge1*Δ, or *dot1*Δ compared to the component single mutants. This both confirms that histone H3 K79 methylation is not involved in PRR and supports the functional separation of PRR from the HRR pathway. Figure 10 shows that the *dot1*Δ *bre1*Δ *rad18*Δ and *dot1*Δ *lge1*Δ *rad18*Δ triple mutants as well as a quadruple mutant involving *bre1*Δ, *lge1*Δ, and *dot1* with *rad18*Δ fall within the range of these doubles, further confirming epistasis

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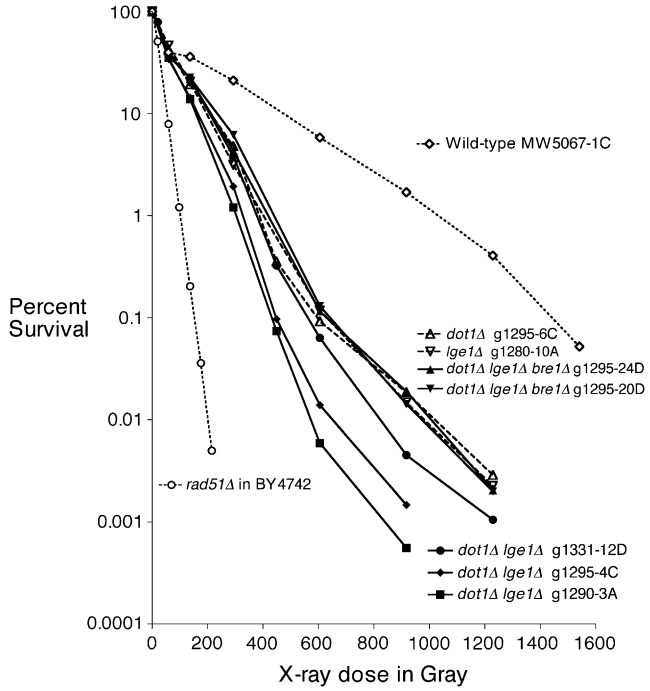

FIGURE 6.—Survival *vs.* X-ray dose for three *lge1*Δ *dot1*Δ double-mutant haploid deletion strains and two *bre1*Δ *lge1*Δ *dot1*Δ triple-mutant strains, shown with a representative *bre1*Δ and *lge1*Δ single mutant (see Figure 2). Wild-type and *rad51*Δ haploids are included for comparison.

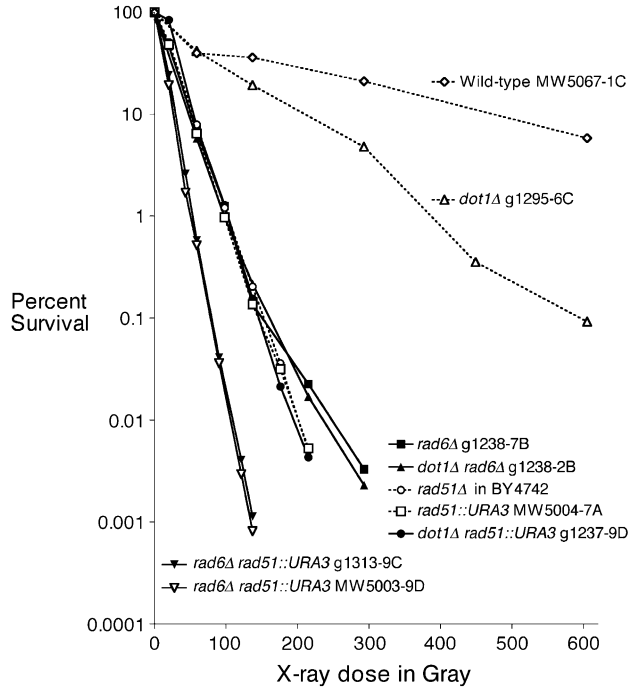

FIGURE 7.—Survival *vs.* X-ray dose for *dot1*Δ *rad6*Δ, *dot1*Δ *rad51::URA3* and two *rad6*Δ *rad51::URA3* double-mutant haploid deletion strains together with *dot1*Δ, *rad6*Δ, and *rad51::URA3* single mutants and a wild-type strain. A *rad51*Δ BY4742 strain carrying the standard deletion library replacement cassette, which exhibits IR sensitivity equivalent to that conferred by the *rad51::URA3* disruption allele, is also shown.

of *bre1*Δ, *lge1*Δ, and *dot1*Δ. It is noteworthy that these strains, and specifically the *bre1*Δ *rad18*Δ double mutant (Figure 8), which is defective in ubiquitination of two separate repair-involved targets of the *RAD6* ubiquitin ligase, resemble the *rad6*Δ single mutant in sensitivity. The median curve in Figure 8 as well as the *rad6*Δ curves in Figure 10 confirm that the additional sensitivity of the *rad6*Δ single mutant compared to the *rad18*Δ mutant can be accounted for by the role of *RAD6* in the *BRE1*-mediated histone ubiquitination step. However, as noted below, we also tested the N-end rule protein ubiquitination activity of *RAD6* for a possible effect on IR resistance by studying *ubr1*Δ mutant strains.

A role in IR repair for the *RAD6*-dependent *UBR1* ubiquitin ligase: The *UBR1* gene encodes the ubiquitin ligase that interacts with Rad6p in its major role in poly-ubiquitinating proteins targeted for degradation according to the N-end rule (DOHMEN *et al.* 1991). This pathway is not specific to DNA repair, but *ubr1*Δ mutants have been found to affect chromosome stability, probably through an indirect effect on sister-chromatid cohesion by affecting the degradation pathway for cohesin (RAO *et al.* 2001). The *ubr1*Δ diploid from the deletion library pool showed a ranking of 38 for relative growth after IR treatment (BROWN *et al.* 2006), consistent with IR sensitivity, and we found a mildly increased sensitivity in *ubr1*Δ haploid survival curves, as shown in Figure 11. A mild sensitivity on plate tests appeared to cosegregate with the *ubr1*Δ allele in crosses (not shown). To determine if the sensitivity is manifested through an effect on the *RAD18* or *BRE1* pathways or perhaps neither of these, we made double and triple mutants involving *ubr1*Δ, *rad18*Δ, and *bre1*Δ. We found little or no increased sensitivity in *bre1*Δ *ubr1*Δ double mutants (Figure 11), but a significant increase in *rad18*Δ *ubr1*Δ doubles (Figure 12). This enhancement of *rad18*Δ sensitivity is consistent with a role for *UBR1* in HRR, as might be expected from the reported effects of *ubr1*Δ on chromosome stability and cohesin degradation (RAO *et al.* 2001). Given the mild sensitivity of *ubr1*Δ, it is less compelling that *BRE1* is really epistatic to *UBR1*. However, the *rad18*Δ *bre1*Δ *ubr1*Δ triple mutants shown in Figure 12 resemble the *rad18*Δ *bre1*Δ double mutant as well as the *rad6*Δ single mutant. We expect this triple-mutant genotype to mimic *rad6*Δ since it should lack all three known ubiquitination activities that *RAD6* mediates, but a potential contribution to IR sensitivity from *ubr1*Δ in the triple mutant might be difficult to discern in the context of the high sensitivity of the *rad18*Δ *bre1*Δ double mutant, which already resembles *rad6*Δ (see above).

The *rad5*Δ mutation is additive for IR sensitivity with *bre1*Δ and with *rad18*Δ: The Rad5 protein acts downstream from Rad18p in the ubiquitination steps of PCNA and thereby plays a major role in postreplication repair (HOEGE *et al.* 2002; TORRES-RAMOS *et al.* 2002).

