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The Bre5/Ubp3 ubiquitin protease complex from budding yeast contributes to the cellular response to DNA damage

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ABSTRACT

The ubiquitination status of proteins can control numerous aspects of protein function through targeted destruction or by altering protein–protein interactions, subcellular localization, or enzymatic activity. In addition to enzymes that mediate the conjugation of ubiquitin moieties to target proteins, there are enzymes that catalyze the removal of ubiquitin, termed ubiquitin proteases. One such ubiquitin protease, Ubp3, exists in a complex with a partner protein: Bre5. This complex has been implicated in a variety of cellular activities, and was recently identified in large-scale screens for genetic interactions with known components of the DNA damage response pathway. We found that this complex plays a role in the cellular response to the DNA damaging agent phleomycin and strains lacking the complex have a defect in non-homologous end joining. Although this complex is also important for telomeric silencing, maintenance of the cell wall, and global transcriptional regulation, we present evidence suggesting that the role of this complex in DNA damage responses is distinct from these other roles. First, we found that Ubp3/Bre5 functions antagonistically with Bul1 in DNA damage responses, but not in its other cellular functions. Additionally, we have generated mutants of Bre5 that are specifically defective in DNA damage responses.

1. Introduction

Protein ubiquitination has been traditionally associated with targeting proteins for degradation. However, it has become clear over recent years that protein ubiquitination might also play an important role in the modulation of protein activity in a manner similar to other covalent modifications such as phosphorylation and acetylation [27]. As well as ubiquitinating enzymes, cells also have numerous de-ubiquitinating enzymes whose roles are relatively still poorly understood [4]. In budding yeast, there are 18 predicted ubiquitin proteases, including Ubp3, which forms a complex *in vivo* with Bre5 (www.yeastgenome.org).

Bre5 has a nuclear transport factor 2 (NTF2) domain in its N-terminus, a putative SH3 binding domain in its central portion, and a RNA recognition motif (RRM) at its C-terminus (Fig. 5A). Importantly, the NTF2 domain of Bre5 was found to interact with Ubp3 [8,27] and this interaction was important for the ability of Ubp3 to deubiquitinate two of its targets; Sec23 [8,9] and Atg19 [4]. Both of these proteins are involved in vesicle

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transport in the cytoplasm. Misregulation of these targets may explain the sensitivity of *bre5Δ* mutant strains to Brefeldin A [32].

More recently, Bre5 and Ubp3 have been found constitutively associated with TFIID [2]. The authors also found the ubiquitin ligase Rsp5 and its co-factor Bul1 in their isolated TFIID complexes. It was observed that the TFIID subunits Taf1 and Taf5 are ubiquitinated and this ubiquitination appeared to be affected by Bul1 and Bre5. Rsp5 and Ubp3 have recently been identified as transcriptional activators using the yeast one-hybrid system [37]. However, there is no evidence to suggest that they play a direct role in transcription activation. It is possible that Ubp3 and Rsp5 might activate transcription in this system by virtue of their association with the TFIID complex. *BRE5* was also identified in a large-scale analysis of 564 *Saccharomyces cerevisiae* deletion strains as one of 10 mutants that increased yeast replicative lifespan, although the mechanism by which *bre5Δ* mutants promote this phenotype is not clear [23]. Finally, Bellaoui et al. [5] and Tong et al. [38] found that *bre5Δ* mutations had a negative synthetic interaction with various genes involved in DNA repair.

Bre5 and Ubp3 have clear homologues in higher eukaryotes; G3BP and Usp10, respectively, which were also found to interact. G3BP has been shown to be a very pleiotropic protein with functions ranging from protein stability to signal transduction [21]. Most of these functions have been ascribed to mRNA modulation and development, although the role of Usp10 has not been thoroughly elucidated. Importantly, G3BP isoforms are commonly found to be massively overexpressed in human cancers [3,13,18], raising the possibility that this complex may affect genomic stability in eukaryotes.

We are constantly exposed to a variety of agents able to generate various forms of DNA damage, which need to be accurately and efficiently sensed and repaired. Of all forms of DNA damage, the most dangerous is the DNA double-strand break (DSB). Eukaryotic cells repair DNA DSBs using either homologous recombination (HR) or non-homologous end joining (NHEJ), both of which are highly conserved throughout eukaryotic evolution.

Given the genetic interactions between the *bre5Δ* mutation and mutations in known DNA repair genes in yeast [5,38], combined with the alterations in the protein expression of Bre5 homologues in human cancers [3,13,18], we decided to investigate whether the Bre5/Ubp3 complex might contribute to DNA damage responses.

We find that this complex is important for facilitating wild-type levels of NHEJ as well as promoting cellular resistance to the DNA DSB-inducing agent phleomycin. Moreover, we find that these functions are genetically antagonistic with the *BUL1* gene; the cofactor of the Rsp5 ubiquitin ligase also found in TFIID [2]. Interestingly, however, global transcriptional responses in *bre5Δ* mutants and *bul1Δ* mutants are not antagonistically regulated, suggesting that the interplay between these two genes is specific to DNA damage responses. Consistent with this, deletion of *bul1Δ* can rescue the sensitivity of a *bre5Δ* mutant strain to phleomycin and restore NHEJ activity, but has no effect on the ability of Bre5 to mediate telomeric silencing or sensitivity to Brefeldin A. Finally, we identify separation of function mutations in the *BRE5* gene that result in impaired DNA damage responses, but not telomeric silencing defects. Together, these data suggest that the Bre5/Ubp3 ubiquitin protease complex has a specific function in DNA damage responses that is alleviated by impairing the Bul1/Rsp5 ubiquitin ligase activity.

