A Conserved RING Finger Protein Required for Histone H2B Monoubiquitination and Cell Size Control

Short Article

William W. Hwang,[1]
Shivkumar Venkatasubrahmanyam,[1]
Alexandra G. Ianculescu,[1] Amy Tong,[2]
Charles Boone,[2] and Hiten D. Madhani[1],*
[1]Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143
[2]Banting and Best Institute
University of Toronto
Toronto, Ontario M5G1L6
Canada

Summary

Monoubiquitination of histone H2B is required for methylation of histone H3 on lysine 4 (K4), a modification associated with active chromatin. The identity of the cognate ubiquitin ligase is unknown. We identify Bre1 as an evolutionarily conserved RING finger protein required in vivo for both H2B ubiquitination and H3 K4 methylation. The RING domain of Bre1 is essential for both of these modifications as is Lge1 (Large 1), a protein required for cell size control that copurifies with Bre1. In cells lacking the euchromatin-associated histone variant H2A.Z, *BRE1*, *RAD6*, and *LGE1* are each essential for cell viability, supporting redundant functions for H2B ubiquitination and H2A substitution in the formation of active chromatin. Notably, analysis of mutants demonstrates a function for Bre1/Lge1-dependent H2B monoubiquitination in the control of cell size.

Introduction

Covalent modifications of histones play crucial roles in several aspects of chromosome behavior, especially transcription (Jenuwein and Allis, 2001). In several instances, a modification at one position of a histone can influence subsequent modification at a second site. For example, in fission yeast and metazoans, methylation of histone H3 K4, a modification associated with active chromatin, is mutually exclusive with the heterochromatin-associated modification of H3 K9 (Jenuwein and Allis, 2001). Moreover, it has recently been shown in the budding yeast, *S. cerevisiae*, that monoubiquitination of K123 of histone H2B is required for methylation of H3 K4, demonstrating that modification of one histone subunit can regulate the subsequent modification of a different subunit (Dover et al., 2002; Sun and Allis, 2002). Despite increasing recognition of the importance of histone ubiquitination and the fact that histones were the first proteins found to be subject to covalent modification by ubiquitin (Hunt and Dayhoff, 1977; Olson et al., 1976), our understanding of the machinery responsible for this modification, its regulation, and its functions remains incomplete.

*Correspondence: hiten@biochem.ucsf.edu

Ubiquitination is catalyzed by a system of three enzymes that includes a generic ubiquitin-activating enzyme or E1, a ubiquitin-conjugating enzyme or E2, and a ubiquitin ligase or E3 (Glickman and Ciechanover, 2002). Cells have many more ubiquitin ligases than ubiquitin-conjugating enzymes; the ligases are the substrate recognition components of the system, and they are generally the target of biological regulation. In *S. cerevisiae*, the Rad6 ubiquitin-conjugating enzyme has been shown to be required for histone H2B monoubiquitination on K123 and consequently methylation of H3 on K4 (Robzyk et al., 2000). Rad6 is known to function in conjunction with one of three RING finger ubiquitin ligase homologs: Ubr1, Rad18, or Rad5 (Bailly et al., 1994; Dohmen et al., 1991; Johnson et al., 1992). Ubr1 functions in the N-end rule protein degradation pathway (Bartel et al., 1990), whereas Rad18 and Rad5 function in DNA repair, where PCNA is a key substrate (Hoege et al., 2002). The ubiquitin ligase for histone monoubiquitination has not previously been reported.