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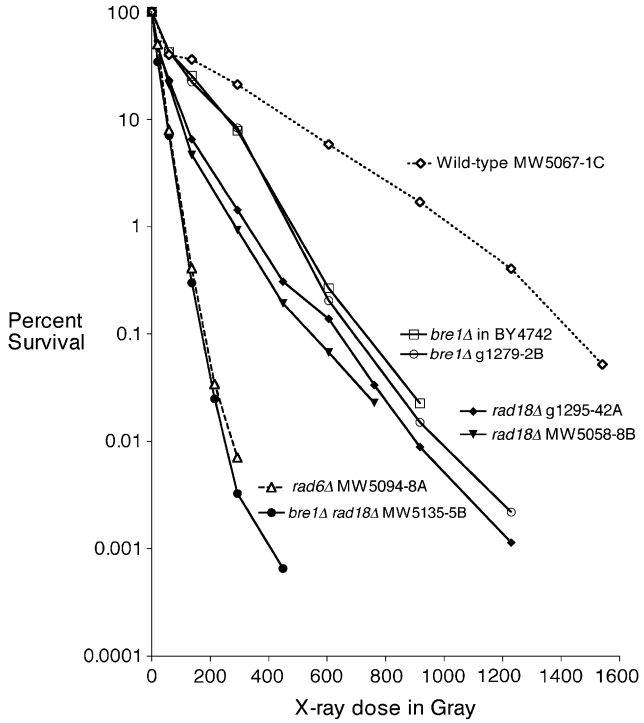

FIGURE 8.—Survival *vs.* X-ray dose for a *bre1*Δ *rad18*Δ double-mutant strain compared to two *bre1*Δ and two *rad18*Δ single mutants. A wild-type and a *rad6*Δ strain are included for comparison. This is the median *rad6*Δ curve of seven obtained; see text.

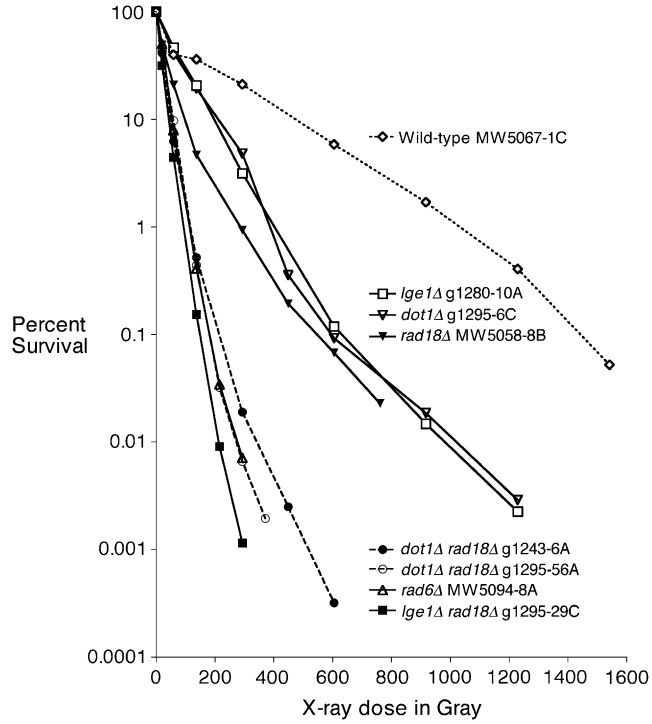

FIGURE 9.—Survival *vs.* X-ray dose for two *dot1*Δ *rad18*Δ and one *lge1*Δ *rad18*Δ double-mutant strain compared to *dot1*Δ, *lge1*Δ, and *rad18*Δ single mutants. A wild-type and a *rad6*Δ strain are included for comparison.

However, while *rad5*Δ and *rad18*Δ interact epistatically with respect to UV sensitivity (JOHNSON *et al.* 1992; this study; data not shown), we observed an additive response for IR sensitivity (Figure 14), in agreement with other reports (FRIEDL *et al.* 2001; CHEN *et al.* 2005). In addition, CHEN *et al.* (2005) presented data showing that *RAD5* has another function that contributes to IR resistance independently of its PCNA-modifying role and is probably related to an *MRE11/RAD50/XRS2*-mediated repair activity. To study *RAD5* in relation to the *BRE1/DOT1* pathway, we constructed *rad5*Δ *bre1*Δ and *rad5*Δ *dot1*Δ double mutants. Figures 13 and 14 show that the *rad5*Δ mutation adds sensitivity to *bre1*Δ and *dot1*Δ as well as to *rad18*Δ. When taken with data for *rad18*Δ combined with *bre1*Δ and *dot1*Δ (Figures 8 and 9), this implies that *RAD5*, *RAD18*, and *BRE1/DOT1* mediate three at least partly independent IR resistance mechanisms. Surprisingly, the *dot1*Δ *rad5*Δ *rad18*Δ triple shows only slight sensitivity beyond each of the component double mutants (Figure 14). It is difficult to assess the significance of this, but it seems less sensitive than would be expected from double-mutant data. Roles for Rad5p in more than one IR repair pathway might account for this, as discussed later.

IR epistasis and colony-size effects of *rad50*Δ with *bre1*Δ and *dot1*Δ: We constructed double and triple mutants involving *bre1*Δ, *dot1*Δ, and *rad50*Δ to test whether the Mre11/Rad50/Xrs2 complex (MRX) is involved in repair affected by histone H3 K79 methylation. Figure 15 shows that combining *bre1*Δ, *dot1*Δ, or both mutants with *rad50*Δ adds no further sensitivity to *rad50*Δ alone, as might be expected from the role of MRX in HRR (BRESSAN *et al.* 1999; GAME 2000) and our double-mutant data with *rad51*Δ. As discussed later, there is no support for a separate IR damage repair role involving nonhomologous end-joining (NHEJ) from these data, since the strains in Figure 15 have survival curves equivalent to those of the *rad51*Δ mutant included for comparison. However, we did observe a strong effect of the *rad50*Δ *bre1*Δ double mutant and the *rad50*Δ *bre1*Δ *dot1*Δ triple-mutant genotypes on the colony size of meiotic spore clones, which was sharply reduced compared to that of other spore clones in the same cross. This implies a slow-growth phenotype presumably caused by interaction of *rad50*Δ with *bre1*Δ and confirms similar findings from large-scale random spore analysis (TONG *et al.* 2004). Since this phenotype was absent in our *rad50*Δ *dot1*Δ double-mutant spore clones, it is presumably not mediated by abrogation of H3 K79 methylation. However, synthetic lethality has been reported (TONG *et al.* 2004) between *rad50*Δ and two mutants for genes in the COMPASS complex, *swd3*Δ and *bre2*Δ, responsible for methylating the histone H3 K4 residue (KROGAN *et al.* 2002). Since di- and trimethylation of this residue depends on histone H2 K123 ubiquitination, it is plausible that the slow-growth phenotype of *rad50*Δ *bre1*Δ double

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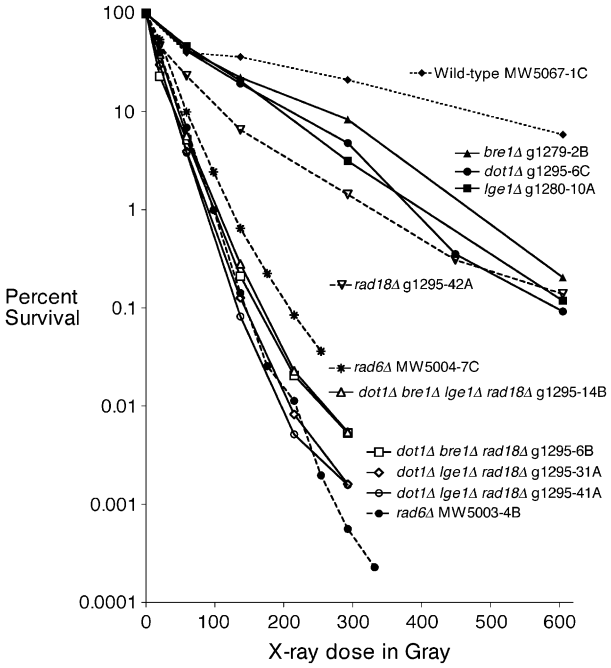

FIGURE 10.—Survival *vs.* X-ray dose for two *dot1*Δ *lge1*Δ *rad18*Δ and one *dot1*Δ *bre1*Δ *rad18*Δ triple-mutant strain and a *dot1*Δ *bre1*Δ *lge1*Δ *rad18*Δ quadruple mutant. These strains are compared with wild type, the four component single mutants, and two *rad6*Δ strains representing the most and least sensitive full curves of seven *rad6*Δ curves obtained; see text.