2. Materials and methods

2.1. Strains and plasmids

Strains were constructed by standard methods and verified by PCR (Table 1). Plasmids were constructed by standard methods and verified by restriction digestion and sequencing (Table 2).

2.2. Telomeric silencing

Logarithmically growing cultures were diluted 10^{-2}, 10^{-3} and 10^{-4} times and 100 μl of each dilution was plated onto 5-FOA or -URA plates. Plates were incubated at 30 °C for 3–4 days when colonies were counted. Results were expressed as the percentage of colonies growing in 5-FOA over the sum of colonies growing on 5-FOA and -URA plates.

2.3. Plasmid repair assay

Logarithmic growing cultures were transformed with 100 ng of pRS416 digested with EcoRI or the same concentration of supercoiled plasmid. After plating, the yeast were incubated at 30 °C for 3–4 days after which colonies were counted. The number of colonies derived from digested plasmids was divided by the number of colonies from intact plasmids to calculate the percentage of repaired plasmid. The ratio obtained for wild-type strains was set as 100% and all other ratios were calculated relative to that standard. The *bre5Δ* strain was assayed nine times, the *ubp3Δ* strain two times, and the *bre5Δyku80Δ* strain three times. WT and *yku80Δ* controls were present in every experiment.

2.4. Chromatin fractionation

Cell lysates were fractionated into chromatin and supernatant fractions according to an adaptation of the protocol developed by Donovan et al. [11]. Cells from 100 ml of logarithmically growing cultures were harvested by centrifugation and resuspended in buffer A (100 mM HEPES pH 8, 25 mM DTT) and incubated at 30 °C for 10 min. Cells were harvested and resuspended in buffer B (0.6 M sorbitol, 25 mM Tris pH 7.4, 4 mg/ml Zymolyase 20T, in YPAD). Suspensions were incubated for a further 40 min at 30 °C. Spheroplasts were centrifuged at 2000 × *g* for 3 min and gently resuspended in buffer C (0.7 M sorbitol, 25 mM Tris pH 7.4, in YPAD). Spheroplasts were allowed to recover at 30 °C for 15 min. Spheroplasts were centrifuged at 2000 × *g* for 3 min and washed in lysis buffer (0.4 M sorbitol, 150 mM KAc, 2 mM MgAc, 20 mM HEPES pH 6.8, protease inhibitors). Spheroplasts were chilled, washed in lysis buffer, and resuspended in 400 μl of lysis buffer. Triton X-100 was added to a final concentration of 1% to lyse the spheroplasts. Ninety microliters aliquots of lysates were taken as whole cell extract controls (WCE). The remaining lysates were centrifuged at 4 °C, 20,000 × *g*, for 15 min. The supernatants

Table 1 – Yeast strains used in the study

Strain	Genotype	Background	Source
W303	*ura3-52 trp1Δ leu2-3 his3Δ200 rad5 matα*		R. Rothstein
JDY120	*BRE5-13Myc::KANMX mec1:: TRP1, sml1-1 mata*	W303	This work
JDY102	*BRE5-13Myc::KANMX, sml1-1 mata*	W303	This work
AEY1017	*TEL-VII-URA3 matα*	W303	[29]
JDY228	*bre5Δ::KANMX TEL-VII-URA3 matα*	W303	This work
JDY589	*ubp3Δ::TRP1 TEL-VII-URA3 matα*	W303	This work
JDY623	*bre5Δ::KANMX ubp3Δ::TRP1 TEL-VII-URA3 matα*	W303	This work
JDY91	*bre5Δ::HISMX matα*	W303	This work
JDY270	*ubp3Δ::HISMX matα*	W303	This work
JDY213	*rad54Δ::LEU2 mata*	W303	This work
JDY268	*bre5Δ::HISMX rad54Δ::LEU2 matα*	W303	This work
JDY269	*ubp3Δ::HISMX rad54Δ::LEU2 matα*	W303	This work
JDY19	*yku80Δ::KANMX matα*	W303	[12]
JDY227	*bre5Δ::HISMX yku80Δ::KANMX matα*	W303	This work
JKM115	*hoΔ hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3 112lys5 trp1::hisG ura3-52 matα*		[31]
JKM179	*hoΔ hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3 112lys5 trp1::hisG ura3-52 ade3::GalHO matα*		[31]
JDY279	*bre5Δ::TRP1 matα*	JKM115	This work
JDY605	*sir4Δ::TRP1 matα*	JKM115	This work
JDY607	*bre5Δ::KANMX sir4Δ::TRP1 matα*	JKM115	This work
JDY281	*bre5Δ::KANMX matα*	JKM179	This work
JDY518	*bul1Δ::TRP1 matα*	W303	This work
JDY520	*bre5Δ::HISMX Δbul1::TRP1 matα*	W303	This work
JDY519	*bul1Δ::TRP1 rad54Δ::LEU2 matα*	W303	This work
JDY517	*bre5Δ::KANMX bul1Δ::TRP1 TEL-VII-URA3 matα*	W303	This work
JDY110	*HRR25-13Myc::KANMX, matα*	W303	This work
JDY603	*bre5-S401*-13Myc::KANMXmatα*	JKM115	This work
JDY624	*bre5-S401*-13Myc::KANMXmatα*	W303	This work
JDY646	*yku80Δ::KANMX rad54Δ::LEU2matα*	W303	This work

(soluble fraction) were removed and their volumes measured. Pellets (chromatin fraction) were washed for three times in 1 ml lysis buffer and resuspended in a volume equivalent to the soluble fractions. SDS page loading buffer was added to all samples which were then boiled and loaded onto 10% SDS page gels or 1% agarose gels for analysis by staining with Coomassie blue or ethidium bromide, or by Western blot analysis with α-Myc.