Here we identify a conserved RING finger domain protein, encoded by *BRE1* in *S. cerevisiae*, that is required for histone H2B monoubiquitination in vivo. A Bre1-associated protein, Lge1, is also required for H2B monoubiquitination. As predicted from recent studies, both Bre1 and Lge1 are required for histone H3 K4 methylation as well. In contrast, the Rad5, Rad18, and Ubr1 RING proteins shown previously to function with Rad6 are dispensable for H2B monoubiquitination and H3 K4 methylation. The *lge1*Δ (large 1) mutant was originally identified in a screen for cell size mutants. We show here that *bre1*Δ mutants and strains harboring a single amino acid change in the H2B monoubiquitination site also display a large cell phenotype. Cells lacking components of the H3 K4 methylase display a size distribution that is intermediate between those of wild-type and *bre1*Δ or *lge1*Δ cells, demonstrating a role for histone H2B monoubiquitination in cell size determination that is in part distinct from its requirement for H3 K4 methylation.

Results and Discussion

We have recently obtained evidence that the universally conserved histone variant, H2A.Z (encoded by *HTZ1* in *S. cerevisiae*), functions to promote the formation of active chromatin (M.D. Meneghini, M. Wu, and H.D.M, submitted). Although *htz1*Δ deletion mutants are viable, we observed that *htz1*Δ *rad6*Δ double mutants are inviable (Figure 1). We systematically screened for other deletion mutants that are synthetically lethal with *htz1*Δ (our unpublished data) and identified a null mutation in *BRE1*, which encodes a protein of unknown function identified previously in a screen for mutants that are hypersensitive to the drug brefeldin A (Muren et al., 2001). *bre1*Δ mutants display synthetic lethality with *htz1*Δ mutants but, like *rad6*Δ mutants, are viable in *HTZ1*+ cells (Figure 1). Database searching and alignment (Figure 2) revealed sequence homologs of Bre1 in *Schizosaccharo*-
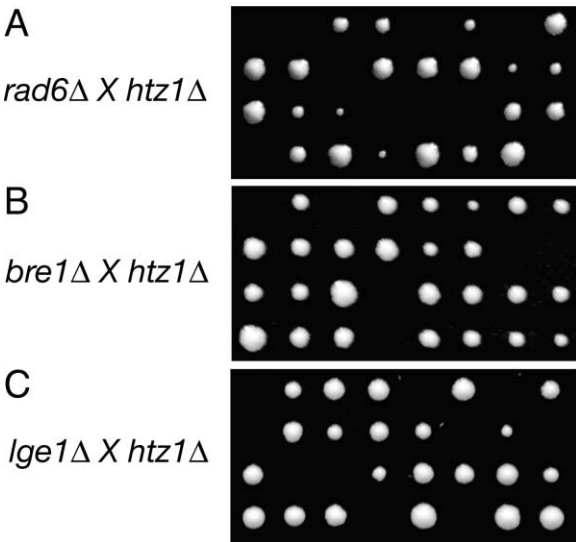
Figure 1. Genetic Identification of *BRE1* and *LGE1*

(A) Shown is tetrad analysis YM1749 (a/α *htz1Δ::natMX4/HTZ1 rad6::kanMX4/RAD6*). Genotyping of these tetrads revealed that the inviable colonies correspond to the *htz1Δ rad6Δ* double mutant.
(B) Synthetic lethality between *htz1Δ* and *bre1Δ*. Shown is tetrad analysis of YM1750 (a/α *htz1Δ::natMX4/HTZ1 bre1::kanMX4/BRE1*). Genotyping revealed that the inviable colonies correspond to the *htz1Δ bre1Δ* double mutant.
(C) Synthetic lethality between *htz1Δ* and *lge1Δ*. Shown is tetrad analysis of YM1751 (a/α *htz1Δ::natMX4/HTZ1 lge1::kanMX4/LGE1*). Genotyping revealed that the inviable colonies correspond to the *htz1Δ lgeΔ* double mutant.