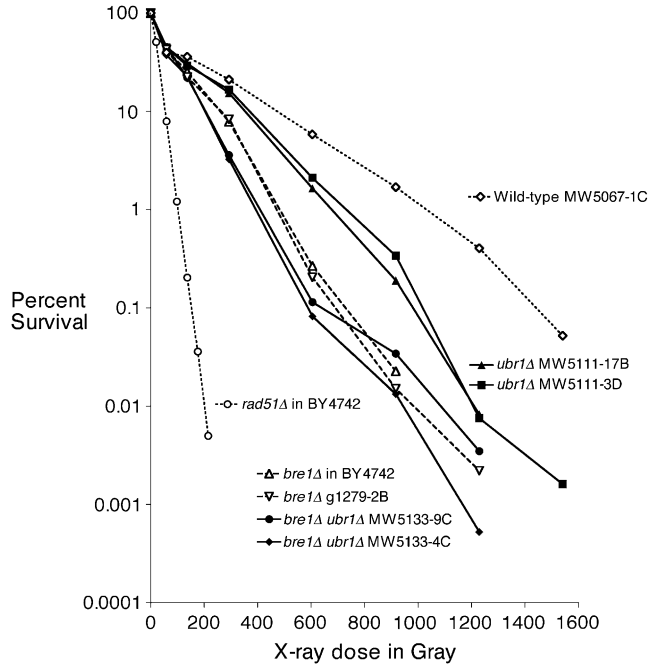

FIGURE 11.—Survival *vs.* X-ray dose for two *ubr1*Δ haploid deletion strains and two *bre1*Δ *ubr1*Δ double mutants, compared with two *ubr1*Δ and two *bre1*Δ single mutants. A wild-type and a *rad51*Δ haploid strain are also shown.

mutants also arises from the impact of *bre1*Δ on H3 K4 methylation.

A role for the *BRE1/DOT1* pathway in IR-damage-induced checkpoint control: While this work was in progress, several reports suggested that histone H2B K123 ubiquitination and histone H3 K79 methylation are important for checkpoint arrest after DNA damage. It was recently shown that the 53BP1 checkpoint protein in mammalian cells recognizes and binds to methylated histone H3 K79 residues and that the methylation is important for attracting 53BP1 to double-strand-break (DSB) sites (HUYEN *et al.* 2004). Saccharomyces Rad9 protein, which has a central role in establishing checkpoint delays after irradiation (WEINERT and HARTWELL 1988, 1989; SIEDE *et al.* 1993), shares homologous domains with 53BP1, including a recently described major domain very similar to the Tudor domain in 53BP1 that interacts with methylated mammalian H3 K79 (ALPHA-BAZIN *et al.* 2005). Hence, the mammalian 53BP1 findings are strongly suggestive of a role for H3 K79 methylation in *RAD9*-mediated checkpoints in yeast. In addition, others have shown directly that *rad6*Δ, *bre1*Δ, and *dot1*Δ mutants reduced or abolished the checkpoint delay seen in wild type after UV- and chemical DNA-damaging treatments in G_1 and intra-S phase cells without affecting the G_2 checkpoint (GIANNATTASIO *et al.* 2005). These authors also showed that phosphorylation of Rad9 protein was reduced or abolished in these mutants after similar DNA-damaging treatments, leading in turn to defective activation of Rad53 checkpoint protein. Most recently, WYSOCKI *et al.* (2005) identified *dot1*Δ in a screen for mutants that abrogate the G_1 damage checkpoint. Surprisingly, however, using

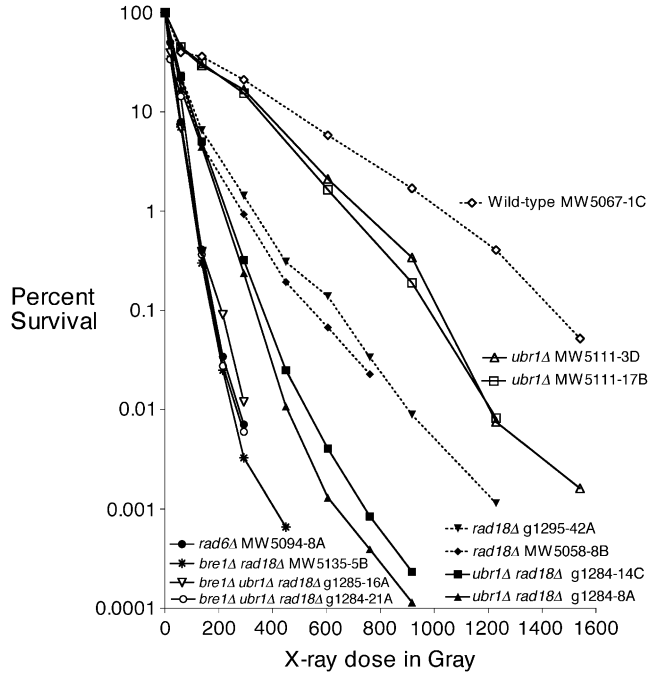

FIGURE 12.—Survival *vs.* X-ray dose for two haploid *ubr1*Δ *rad18*Δ double mutants and two *bre1*Δ *ubr1*Δ *rad18*Δ triple-mutant strains, compared with two *rad18*Δ and two *ubr1*Δ single mutants. A wild-type strain, a *bre1*Δ *rad18*Δ double mutant, and a *rad6*Δ strain (see also Figure 8) are also shown.

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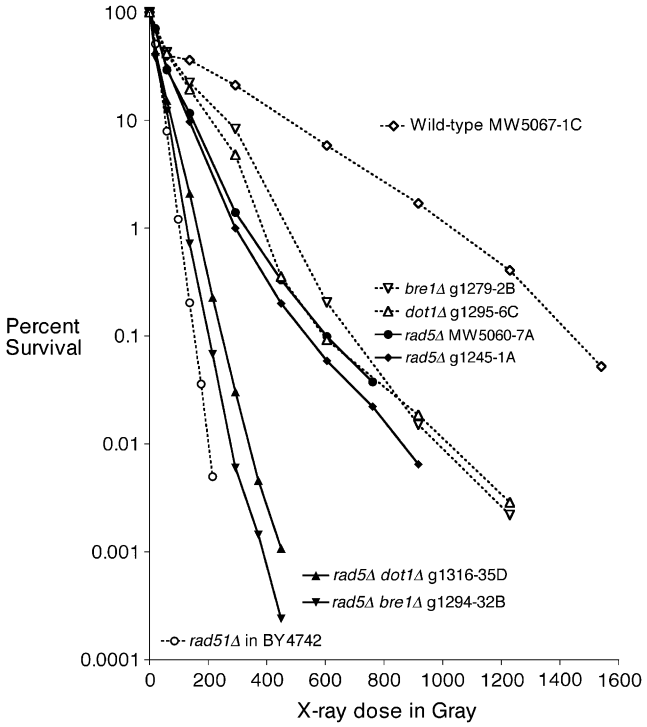

FIGURE 13.—Survival *vs.* X-ray dose for two haploid *rad5*Δ mutant strains, a *bre1*Δ *rad5*Δ double mutant and a *dot1*Δ *rad5*Δ double mutant. A *bre1*Δ and a *dot1*Δ mutant are shown for comparison, together with wild type and a *rad51*Δ strain.