2.5. *MNase assay*

Chromatin was digested with MNase following a protocol adapted from Kent and Mellor [24]. Cells from 100 ml of logarithmically growing cultures were cooled, harvested by centrifugation and washed in 40 ml of H_2O. The pellets were then ressuspended in 1 ml YLE buffer (1 M sorbitol, 5 mM β-mercaptoethanol, 9.5 mg Zymolyase 20 T) and incubated at room temperature for 15 min with occasional mixing. The resulting spheroplasts were collected by gentle centrifugation and washed 2× with 1 M sorbitol. Spheroplasts were then resuspended in 1.2 ml of SDB (1 M sorbitol, 50 mM NaCl, 10 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.5 mM spermidine, 0.0075% NP_40) and 15 μl of 2.86 U/μl of micrococcal nuclease was added to each sample. Reactions were incubated at 37 °C. At timepoints 0, 5, 10, 15, 20 and 25 min, aliquots were taken from the reaction mixtures and added to tubes containing 20 μl stop solution (0.25 M EDTA, 5% SDS). The reaction products were then phenol:chloroform extracted, RNase A treated, phenol extracted again, and isopropanol precipitated. The digested DNA pellets were resuspended in TE and analyzed on 1% agarose gels by staining with ethidium bromide.

Table 2 – Plasmids used in the study

Plasmid	Features	Source
JD444	*UBP3* from −576 to 2970 in pRS416	This work
JD445	*ubp3-C469A* from −576 to 2970 in pRS416	This work
JD446	*UBP3* from −576 to 2970 in pRS426	This work
JD447	*BRE5* (HindIII fragment) in pRS416	This work
JD448	*bre5-P322A* in pRS416	This work
JD449	*BRE5* (HindIII fragment) in pRS415	This work
JD450	*bre5-P322A* in pRS415	This work
JD453	*bre5-T351A, S401** in pRS415	This work
JD522	MYC tagged *BRE5* in pRS415	This work
JD556	MYC tagged *bre5-P322A* in pRS415	This work
pGal-HO	*HO* gene under the regulation of the *GAL1-10* promoter	S. Buratowski

2.6. Co-immunoprecipitation

Cells from 100 ml of logarithmically growing cultures were cooled to 4 °C, harvested by centrifugation and washed in 25 ml of IP buffer (40 mM HEPES, 500 mM NaCl, 0.1% Tween-20, 10% glycerol). The pellets were resuspended in 1 ml of IP buffer with protease inhibitors. Cell suspensions were transferred to 2 ml screw cap tubes containing 400 μl of glass beads. Cells were disrupted using the FastPrep system (MP Biomedicals) at speed 6 for 2× 45 s. Supernatants were transferred to new tubes and cleared by centrifugation at 18,000 × *g* for 15 min. Lysates was pre-cleared for 1 h at 4 °C with 80 μl of 1:1 Sepharose A beads (Amersham) equilibrated in IP buffer. Pre-cleared lysates were incubated for 2 h at 4 °C with 1.5 μl of α-Myc antibody (Sigma). Eighty microliters of 1:1 Sepharose A beads were added to the lysates and these were incubated for another hour. Beads were then washed three times with 1 ml of IP buffer. One hundred microliters of SDS loading buffer was added to the samples that were then boiled and electrophoresed in 7.5% SDS polyacrylamide gels. Gels were analyzed by Western blotting, silver staining and Coomassie blue staining.

2.7. Survival assays after HO endonuclease induction

JKM115 and JKM179 derived strains were grown in YPAD, re-inoculated into YEP-lactate at an OD_{600} of 0.1. When cultures reached and OD_{600} of 0.5, galactose was added to 2%. Samples were taken at 0 and 2 h after galactose induction and 100 μl of 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on to YPAD. The relative repair of each mutation was calculated as a percentage between colony formation from: (JKM179 background with 2 h induction/JKM179 background with 0 h induction)/(JKM115 background with 2 h induction/JKM115 background with 0 h induction) × 100. Each strain was assayed three times.

Alternatively JKM115 derived strains were transformed with pGAL-HO plasmids and grown in YNB with the required supplements, re-inoculated into YNB-lactate at an OD_{600} of 0.1. When cultures reached and OD_{600} of 0.5, galactose was added to 2%. Samples were taken at 0 and 3 h after galactose induction and 100 μl of 10^{-3} dilutions were plated on to YPAD. The relative repair of each mutation was calculated as a percentage between colony formation from induced cultures relative to uninduced cultures.

2.8. Microarray analysis

Total RNA from at least two independent experiments was isolated from logarithmically growing cells of W303-1B (WT), JDY91 *bre5Δ* and JDY518 (*bul1Δ*) strains using the RiboPure-Yeast kit (Ambion) or from the RNeasy kit (QIAGEN) according to the manufacturers' instructions. For phleomycin treatment, 10 μg/ml was added in the culture medium 1 h before RNA isolation. A mixture of oligo(dT)$_{20}$ and random hexameric oligonucleotides were used to prime cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) in the presence of Cy3-dUTP or Cy5-dUTP (GE Healthcare) according to standard protocols (cmgm.stanford.edu/pbrown) except that synthesis was allowed to proceed in the dark for 2 h at 50 °C.