myces pombe (SpBre1A, SpBre1B), *Dictyostelium discoidium* (DictBre1), *Arabadopsis thaliana* (AtBre1), *Caenorabditis elegans* (CeBre1), *Drosophila melanogaster* (DmBre1), and humans (HuBre1A, HuBre1B). Multiple domains in Bre1 and its homologs yield strong predictions of α-helical coiled-coil structure, but the highest sequence conservation is apparent in a canonical RING domain that lies at the extreme C terminus of each family member. Phylogenetic analysis of the similarity between Bre1 homologs from different organisms recapitulates the expected relationships between species, further supporting their assignment as a family of proteins (data not shown). RING domains are characteristic of a large class of ubiquitin ligases and are likely to be diagnostic for this activity (Joazeiro and Weissman, 2000). Since *BRE1* shares genetic properties with *RAD6* and since the protein has a domain found in ubiquitin ligases, we hypothesized that Bre1 is a component of the ubiquitin ligase that cooperates with Rad6 in the ubiquitination of histone H2B.

As shown in Figure 3A, monoubiquitination of H2B can be readily detected in wild-type cells harboring a functional FLAG-tagged allele of the *HTB1* gene (encoding histone H2B) as a slower-migrating form upon SDS-PAGE and immunoblotting of whole-cell extracts with anti-FLAG antibodies. Consistent with previous studies, the ubiquitinated species is absent in cells containing a tagged *htb1-K123R* mutant in which the lysine at the monoubiquitination site has been changed to a nonmodifiable arginine residue; moreover, ubiquitination is absent in cells lacking the E2 Rad6 (Figure 3A). In cells lacking Bre1, monoubiquitination of H2B is similarly
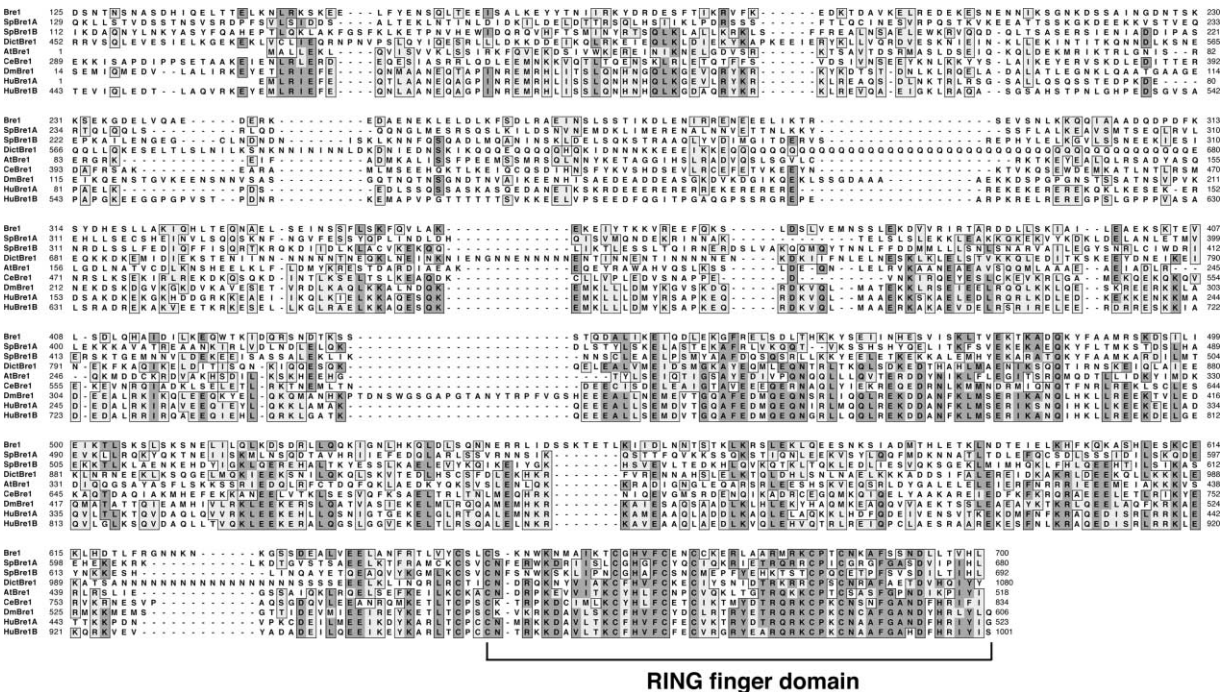
Figure 2. Conservation of Bre1 across Eukaryotic Evolution