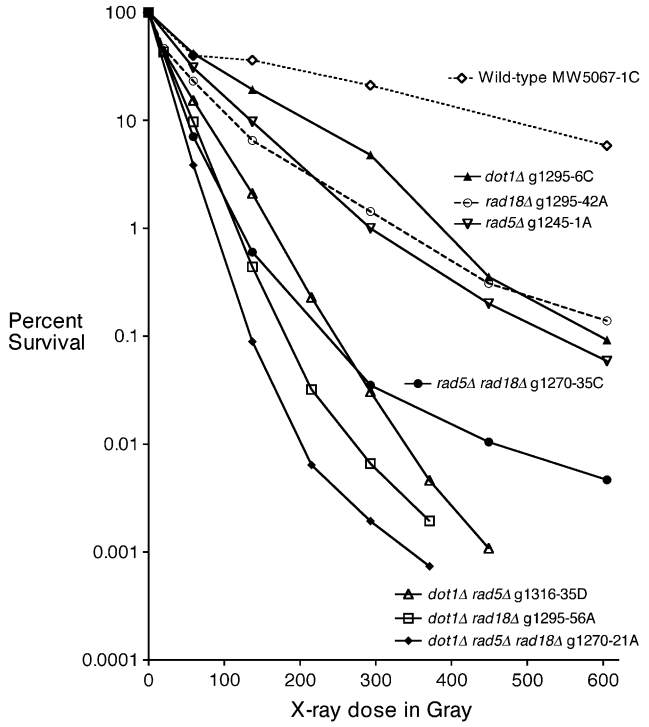

FIGURE 14.—Survival *vs.* X-ray dose for a *rad5*Δ *rad18*Δ double mutant and a *dot1*Δ *rad5*Δ *rad18*Δ triple mutant, compared to the component single mutants and a *dot1*Δ *rad18*Δ and *dot1*Δ *rad5*Δ double mutant (see also Figures 9 and 13). A wild type and a *rad51*Δ curve are also shown.

more qualitative tests, these authors found little evidence of IR sensitivity in the *dot1*Δ mutant. Genetic differences in the strain backgrounds used probably account for these different results (see DISCUSSION). To investigate whether the substantial IR sensitivity of *bre1*Δ and *dot1*Δ mutants in our strains is conferred through an effect on checkpoint controls, and to extend the epistasis analysis, we studied double mutants involving *rad9*Δ as well as tested directly for abrogation of checkpoints in *bre1*Δ and *dot1*Δ mutants using IR.

Double-mutant analysis with *rad9*Δ: Since *RAD9* has a well-established role in DNA-damage-induced checkpoint controls (WEINERT and HARTWELL 1988, 1989; SIEDE *et al.* 1993), we anticipated that if *bre1*Δ confers sensitivity through abrogating one or more *RAD9*-dependent checkpoints, epistasis between *rad9*Δ and *bre1*Δ would be observed, whereas an additive response would suggest a different mechanism. Mutations in the *RAD9* gene are known to be IR sensitive (COX and PARRY 1968; GAME and MORTIMER 1974), but surprisingly, we could find little published information about the epistasis relationships of *rad9* in combination with mutants in other *RAD* genes. An exception is the *rad9*Δ *rad6*Δ combination, which has been reported to show additive sensitivity compared to the single mutants (SCHIESTL *et al.* 1989). It has also been shown that activation of some Saccharomyces HRR proteins after radiation is dependent on an intact *RAD9* gene (BASHKIROV *et al.* 2000) and that the ATM-mediated checkpoint is required for normal function of the *RAD54* HRR gene in chicken DT40 cells (MORRISON *et al.* 2000). We tested the double-mutant *rad9*Δ *bre1*Δ and the triple-mutant *rad9*Δ *bre1*Δ *dot1*Δ in the deletion library background, where each of the single mutants is sensitive, to determine whether *RAD9* affects the same pathway as *BRE1* and *DOT1*, as might be expected on the basis of results with mammalian cells (HUYEN *et al.* 2004).

We found that the *rad9*Δ single mutant is significantly more sensitive than the *bre1*Δ or *dot1*Δ strains, but that double or triple mutants involving *rad9*Δ with either or both of these two are no more X-ray sensitive than *rad9*Δ alone (Figure 16). This agrees with findings by WYSOCKI *et al.* (2005), who observed qualitatively that *dot1*Δ did not potentiate the sensitivity of *rad9*Δ in a different genetic background where there was little or no IR sensitivity of the *dot1*Δ single mutant. The epistasis implies either that the X-ray resistance mediated by *BRE1* and *DOT1* is dependent on a *RAD9*-mediated checkpoint or that the *RAD9* checkpoint itself is partially dependent on an intact *BRE1/DOT1*-mediated H3 K79 methylation pathway. In the latter case, which is consistent with data from mammalian cells (HUYEN *et al.* 2004), *RAD9* function is presumably only partly dependent on H3 K79 methylation, since substantial *RAD9*-dependent resistance remains in *bre1*Δ and *dot1*Δ single mutants (Figure 16).

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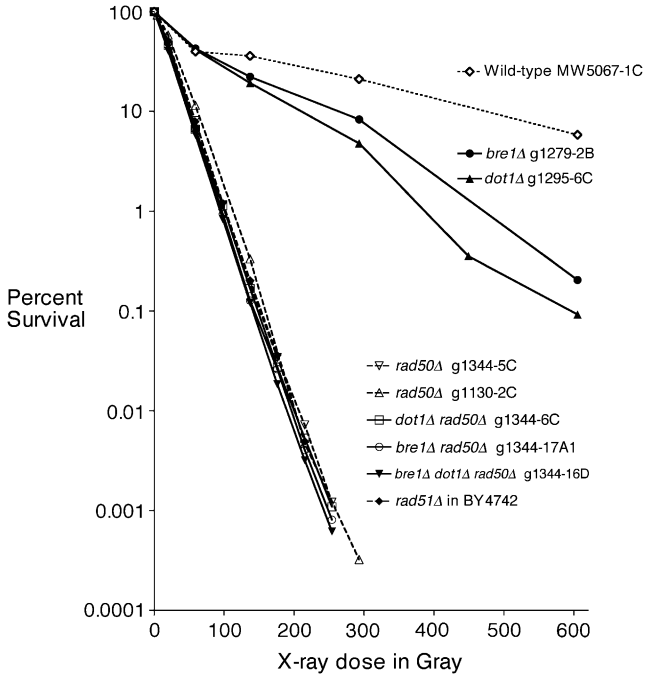

FIGURE 15.—Survival *vs.* X-ray dose for two *rad50*Δ mutants and a *dot1*Δ *rad50*Δ and a *bre1*Δ *rad50*Δ double mutant. A *rad51*Δ mutant, a wild type, and the *dot1*Δ and *bre1*Δ single mutants are included for comparison.