Yeast 6.4 K slides containing approximately 6200 *S. cerevisiae* ORFs spotted in duplicate on each slide were purchased the University Health Network Microarray Centre, Canada (www.microarrays.ca/home.html). Material from either untreated or phleomycin-treated *bre5Δ* or *bul1Δ* mutants were compared to similarly treated wild-type on the same slide. Targets to be hybridized on the same slide were mixed and purified using the CyScribe GFX Purification Kit (GE Healthcare). Twenty microliters of mouse Cot-I DNA was added to the purified probes, and samples were dissolved in 80 μl of DIG EasyHyb solution (Roche). After denaturation, samples were added to the slides and hybridized over night at 42 °C in the dark. The slides were washed for 30 min in 2× SSC, 0.1% SDS at 50 °C; 15 min in 1× SSC at rt; 15 min in 0.1× SSC at rt.

Every slide was scanned at saturated and sub-saturated total signal intensities at 10 μm resolution in a VersArray ChipReader System 3.1 (BioRad). Technical replicates including repeated hybridizations and swapping of Cy3 and Cy5 dyes yielded a total of 4–8 measurements per condition.

Array images were quantified and normalized by the LOWESS subgrid method on $\log_2$ transformed signal intensity ratios, using ImaGene 6.0 software (BioDiscovery). Analysis of Gene Ontology terms and of similarity of gene expression profiles was done with tailor-made software at the Yeast Microarray Global Viewer (www.transcriptome.ens.fr/ymgv/) and Saccharomyces Genome Database (www.yeastgenome.org/) Clustering of arrays was performed using Hierarchical Cluster Explorer (www.cs.umd.edu/hcil/hce) or Cluster 3.0 [10] and visualized with Mapletree (www.rana.lbl.gov/EisenSoftware.htm).

3. Results

3.1. The Bre5/Ubp3 complex is important for resistance to DNA damage

It has been reported that Bre5 and Ubp3 form a complex, and that this is critical for the ability of Ubp3 to catalyze the deubiquitination of its substrates [8,15,22,25]. We independently investigated this by creating a strain in which the genomic copy of *BRE5* was C-terminally tagged with a Myc epitope. Bre5 was immunoprecipitated under stringent conditions and the associated proteins examined by SDS-PAGE and Coomassie staining. We found only one significant and reproducible associating protein in these preparations (Fig. 1A), which we identified as Ubp3 by mass spectrometry analysis, consistent with the conclusion that Bre5 and Ubp3 constitutively associate with each other *in vivo*. To verify the specificity of the interaction, immunoprecipitation of Bre5-Myc was repeated in parallel with the immunoprecipitation of an unrelated tagged protein; Hrr25-Myc. We examined the associated proteins by SDS-PAGE and silver staining and detected the presence of an Ubp3 band only when Bre5-Myc was present (Fig. 1A). We investigated the possibility that this interaction may dynamically change after cells are treated with DNA damage, but we found no significant or reproducible differences in the association of Ubp3 with Bre5 (Fig. 1A and data not shown).