Shown is a Clustal W alignment of Bre1 sequence homologs. The RING domain is indicated. Regions upstream of the RING domain of each of the homologs yield strong coiled-coil predictions over multiple regions. Database accession numbers: CAA98640 (Bre1), NP_587845 (SpBre1A), CAA22646 (SpBre1B), AAL93605 (DictBre1), AAL91211 (AtBre1), AAK21443 (CeBre1), AAF50744 (DmBre1), BAB14005 (HuBre1A), AAH18647 (HuBre1B).
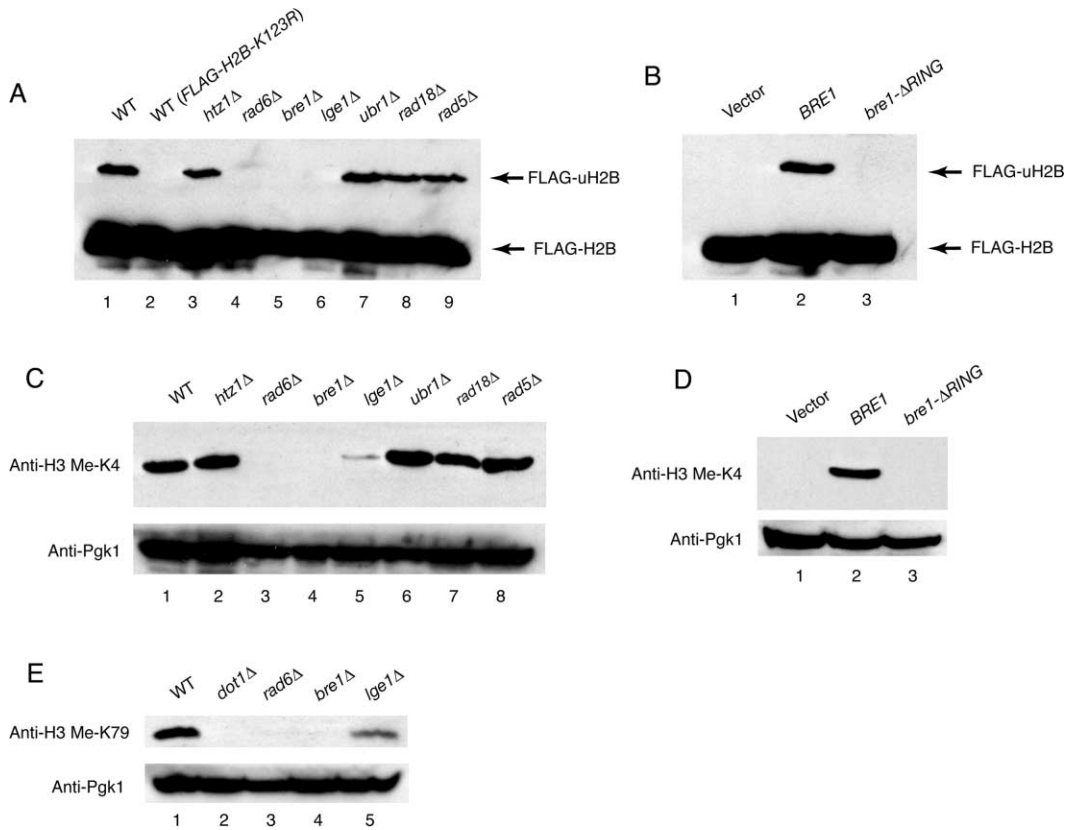
Figure 3. Analysis of H2B Monoubiquitination and H3 Methylation

(A) Determination of H2B monoubiquitination in selected wild-type and mutant strains. Shown is an immunoblot using anti-FLAG antibodies of extracts from strains of the indicated genotypes harboring *FLAG-H2B* on a *CEN-ARS* plasmid.