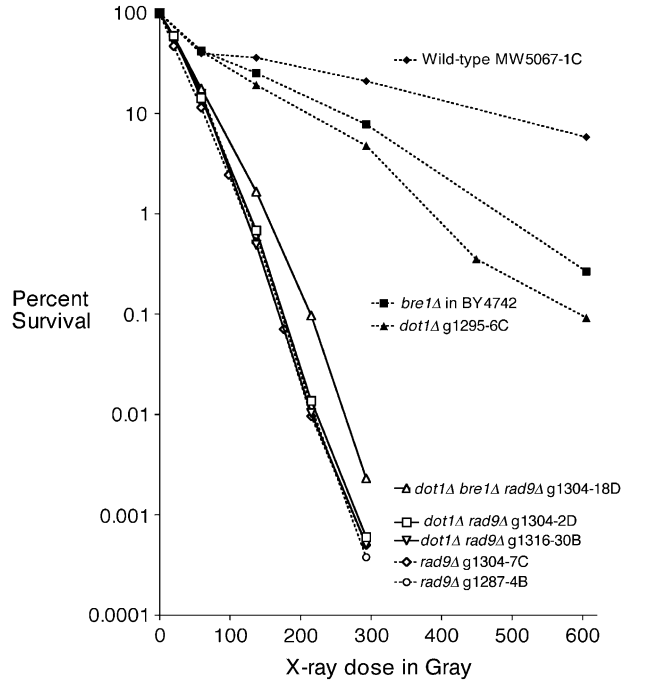

FIGURE 16.—Survival *vs.* X-ray dose for two *dot1*Δ *rad9*Δ double-mutant strains and a *dot1*Δ *bre1*Δ *rad9*Δ triple mutant, compared to two *rad9*Δ single mutants. Wild type and the *bre1*Δ and *dot1*Δ single mutants are included for comparison.

On the basis of our observations that *rad51null* and *rad9*Δ are each epistatic to *bre1*Δ and *dot1*Δ, we expected that *RAD9* itself would fall into the *RAD51* epistasis group with respect to X-ray sensitivity. This would also be consistent with reported additivity between *rad9*Δ and *rad6*Δ (SCHIESTL *et al.* 1989) since known mutants in HRR such as *rad51null* show increased sensitivity in combination with *rad6*Δ (Figure 17; see also MCKEE and LAWRENCE 1980; GAME 2000). Since we find no additivity between *bre1*Δ and *rad9*Δ, increased sensitivity contributed by *rad6*Δ in the *rad6*Δ *rad9*Δ double mutant seems likely to arise from the *RAD18*-dependent aspect of *RAD6* repair. To test this, we studied double mutants involving *rad9*Δ with *rad18*Δ, *rad51null*, and *rad5*Δ as well as retesting the *rad6*Δ *rad9*Δ combination.

We found a sharp increase in sensitivity in *rad9*Δ *rad18*Δ (Figure 18), a slightly lesser increase in *rad9*Δ *rad5*Δ (Figure 18), and, at most, only a slight increase in the *rad9*Δ *rad51null* strains (Figure 17), compared to the single mutants in each case. Hence *RAD18*-mediated repair seems to be largely independent of the *RAD9*-mediated checkpoint, whereas *RAD51*-mediated HRR is heavily dependent on *RAD9* function. As expected from these observations as well as previous work (SCHIESTL *et al.* 1989), we found that the *rad6*Δ *rad9*Δ double mutant also shows strongly increased IR sensitivity compared to the single mutants. In fact, this double mutant is equivalent to *rad6*Δ *rad51null* double mutants (Figure 17), again suggesting that *RAD9* is required for most or all *RAD51*-mediated IR recovery. The equivalent sensitivity of the *rad9*Δ *rad18*Δ double mutant to *rad9*Δ *rad6*Δ and *rad6*Δ *rad51null*, despite the lower sensitivity of *rad18*Δ compared to *rad6*Δ, also implies that *RAD9* is required for the part of *RAD6*-mediated resistance that is independent of *RAD18* and that this, in turn, is dependent on *RAD51*. The *rad9*Δ *rad6*Δ *rad51*Δ triple mutants in Figure 17 are possibly slightly more sensitive than the *rad6*Δ *rad51*Δ double mutants; hence a minor additional role for *RAD9* outside either PRR or HRR cannot be excluded. Interestingly, in double-mutant combinations both *rad9*Δ and *rad18*Δ also show increased sensitivity with *rad5*Δ, but the *rad5*Δ *rad9*Δ *rad18*Δ triple mutants in Figure 18 are no more sensitive than the *rad9*Δ *rad18*Δ double mutants, perhaps implying that *RAD5* acts in more than one pathway, as discussed later.

The *dot1*Δ and *bre1*Δ mutants are defective in the G_1 but not the G_2 IR-induced cell cycle checkpoint: Wild-type yeast cells irradiated in the G_2 phase of the cell cycle become arrested before proceeding through cell division. This arrest is dependent on the *RAD9* gene and is important for subsequent cell survival: *rad9* mutants substantially fail to arrest in G_2 after irradiation, and this is thought to be part of the reason for their increased killing by IR compared to wild type (WEINERT and HARTWELL 1988). Using the *rad9*Δ mutant and wild type as controls, we tested haploid *dot1*Δ and *bre1*Δ mutants for an effect on the IR-induced G_2 checkpoint by monitoring their ability to progress into mitosis following a nocodazole-induced accumulation in G_2. Figure 19 shows that, as expected, without irradiation, all four

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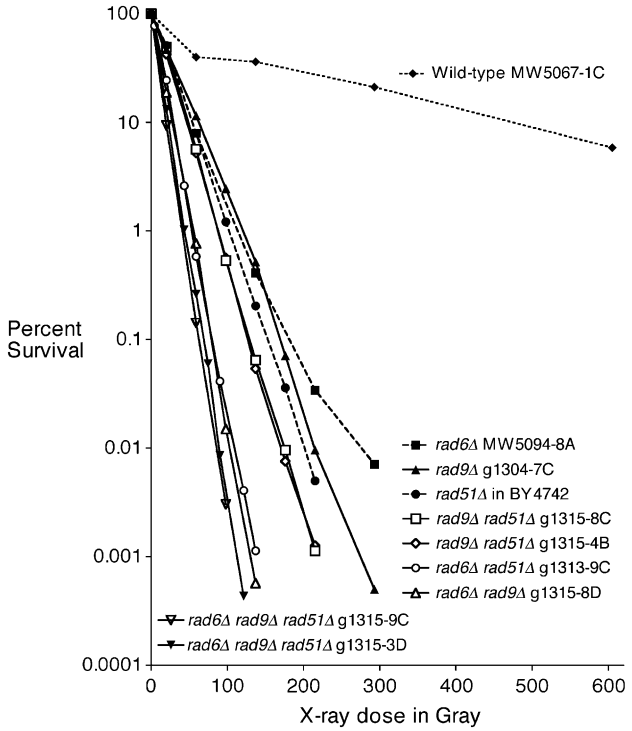

FIGURE 17.—Survival *vs.* X-ray dose for two *rad9*Δ *rad51::URA3* double mutants and one *rad9*Δ *rad6*Δ double-mutant and two *rad9*Δ *rad6*Δ *rad51::URA3* triple-mutant strains, compared with a *rad6*Δ *rad51::URA3* double mutant (see also Figure 5), a wild type, and the three component single mutants.