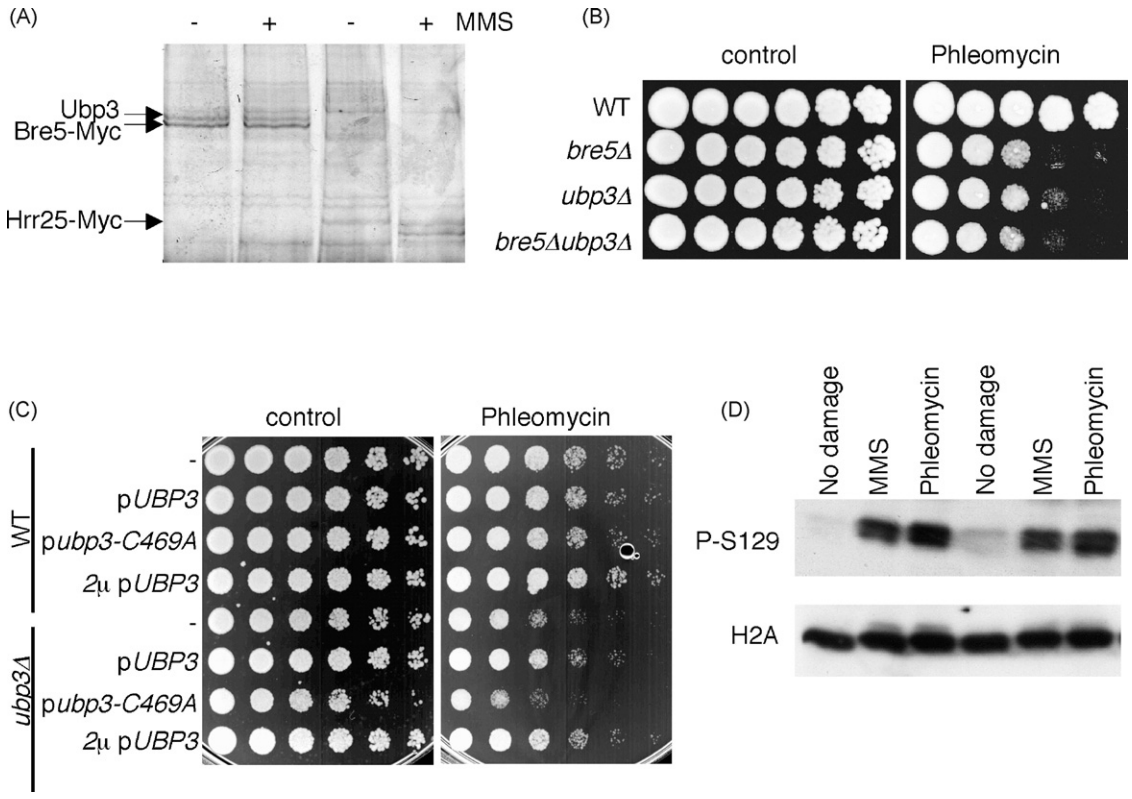


Fig. 1 – The Bre5/Ubp3 complex is important for resistance to DNA damage. (A) Coomassie stained SDS page gel of α-Myc immunoprecipitation (IP) product from JDY120 strain (Bre5-myc; lanes 1 and 2) or JDY110 (Hrr25-myc; lanes 3 and 4). IPs in lanes 2 and 4 were isolated from cultures treated with 0.1% MMS for 1 h prior to lysis. Ubp3 was identified by mass spectrometry. (B) Five-fold serial dilutions of AEY1017 (WT), JDY228 (*bre5Δ*), JDY589 (*ubp3Δ*) and JDY623 (*bre5Δubp3Δ*) were spotted onto media containing no damaging agent (control) or 0.1 μg/ml of phleomycin. (C) W303α (WT) and JDY270 (*ubp3Δ*) strains were transformed with pRS416 (empty vector), JD444 (*UBP3*), JD445 (*ubp3-C469A*), or JD446 (*UBP3* on a 2 μm plasmid). Five-fold serial dilutions of transformed strains were spotted onto media containing no damaging agent (control) or 0.5 μg/ml of phleomycin. (D) Western blot analysis of samples prepared from WT or *bre5Δ* mutant yeast strains grown in the presence of no damaging agent, phleomycin or MMS, and analyzed with an antibody against P-S129 H2A (top panel) or H2A (lower panel).

In order to investigate the potential role of this complex in DNA damage responses, we constructed strains in which each gene was disrupted to create null mutant strains. We found that both the *bre5Δ* and *ubp3Δ* mutant strains are hypersensitive to the presence of phleomycin when compared to the isogenic wild-type control (Fig. 1B), suggesting a potential role in mediating DNA damage responses. Importantly, the double *bre5Δubp3Δ* mutant strain was as sensitive to phleomycin as either single mutant, suggesting that these proteins are working together to mediate wild-type levels of survival after treatment with phleomycin. While this complex is known to act as a ubiquitin protease, it is conceivable that the role of the two proteins in mediating cellular resistance to phleomycin is due to a function unrelated to the enzymatic activity of Ubp3. We therefore constructed plasmids encoding either wild-type *UBP3* or the *UBP3* gene containing a mutation in the catalytic domain (*ubp3*-C469A [8]) and used these or an empty vector to complement the *ubp3Δ* mutant strain. We found that while the wild-type *UBP3* gene was able to restore wild-type levels of survival in the presence of phleomycin, the catalytic dead *ubp3*-C469A mutant was not, suggesting that the ability of the Bre5/Ubp3 complex to facilitate resistance to phleomycin is dependent on its enzymatic activity (Fig. 1C).

One possible explanation for the hypersensitivity of the *bre5Δ* and *ubp3Δ* mutant strains to phleomycin is a loss of the ability to detect or signal the presence of the DNA damage. One of the first cellular responses to the presence of DNA damage is the activation of the DNA damage dependent kinases Mec1 and Tel1. These kinases are required for the DNA damage checkpoint responses and their targets include histone H2A and Rad53. We therefore examined these responses in the *bre5Δ* mutant cells to determine whether the DNA damage was being appropriately sensed and responded to. We found no significant differences in the G2/M DNA damage checkpoint response or the level and timing of H2A and Rad53 phosphorylation in *bre5Δ* mutant cells in response to either phleomycin or MMS (Fig. 1D and data not shown). These data indicate that the absence of Bre5 does not affect cellular sensing and initial responses to DNA damage.

3.2. *The Bre5/Ubp3 complex contributes to NHEJ*

Because phleomycin results in the generation of DNA double-strand breaks, the Bre5/Ubp3 complex could be mediating survival in the presence of phleomycin by contributing to the DNA DSB repair pathways. In order to investigate this, we made strains with mutations in *BRE5* or *UBP3* in combination with strains lacking either components of the HR (*rad54Δ*) or NHEJ (*yku80Δ*) DNA DSB repair pathways. As seen in Fig. 2A, a *bre5Δrad54Δ* double mutant strain is more sensitive to