(B) Bre1 RING domain is essential for H2B monoubiquitination. *bre1Δ* cells harboring either *pADH-BRE1* or *pADH-bre1ΔRING* were analyzed as in (A).

(C) Determination of H3 K4 methylation in selected wild-type and mutant strains. (Upper panel) Shown is an immunoblot using anti-H4 methyl-K4 antibodies of extracts from strains of the indicated genotypes fractionated by SDS-PAGE. (Lower panel) Reprobing of the blot in the upper panel with antibodies to Pgk1.

(D) Bre1 RING domain is essential for H3 K4 methylation. *bre1Δ* cells harboring either *pADH-BRE1* or *pADH-bre1Δ RING* were analyzed as in (A).

(E) Determination of H3 K79 methylation in selected wild-type and mutant strains. (Upper panel) Shown is an immunoblot using anti-H4 methyl-K79 antibodies of extracts from strains of the indicated genotypes fractionated by SDS-PAGE. (Lower panel) Reprobing of the blot in the upper panel with antibodies to Pgk1.

eliminated (Figure 3A). In contrast, cells lacking the RING finger proteins Ubr1, Rad5, or Rad18, which have been shown previously to act with Rad6, display no defect in H2B monoubiquitination (Figure 3A). Cells harboring a *BRE1* allele containing a truncation of 54 codons corresponding to the C-terminal RING domain (*bre1-ΔRING*) are also defective in ubiquitination, consistent with the known requirement of RING domains for activity of this family of ubiquitin ligases (Figure 3B).

A large-scale protein complex purification study has previously identified two polypeptides associated with Bre1: Lge1 and Yhr149c (Ho et al., 2002). Interestingly, *lge1Δ* displays synthetic lethality with *htz1Δ* (Figure 1; our unpublished data). Lge1 has a strongly predicted coiled-coil domain at its C terminus, which may mediate its interaction with Bre1 (our unpublished data). We therefore examined H2B ubiquitination in *lge1Δ* cells. As shown in Figure 3A, the *lge1Δ* mutant is defective in H2B ubiquitination, suggesting that Bre1 functions as part of a multiprotein complex.