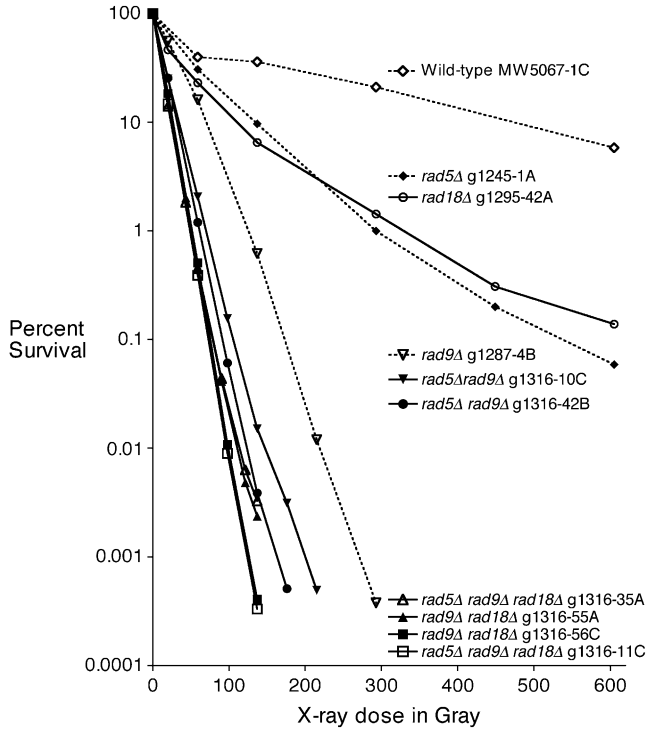

FIGURE 18.—Survival *vs.* X-ray dose for two *rad9*Δ *rad5*Δ and one *rad9*Δ *rad18*Δ double mutant and two *rad9*Δ *rad5*Δ *rad18*Δ triple mutants. Each single mutant and wild type are also shown for comparison.

strains promptly enter nuclear division when released from nocodazole. After 500 Gy of ^{137}Cs gamma irradiation, however, wild-type, *dot1*Δ, and *bre1*Δ cells remain arrested, with little sign of division up to 90 min following irradiation. In contrast, *rad9* shows significant escape from the G_2 checkpoint, although at this dose it too shows delayed division compared to the unirradiated control. Results are similar after 1000 Gy of radiation (not shown), and together these data indicate that *dot1*Δ and *bre1*Δ mutations do not significantly abrogate the IR-induced G_2 cell cycle checkpoint.

To assess radiation-induced G_1 arrest, we followed the cell cycle progression of *dot1*Δ and *bre1*Δ mutants released from α-factor-induced synchrony with and without 500 Gy of ^{137}Cs gamma irradiation and, as reported by others (GIANNATTASIO *et al.* 2005; WYSOCKI *et al.* 2005), observed a significant effect of both mutants in abrogating the arrest response seen in wild type (see Figure 20). In fact, both mutants are essentially equivalent in this phenotype to the *rad9* strain. We used *rad9* as a positive control because previous work (SIEDE *et al.* 1993) has shown that *RAD9* is involved in the G_1 checkpoint as well as in the G_2 checkpoint. The three strains differ from wild type, where IR-induced arrest can clearly be seen. Despite differences in IR sensitivity, our findings of a G_1 but not a G_2 checkpoint defect in *dot1*Δ or *bre1*Δ mutants in the deletion library background are thus consistent with recent findings by others in the W303 background (GIANNATTASIO *et al.* 2005; WYSOCKI *et al.* 2005).

DISCUSSION

We have found that Saccharomyces mutants that are unable to ubiquitinate the histone H2B lysine 123 residue are substantially sensitive to ionizing radiation. It has been shown elsewhere that ubiquitination of H2B K123 is required for completion of the subsequent methylation of histone H3 at its lysine 4 and lysine 79 (but not lysine 36) residues (BRIGGS *et al.* 2002; DOVER *et al.* 2002; NG *et al.* 2002a; SUN and ALLIS 2002; SHAHBAZIAN *et al.* 2005). We reported earlier (GAME *et al.* 2005) that abolishing methylation at H3 K79 also confers IR sensitivity, and it seems clear from data reported here and elsewhere that the IR sensitivity of mutants that abolish H2B K123 ubiquitination arises from this downstream effect on histone H3 K79 methylation. This is supported by the finding that the *bre1*Δ *dot1*Δ double mutant has the same sensitivity as the *bre1*Δ and *dot1*Δ single mutants (Figure 5). Moreover, the equivalent sensitivity of the *bre1*Δ and *dot1*Δ single mutants implies that all the sensitivity of mutants affected in H2B K123 ubiquitination can be accounted for in our strains by their secondary effects on H3 K79 methylation. This is consistent with the absence of the sensitivity of

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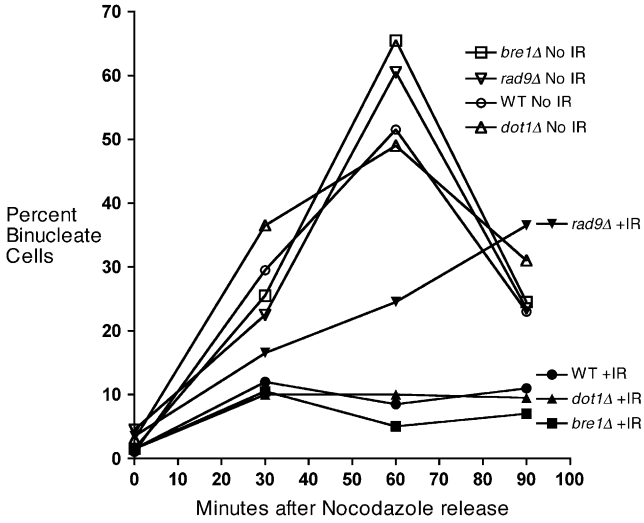

FIGURE 19.—Effect on the IR-induced G_2/M checkpoint of the *bre1*Δ, *dot1*Δ, and *rad9*Δ mutations compared to wild type. Cells were released from nocodazole synchronization into fresh medium at time zero after 500 Gy of ^{137}Cs gamma irradiation, and in parallel without irradiation. The percentage of cells that have undergone nuclear division is shown for each culture at 30-min intervals thereafter. Strains used were g1295-21C *bre1*Δ, g1295-6C *dot1*Δ, g1304-19C *rad9*Δ, and BY4741 wild-type *MAT***a**.

COMPASS mutants, which impact the other H3 methylation (K4) that is dependent on H2B K123 ubiquitination, and with separate IR sensitivity of *set2*Δ mutants, which impact H3 K36 methylation independently of H2B K123 ubiquitination. Since deleting *BRE1* impacts di- and trimethylation rather than mono-methylation of H3 K79 (SHAHBAZIAN *et al.* 2005), the IR sensitivity of the *bre1*Δ mutant indicates that wild-type IR resistance depends on di- or trimethylation rather than on mono-methylation of H3 K79 by Dot1p.

Surprisingly, the *paf1*Δ mutant fails to show increased IR sensitivity although it too has been reported to block H2B K123 ubiquitination (WOOD *et al.* 2003b) as well as H3 K79 methylation (KROGAN *et al.* 2003). If checkpoint defects are responsible for the sensitivity of the other mutants that abolish H2B K123 ubiquitination, then possibly the more severe slow-growth phenotype of *paf1*Δ strains compared to related mutants (MUELLER and JAEHNING 2002; this study) relieves their IR sensitivity by providing adequate time for repair even in the absence of normal checkpoints. In the case of *rad9* mutants, delaying the cell cycle artificially alleviates their IR sensitivity (WEINERT and HARTWELL 1988), and perhaps an analogous effect occurs spontaneously in *paf1*Δ strains. Alternatively, one of the other *paf1*Δ phenotypes may impact the need for H2B K123 ubiquitination or H3 K79 methylation in IR resistance in an unknown way. Although both Paf1p and Rtf1p are members of the PAF1/RNA polymerase II complex, their mutants differ in several aspects of their phenotypes, and surprisingly, it has been reported that knocking out *RTF1* in a *paf1*Δ background substantially reverses the slow-growth phenotype (MUELLER and JAEHNING 2002). Work with double mutants involving *paf1*Δ may clarify the reason for its IR resistance.