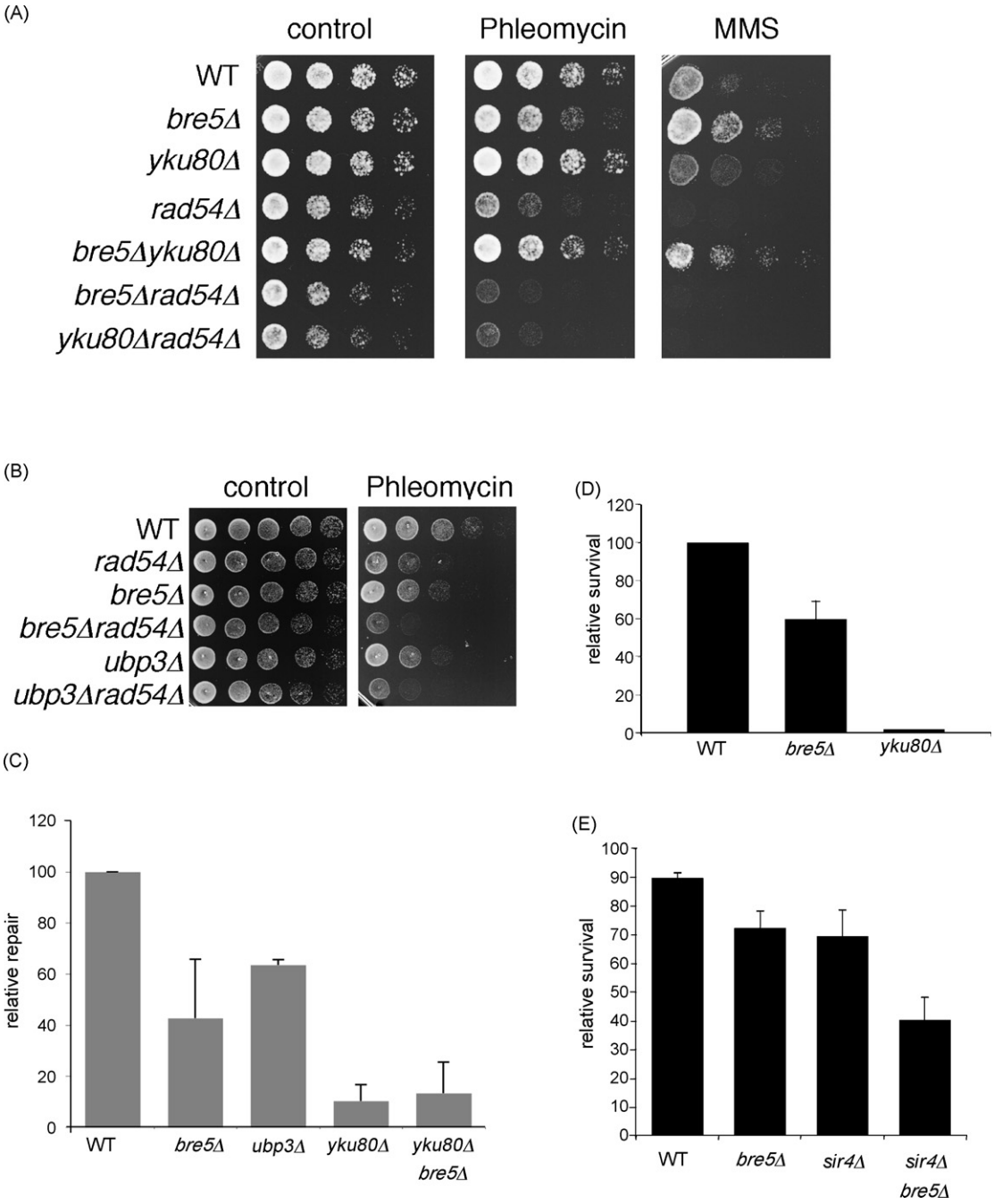


Fig. 2 – The Bre5/Ubp3 complex contributes to NHEJ. Five-fold serial dilutions of W303α (WT), JDY91 (*bre5Δ*), JDY213 (*rad54Δ*), JDY268 (*bre5Δrad54Δ*), JDY19 (*yku80Δ*), JDY227 (*bre5Δyku80Δ*), JDY270 (*ubp3Δ*), JDY646 (*yku80Δrad54Δ*), and JDY269 (*ubp3Δrad54Δ*) were spotted on to media containing (A) no damaging agent (control), 0.08 μg/ml of phleomycin, or 0.005% MMS or (B) no damaging agent (control) or 0.2 μg/ml of phleomycin. (C) Relative plasmid repair activity of W303α (WT), JDY91 (*bre5Δ*), JDY270 (*ubp3Δ*), JDY19 (*yku80Δ*) and JDY227 (*bre5Δyku80Δ*) strains transformed with supercoiled or EcoRI-digested pRS416. (D) Survival of WT (JKM179), *bre5Δ*, and *yku80Δ GAL1*-HO endonuclease containing strains relative to isogenic WT (JKM115), *bre5Δ*, and *yku80Δ* strains lacking *GAL1*-HO following galactose induction. Survival is shown relative to WT. (E) Survival of JKM115 (WT), JDY279 (*bre5Δ*), JDY605 (*sir4Δ*) and JDY607 (*bre5Δsir4Δ*) strains, transformed with pGal-HO, following galactose induction of the HO endonuclease.

phleomycin than either single mutant strain. This is similar to the additive effect seen in a *yku80Δrad54Δ* mutant strain (Fig. 2A). In contrast, we saw no significant additive effect when *bre5Δ* was combined with *yku80Δ* (Fig. 2A). The same additive effect was found when we combined the *ubp3Δ* mutation with *rad54Δ* (Fig. 2B). Together, these data suggest that the Bre5/Ubp3 complex is acting to facilitate survival after exposure to phleomycin in a manner that is distinct from HR and indicate a potential role in NHEJ. In order to more directly investigate this possibility, we performed plasmid repair assays by transforming wild-type and mutant strains with either supercoiled or EcoRI-digested linear plasmids in order to quantitate the repair efficiency. Notably, we observed a marked reduction in repair efficiency using this assay in *bre5Δ* and *ubp3Δ* mutants, although not to the same extent as that seen in a *yku80Δ* mutant strain (Fig. 2C). In addition, we performed the plasmid repair assay on *bre5Δyku80Δ* double mutant strains and saw no additional defect in repair, suggesting that *BRE5* and *YKU80* are functioning in the same pathway to mediate NHEJ.

It has been demonstrated that, while repair of linear plasmids with EcoRI-generated overhangs in wild-type yeast is accurate, the residual repair detected in *yku* mutants results in the loss of genetic information at the site of the break [7]. To investigate whether Bre5 contributes to the accurate repair of DNA ends by NHEJ, we extracted the repaired plasmids from WT, *bre5Δ*, and *yku80Δ* strains and re-digested them with EcoRI. We found that the majority of plasmids re-ligated by a *bre5Δ* mutant strain were repaired in an error free manner whereas the repair in *yku80Δ* mutants, as previously established, was error prone (data not shown). Thus, *bre5Δ* mutants had a lower efficiency of repair but uncompromised accuracy.