As in other species, H3 K4 methylation in *S. cerevisiae* by the Set1 complex is associated with transcriptionally active chromatin (Bernstein et al., 2002; M. D. Meneghini, M. Wu, and H.D.M., submitted). Since Rad6-dependent monoubiquitination of histone H2B has been demonstrated to be required for H3 K4 methylation (Dover et al., 2002; Sun and Allis, 2002), we predicted that Bre1 and Lge1 should likewise be important for methylation of H3 K4. SDS-PAGE fractionation and immunoblotting of whole-cell extracts using antibodies specific for H3 methyl-K4 revealed a robust band corresponding to methyl-K4 H3 in wild-type cells (Figure 3C). As predicted, *bre1Δ* mutants, like *rad6Δ* mutants, were completely defective in H3 K4 methylation. Moreover, the *bre1-ΔRING* allele also abolished H3 K4 methylation (Figure 3D), whereas cells lacking Lge1 showed greatly reduced methylation (Figure 4A). Finally, we examined whether *bre1Δ* and *lge1Δ* are required for histone H3 K79 methylation, which has recently been shown to require H2B monoubiquitination (Briggs et al., 2002; Ng
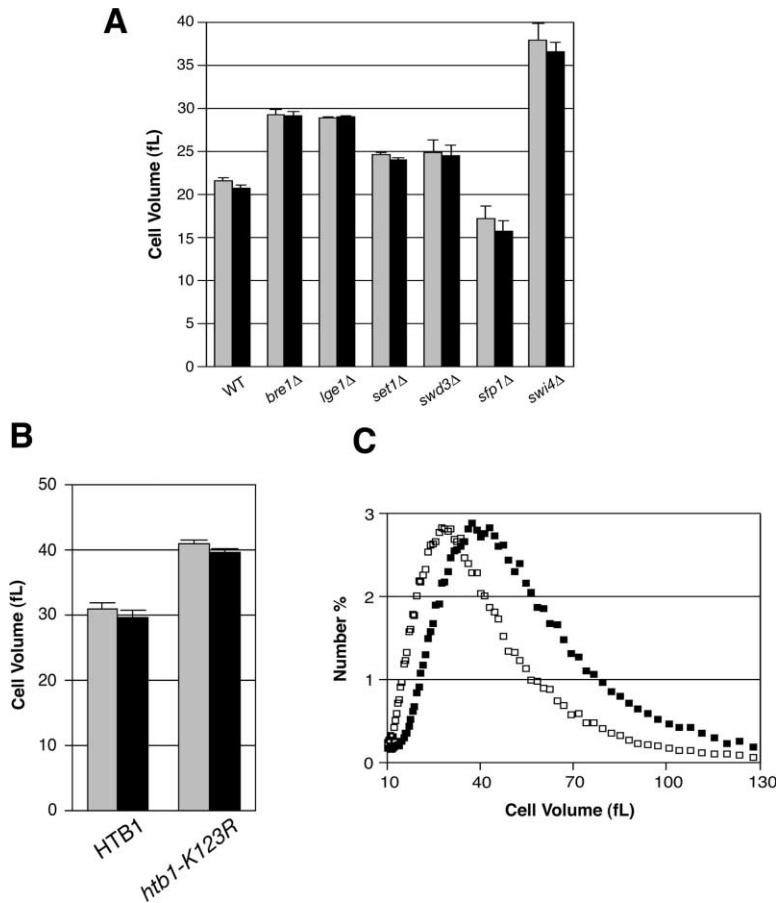
Figure 4. Analysis of Cell Size

(A) Cell size measurements of selected haploid knockout strains in the S288C strain background. Shown is a graph of mean (gray bars) and median (black bars) cell volumes of the indicated strains. Each measurement was performed on three cultures. Error bars indicate standard deviations.

(B) Cell size measurements of *htb1Δ htb2Δ* cells (W303 strain background) harboring plasmids encoding wild-type *HTB1* versus *htb1-K123R* alleles. Scale and legend are as in (A).

(C) Histogram of cell size distribution of strains shown in (B). Shown are the population cell size distributions of *htb1Δ htb2Δ* cells harboring plasmids encoding wild-type *HTB1* (open boxes) versus the *htb1-K123R* allele (filled boxes).

et al., 2002). As with K4 methylation, *bre1Δ* cells lack methylation of K79, whereas *lge1Δ* cells display a partial defect in K79 methylation that is weaker than their defect in K4 methylation (Figure 3E). The residual methylation present in *lge1Δ* cells could be due to a very low amount of residual H2B monoubiquitination (below our detection limit) in the *lge1Δ* mutant, or possibly, Lge1 could play an inhibitory role in methylation that is overcome by ubiquitination.

The *lge1Δ* mutant was originally identified in a large-scale screen for mutants with defective cell size control (Jorgensen et al., 2002). Cells lacking *LGE1* display a large cell phenotype. Inspection of the published genome-wide dataset revealed that *bre1Δ* mutants also display a large cell phenotype. To confirm these data, we examined the population size distributions of wild-type, *bre1Δ*, and *lge1Δ* cells. As controls, we examined the cell sizes of *sfp1Δ* (a *whi* or small size mutant) and *swi4Δ* (a *lge* or large size mutant) strains (Jorgensen et al., 2002). As shown in Figure 4A, both *bre1Δ* and *lge1Δ* strains displayed a large cell phenotype, consistent with previous measurements (Jorgensen et al., 2002). The phenotype of *bre1Δ* and *lge1Δ* mutant data raised several questions. First, is the large cell phenotype observed in *lge1Δ* and *bre1Δ* mutants due to a defect in H2B monoubiquitination? To test this possibility, we examined the cell size distributions of isogenic *S. cerevisiae* strains containing a deletion of both H2B genes (*htb1Δ htb2Δ*) complemented by plasmid-borne alleles