A strong increase in IR sensitivity is seen when *bre1*Δ, *dot1*Δ, or *lge1*Δ are combined with *rad18*Δ, but no such increase is seen in double mutants that we tested with *rad50*Δ or *rad51*Δ. This represents convincing evidence that these histone modification genes are not required for any of the several aspects of postreplication repair/translesion synthesis that are dependent on *RAD18*-mediated ubiquitination of PCNA. Rather, they are required for effective homologous recombination repair as mediated by the *RAD51* pathway, although clearly they are needed only for a subset of such repair, since their mutant strains are consistently less sensitive than *rad51*Δ or *rad50*Δ strains (Figures 2 and 15) and similar mutants (GAME 2000).

There is also strong evidence from this study and others that *bre1*Δ and *dot1*Δ mutations abrogate the radiation-induced checkpoint control in G_1 cells but leave the G_2 checkpoint largely unaffected. As a cause of IR sensitivity, this seems paradoxical, given that HRR does not occur in haploid G_1 cells. Also, our data suggest that in log-phase cultures the histone modification mutants, like others involved in HRR, are less affected in the initial slope of the curves representing mainly cells in G_1 than they are in what is the tailed part of the curve in wild type, which represents mainly G_2 cells (see Figures 2, 3, and 14; see also GAME 1983 for review). However, an effect on the intra-S checkpoint, as has been reported elsewhere (GIANNATTASIO *et al.* 2005; WYSOCKI *et al.* 2005), may contribute to the lethality that we observed, since HRR can occur not only in G_2 but also in S-phase. Alternatively, an additional mutant effect may cause lethality through impacting HRR directly without being strictly dependent on checkpoint controls. The epistasis between *rad9*Δ and *dot1*Δ indicates that all the IR resistance conferred by Dot1p depends on Rad9p, but on the basis of our data and that of others (BASHKIROV *et al.* 2000; MORRISON *et al.* 2000), *RAD9* itself may perhaps be considered a player in HRR as well as a checkpoint gene, since *rad9*Δ strains in our hands are as sensitive as mutants such as *rad51*Δ that effectively abolish HRR. In mammalian cells, the product of the 53BP1 gene (an ortholog of Saccharomyces *RAD9*) associates through its Tudor domain with methylated H3 K79, and it has been proposed that when DSBs occur, the preplaced methyl groups on this residue in the core of H3 become exposed near the DSBs, serving as a signal to bring Rad9p to the site (HUYEN *et al.* 2004). Clearly, Rad9p function is not exclusively dependent on this signal, since much Rad9p-dependent resistance remains in *dot1*Δ strains. Present results suggest that alternative mechanisms may exist at different stages of the cell cycle, but it is also possible that multiple DSB-signaling pathways occur in parallel or that only certain

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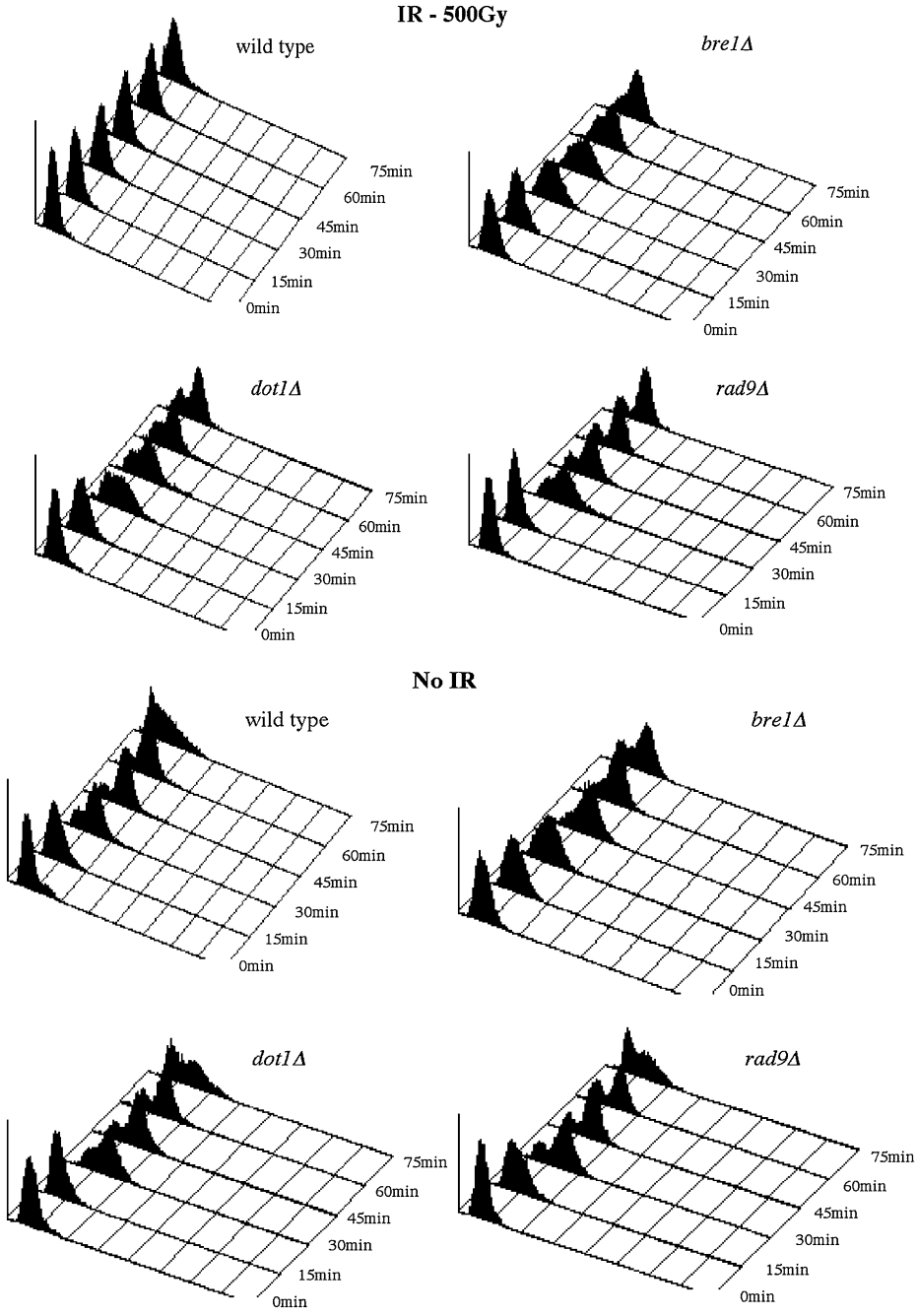

FIGURE 20.—Effect on the IR-induced G_1/S checkpoint of the *bre1*Δ, *dot1*Δ, and *rad9*Δ mutations compared to wild type. Cells were released from α-factor synchronization into fresh medium at time zero after 500 Gy of ^{137}Cs gamma irradiation, and in parallel without irradiation, and fixed and analyzed for DNA content with flow cytometry at 15-min intervals thereafter. Strains used for each genotype were as in Figure 19.

subsets of DSBs are dependent on H3 K79 trimethylation to bring about interaction with Rad9p. The Dot1p methylase is important in differentiating euchromatin from heterochromatin (NG *et al.* 2003b), and it seems possible that there are positional impacts on DSB repair that depend on this effect. Currently, rapid progress is being made in understanding the early steps in chromatin changes that occur at DSB sites and lead to the formation of foci containing phosphorylated histone H2A (in Saccharomyces) or H2A.X (in mammals) (TSUKUDA *et al.* 2005; VAN ATTIKUM and GASSER 2005; NUSSENZWEIG and PAULL 2006). However, the spatial, temporal, and functional relationships among these foci and H3 K79 trimethylation in determining IR resistance still remain unclear.