To further confirm the role of Bre5 in the NHEJ pathway, we deleted *BRE5* in the JKM179 background where one can induce the expression of the HO endonuclease but the donor cassettes for repairing the DSB induced by the HO endonuclease by HR are deleted [31], resulting in a DNA DSB break that has to be repaired by NHEJ. In agreement with the results observed in the plasmid repair assays, we detected decreased survival of the *bre5Δ* mutant strain when compared to wild-type after induction of the HO endonuclease, and again, the decrease in survival was not as severe as that seen in a *yku80Δ* mutant strain (Fig. 2D).

It has been reported that a *bre5Δ* mutant strain has a defect in telomeric silencing [30], discussed in more detail below. Silencing at telomeres and mating type loci has been linked to NHEJ activity, and the disruption of silencing by loss of the SIR genes results in defective end joining. This was shown to be due, at least in part, to the loss of silencing at the silent mating cassettes which results in misregulation of haploid specific genes resulting in the down-regulation of NHEJ factors [1,26]. Moreover, Ubp3 was reported to interact with Sir4 [30]. These data raise the possibility that the Bre5/Ubp3 complex may be affecting NHEJ activity via altered silencing. However, the *bre5Δ* mutant strain has increased, rather than decreased, telomeric silencing. This suggests that the alteration of silencing is unlikely to be mechanistically linked to the defect in NHEJ that we detect. Nevertheless, we investigated the possibility that the defect in NHEJ in the *bre5Δ* mutant was linked to Sir4. We therefore deleted *SIR4* and *BRE5* in the JKM115 background and transformed the derived strains with gal HO expression plasmids. HO generated DSB in this background has to be repaired by NHEJ. We found that the double *bre5ΔsirΔ* strain had and additive sensitivity to a genomic DSB (Fig. 2E), indicating that the functions provided by *BRE5* and *SIR4* in NHEJ are distinct and that Bre5/Ubp3 is not likely to be mediating NHEJ via its affect on transcriptional silencing (discussed in more detail below).

3.3. *Bre5 is associated with chromatin*

The Bre5/Ubp3 complex clearly has functions in the cytosol [4,8], and not surprisingly, GFP-tagged proteins appear to localize predominantly to the cytosol [20]. However, Bre5 was found to interact with Sir4, and the complex was recently found

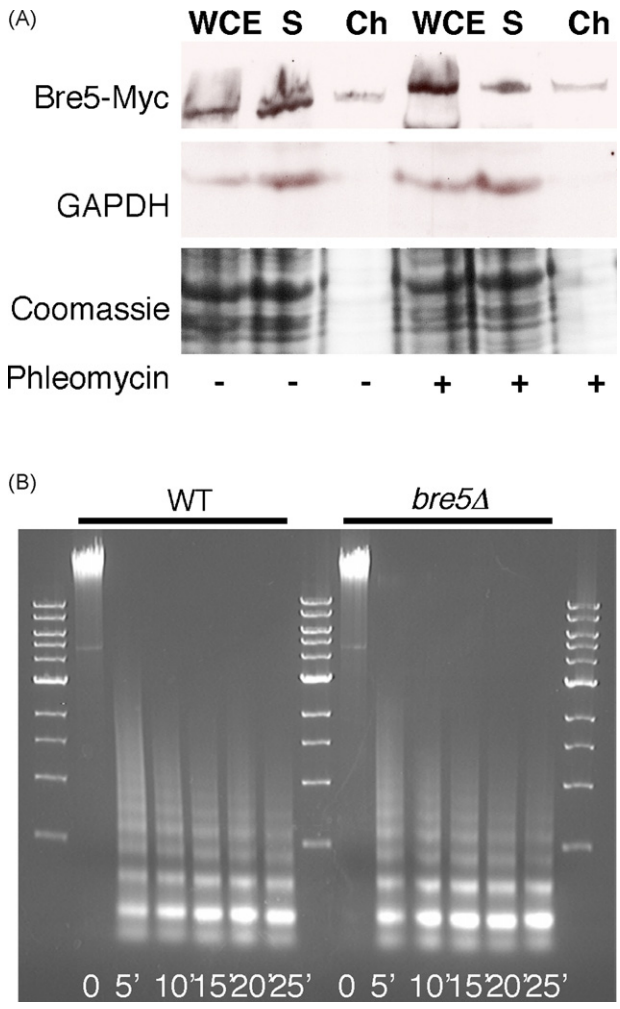


Fig. 3 – Bre5 is constitutively associated with chromatin. (A) JDY102 (Bre5-Myc) cultures were grown to log phase and treated with either no DNA damaging agent or 20 μg/ml phleomycin for 2 h. Cells were fractionated into either whole cell extracts (WCE), soluble proteins (S) and chromatin associated proteins (Ch). Fractions were analyzed by Western blot analysis with αGAPDH or α-Myc, or by Coomassie staining. (B) Spheroplasts derived from W303α (WT) and JDY91 (*bre5Δ*) strains were treated with MNase for 0–25 min and their DNA was analyzed on agarose gels stained with ethidium bromide.

associated with TFIID [2], suggesting that at least a subset of the complex is present in the nucleus. If this complex is acting directly in DNA DSB repair, we postulated that the complex would be associated with chromatin and that this association may change after treatment with phleomycin. We therefore fractionated soluble and chromatin bound proteins [11] from the strain containing Myc-tagged Bre5, and found that Bre5 is present in both fractions (Fig. 3A). In contrast, GAPDH is only found in the soluble fraction, suggesting that the chromatin fraction was not contaminated with abundant cytosolic proteins. When the same fractionation was performed after cells were treated with phleomycin, we found no significant or reproducible difference in the amount of Bre5 present in the chromatin fraction (Fig. 3A), suggesting that there is no dramatic relocalization of Bre5 to chromatin after DNA damage.