of either wild-type H2B or H2B-K123R. Strikingly, cells harboring the monoubiquitination site mutant displayed a large cell phenotype relative to the wild-type H2B control (Figure 4B). (We note that the strains harboring the wild-type H2B allele are larger than that of the wild-type control for the experiments in Figure 4A; this may result from differences in the strain background used [W303 for the *HTB1* experiments versus S288C for the deletion mutant experiments]). The second question raised by the cell size phenotypes of *bre1Δ* and *lge1Δ* mutants is whether the size defect is due to a defect in H3 K4 methylation, which requires H2B ubiquitination. To test this hypothesis, we examined the cell size distributions for mutants in the Set1 H3-K4 methylase complex (*set1Δ* and *swd3Δ*), in which H3 K4 methylation is absent (Krogan et al., 2002; Roguev et al., 2001). These mutants displayed a median and mean size that was intermediate between those of wild-type and H2B monoubiquitination-defective mutants (Figure 4A). Thus, the large cell phenotype of *bre1Δ*, *lge1Δ*, and *H2B-K123* mutants can only partially be explained by a loss of H3 K4 methylation.

We have identified Bre1 as a RING finger protein required for monoubiquitination of histone H2B on K123 and methylation of histone H3 on K4. We also show that the Bre1-associated protein Lge1 is required for normal levels of H2B ubiquitination and H3 K4 methylation, suggesting that both proteins are components of a multisubunit E3. Although we cannot rule out the formal pos-

sibility that Rad6 and Bre1-Lge1 indirectly activate an unknown ubiquitin ligase that promotes H2B monoubiquitination, their specific requirement in vivo for H2B ubiquitination and the necessity for the Bre1 RING domain for the modification are most simply explained by a direct role. However, demonstration of H2B-specific in vitro ubiquitin ligase activity of the Bre1 complex in a purified system will be necessary to show that it functions as an E3. As noncatalytic subunits of ubiquitin ligase complexes can serve a regulatory function (Carroll and Morgan, 2002), we speculate that Lge1 may function to modulate the activity of the complex in response to regulatory inputs and/or to control substrate selection.

Our analysis of cell size distributions of mutants lacking histone H2B monoubiquitination revealed an unanticipated role for this chromatin modification in cell size control. Moreover, our finding that blocking H3 K4 methylation by deletion of genes encoding essential components of the H3 K4 methylase only partially recapitulates the cell size defects of *bre1Δ* and *lge1Δ* mutants indicates that H2B monoubiquitination has a role in size control independent of its function in H3 K4 methylation. Methylation of H3 on K79 has been recently shown to require H2B monoubiquitination (Briggs et al., 2002; Ng et al., 2002) and may explain the intermediate cell size phenotype of cells lacking K4 methylation. This requirement for H2B monoubiquitination for size control could reflect an effect on the cell cycle transcriptional program. An intriguing alternative model would be a role for H2B monoubiquitination which is independent of transcription; one possibility is that cells measure the modified histone content of chromatin as part of the still-mysterious biochemical calculation of cell size which is used to time critical transitions in the cell cycle such as START. Such a model would be consistent with the known dependence of cell size on chromosome ploidy that has been established in organisms from yeast to man (Galitski et al., 1999; Su and O'Farrell, 1998).