This study and recent related work (DOVER *et al.* 2002; NG *et al.* 2002a; GIANNATTASIO *et al.* 2005; WYSOCKI *et al.* 2005) have clarified the role of *RAD6* in radiation resistance. The high IR and UV sensitivity of *rad6* mutants has usually been attributed to the role of Rad6p in at least three forms of postreplication repair or translesion synthesis mediated by ubiquitination of PCNA through an interaction with Rad18p (XIAO *et al.* 2000; BROOMFIELD *et al.* 2001; HOEGE *et al.* 2002). However, this fails to account for the substantially greater IR sensitivity of *rad6*Δ strains compared to *rad18*Δ strains.

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The IR sensitivity of *bre1*Δ suggests that this extra sensitivity arises from the role of Rad6p in H2B K123 ubiquitination. This is convincingly confirmed by double-mutant analysis, since *rad6*Δ is epistatic to both *bre1*Δ and *rad18*Δ, while *bre1*Δ is additive with *rad18*Δ and the *rad18*Δ *bre1*Δ double mutant mimics the sensitivity of *rad6*Δ alone. The *RAD6/BRE1/DOT1* pathway also provides a clear link for Rad6p to HRR, as indicated by epistasis of *rad51*Δ to *dot1*Δ. It is not yet clear if this role impacts HRR itself or is mediated entirely through checkpoint controls that may be prerequisites for HRR. Recently, Zhang and Lawrence (2005) have reported that the error-free mode of *RAD18*-dependent postreplicational repair frequently involves recombination between sister strands, at least in plasmid DNA. However, this *RAD18*-dependent process may perhaps be better regarded as an aspect of postreplication repair that depends on recombination, rather than part of HRR *per se*, in contrast to the separate *RAD6/BRE1/DOT1* pathway, which is independent of *RAD18*.

The availability of strains separately mutant for *UBR1*, *BRE1*, and *RAD18* enables us to determine which of the many phenotypes of *rad6*Δ mutants arise from each of the pathways that *rad6*Δ impacts. It will be instructive to determine if the *ubr1*Δ *bre1*Δ *rad18*Δ triple mutant truly mimics all the phenotypes of *rad6*Δ; if not, this will imply more roles for the Rad6 ubiquitin conjugase. Homozygous *rad6* mutant diploids are able to undergo premeiotic DNA synthesis but are completely defective in sporulation, meiotic division, and in commitment to meiotic recombination (Cox and Parry 1968; Game *et al.* 1980), but little information has been available about the specific nature of the meiotic defect. There is evidence that the H2B ubiquitination function of Rad6p is important for the role of *RAD6* in meiosis, since both *rad6*Δ and *bre1*Δ, as well as a histone H2B K123 substitution mutant, reduced the frequency of meiotic DSBs, at least in the SK1 genetic background (Yamashita *et al.* 2004). The *set1*Δ deletion mutant has been shown to confer meiotic defects broadly similar to those of *bre1*Δ (Sollier *et al.* 2004), implying that the meiotic phenotype of H2B ubiquitination mutants may be manifested through their effect on *SET1*-mediated histone H3 K4 methylation. Studying the *dot1*Δ mutant in meiosis should reveal whether H3 K79 methylation is similarly involved.

Uncertainty remains about the role of the *RAD5* gene in IR resistance despite the recent finding that this role is independent of the Rad5p function in poly-ubiquitinating PCNA and results instead from a separate ATPase activity in the protein (Chen *et al.* 2005). These authors found a lack of additivity for IR sensitivity between *rad5*Δ and each of the MRX deletion mutants, but they found additivity between *rad5*Δ and *rad51*Δ and *rad5*Δ and *rad52*Δ (Chen *et al.* 2005). From this, one might expect that MRX deletion strains by themselves would be more IR sensitive than *rad51*Δ strains, but in fact the curves are equivalent (Game 2000; Chen *et al.* 2005; see Figure 15), implying complexity in pathway interactions. We have confirmed that *rad5*Δ adds sensitivity to *rad51*Δ (not shown) and find that it also adds sensitivity to *rad9*Δ and *rad18*Δ as well as to *bre1*Δ and *dot1*Δ (Figures 13 and 18). However, the *rad5*Δ *rad18*Δ *dot1*Δ triple mutant (Figure 14) is less sensitive than expected from these double-mutant combinations, and *rad5*Δ fails to add significant sensitivity to *rad6*Δ (not shown), again presenting complexity in interpretation. Finally, as shown in Figures 14 and 18, each of the three double mutants involving *rad5*Δ, *rad9*Δ, and *rad18*Δ are more sensitive than the component single mutants, yet the *rad5*Δ *rad9*Δ *rad18*Δ triple mutant is no more sensitive than the *rad9*Δ *rad18*Δ double mutant. Thus, the additivity between *rad5*Δ and *rad18*Δ is abolished in a *rad9*Δ background. Some of these paradoxical findings can be explained if *RAD5* contributes to IR resistance through more than one pathway. If it partially impacts two pathways, then its deletion should cause at least some increased sensitivity when combined with single mutants in either pathway, but no further increase in a triple-mutant combination where both the pathways are already blocked by other mutations.

Our data differ from those of Wysocki *et al.* (2005) with respect to IR sensitivity of the *dot1*Δ single mutant. Using qualitative plate tests, these authors found little difference between wild type and *dot1*Δ at doses up to 900 Gy. The most likely explanation for the different findings is the different strain backgrounds used, which were W303 (Thomas and Rothstein 1989) in the work by Wysocki *et al.* (2005) *vs.* the S288C/deletion library background used here. There is good agreement with respect to the mutants' effects on the damage checkpoints themselves; hence it is likely that the two backgrounds differ in the relative influence of checkpoint defects on survival.

Our data do not address the role of the *BRE1* and *DOT1* in nonhomologous end-joining, since wild-type Saccharomyces repairs little if any X-ray-induced damage by NHEJ. Although Saccharomyces lacks the catalytic subunit of DNA protein kinase that is involved in mammalian NHEJ, it is still able to process some types of DSBs by NHEJ, such as those induced by restriction endonucleases (Boulton and Jackson 1996). However, mutants such as *yku70*Δ that are defective in NHEJ but not in HRR or PRR confer little or no IR sensitivity when they are present as single mutants, and only mild (Boulton and Jackson 1996; Siede *et al.* 1996) or no additional IR sensitivity (Milne *et al.* 1996; J. Game, unpublished observations) when present in double-mutant combinations with HRR mutants. Thus, an NHEJ defect, if present in *bre1*Δ or *dot1*Δ strains, would be unlikely to impact IR sensitivity.

We reported earlier that the *dot1*Δ deletion does not by itself confer any substantial UV sensitivity on our strains, and during this study we found that *bre1*Δ,

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*rtf1*Δ, and *paf1*Δ also confer no (or minimal) sensitivity. However, preliminary data (not shown) indicate a probable synergistic increase in UV sensitivity in a *rad18*Δ *dot1*Δ double mutant compared to either single mutant. HRR mutants as well as excision repair mutants are well known to interact synergistically with mutants in the *RAD18* pathway with respect to UV sensitivity (Cox and Game 1974; Game 1983), so this observation is consistent with our other data indicating a role for *DOT1* in HRR. Further understanding of the role of the *BRE1* and *DOT1* genes in UV repair requires studying double-mutant combinations with excision repair genes, since excision repair is the major mechanism of UV resistance in Saccharomyces.

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