Both *BRE1* and *LGE1* are essential for cell viability in cells lacking the conserved histone variant H2A.Z, and they are required for H3-K4 methylation, a modification associated with active chromatin. Moreover, we have recently shown that H2A.Z promotes the formation of active chromatin (M.D. Meneghini, M. Wu, and H.D.M., submitted). We speculate that Rad6, Bre1, and Lge1 act upstream of H3 K4 methylation in a pathway parallel to that of H2A.Z which also functions to establish and/or maintain the euchromatic state. Our identification of homologs of Bre1 in diverse eukaryotic species suggests that the fundamental elements of the histone-based regulatory circuitry described in this and other recent studies have been conserved during evolution.

Experimental Procedures

Yeast Strains
S. cerevisiae strains used in this study are listed in Supplemental Table S1 at http://www.molecule.org/cgi/content/full/11/1/261/DC1.

Yeast Methods
Standard procedures were used for cultivation and genetic manipulations (Guthrie and Fink, 2002). Strains were of the S288C background. Knockouts were obtained from the Yeast Deletion Consortium collection (Research Genetics), and their genotypes are indicated in the figure legends.

Plasmid Constructions
p*ADH1-BRE1* was constructed as follows. The coding region of *BRE1* was amplified using genomic DNA as a template and cloned into pZeroBluntII (TOPO cloning kit; Invitrogen) and then subcloned into p415-*ADH1* (*LEU2, CEN*) using PstI and BamHI. The p*ADH1-bre1ΔRING* allele was generated using the identical strategy except that *BRE1* was truncated to remove the C-terminal 54 codons.

Histone Modification Assays
For histone monoubiquitination assays, extracts were prepared as described (Sun and Allis, 2002). Samples were fractionated by SDS-PAGE (12%). After transfer, PVDF membranes were washed for 3 min in water and blocked in 3% milk in ATBS (50 mM Tris [pH 8], 138 mM NaCl, 2.7 mM KCl). Following a 5 min wash in ATBS, membranes were incubated with a mouse anti-FLAG monoclonal antibody (Sigma, 1:1000) for 30 min in 3% milk in ATBS. Membranes were washed for 5 min with TBS and incubated for 30 min with HRP-conjugated anti-mouse secondary antibodies and then washed eight times for 2.5 min in ATBS + 0.05% Tween. Blots were visualized by ECL (Pierce Pico Kit).

Histone H3 K4 methylation was assayed as follows. Cells were grown to mid-log phase (OD_{600} of 0.6 to 0.8). Three OD units of cells were pelleted and resuspended in 100 μl of 2× SDS sample buffer and boiled for 2 min. Fifty microliters of glass beads was added, and samples were vortexed and then boiled for an additional 2 min. Extracts were clarified by centrifugation for 10 min at 21 kg, and the supernatants were boiled again for 3 min. Samples were fractionated by SDS-PAGE (12%) and transferred to nitrocellulose. Blots were blocked for 30 min in 5% milk in TBST (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween), and then incubated with rabbit anti-histone H3 methyl-K4 antibodies (Abcam, 1:1000) for 1 hr in 5% milk in TBST. Following five washes for 5 min each in TBST, blots were incubated with HRP-conjugated anti-rabbit secondary antibodies for 30 min in 5% milk in TBST. Following four washes for 5 min each in TBST and one 5 min wash in TBS, blots were visualized using ECL (Pierce Pico ECL kit). The same procedure was used to assay H3 K79 methylation using antibodies generously provided by Frank van Leeuwen and Dan Gottschling.

Cell Size Distribution Determinations
Strains were grown in YPD medium to mid-log phase (OD 0.6–0.8). Two percent formaldehyde was added, and cells were incubated for 20 min at 30°C. Cells were collected by centrifugation and washed twice with 0.1 M potassium phosphate (pH 6.5) and stored in 1 ml of solution P (0.1 M potassium phosphate [pH 6.5], 1.2 M sorbitol) at 4°C. Twenty microliters of the fixed cell suspension was diluted into 20 ml Isoton (Beckman-Coulter). Cell size distributions were determined using a Beckman-Coulter Multisizer 3 instrument fitted with a 100 uM aperture. Approximately $3-5 \times 10^7$ cells were counted for each culture.

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