To investigate whether Bre5 has a global effect on chromatin structure, we examined the profile of micrococcal nuclease (MNase) digested chromatin in wild-type and *bre5Δ* mutant strains. There was no significant difference in the digestion patterns detected (Fig. 3B), suggesting that the presence of Bre5 in chromatin does not mediate phleomycin resistance by globally affecting chromatin architecture.

3.4. *Genetic interactions between BRE5 and BUL1*

As previously discussed, the Bre5/Ubp3 complex was recently found associated with TFIID [2]. This raises the possibility that the role of the ubiquitin protease complex in NHEJ is mediated through its effects on global transcriptional regulation. In addition to Bre5/Ubp3, Auty et al. found the Rsp5/Bul1 E3 ubiquitin ligase complex in their purified TFIID complexes [2]. Moreover, they found that these two complexes appeared to antagonistically regulate the ubiquitination status of the Taf1 and Taf5 subunits within TFIID. We therefore investigated whether Rsp5/Bul1 functioned antagonistically with Bre5/Ubp3 in DNA damage responses. While *RSP5* is an essential gene, *BUL1* is not, so we generated a *bul1Δ* mutant strain, and examined the ability of this strain to survive in the presence of phleomycin. Strikingly, we found that, in contrast to the *bre5Δ* mutant strain, the *bul1Δ* mutant strain was resistant to phleomycin when compared with the wild-type control (Fig. 4A), consistent with the possibility that these two complexes are functioning antagonistically in DNA damage responses. To further test this hypothesis, we created a double *bre5Δ/bul1Δ* mutant strain and examined the behaviour of this strain. Notably, we found that the deletion of *bul1Δ* rescues the phleomycin hypersensitivity of the *bre5Δ* deletion

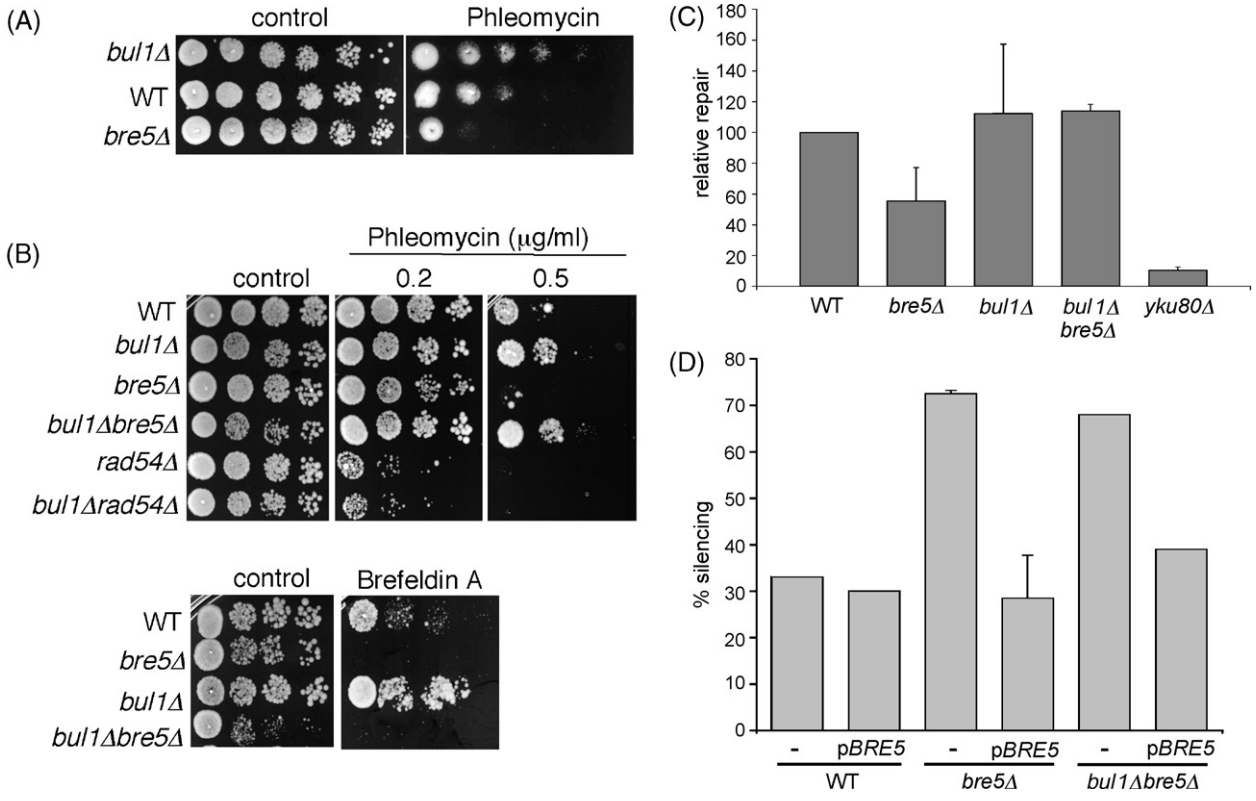


Fig. 4 – The *bul1Δ* mutation is resistant to phleomycin and rescues a subset of defects of the *bre5Δ* mutant strain. (A) Five-fold serial dilutions of JDY518 (*bul1Δ*), W303α (WT), and JDY91 (*bre5Δ*) log phase cultures were spotted onto YPAD containing no damaging agent (control), or 0.5 μg/ml of phleomycin. (B) W303α (WT), JDY518 (*bul1Δ*), JDY91 (*bre5Δ*), JDY520 (*bul1Δbre5Δ*), JDY213 (*rad54Δ*), and JDY519 (*bul1Δrad54Δ*) were assayed on plates containing no DNA damaging agent (control), phleomycin or Brefeldin A. (C) Relative plasmid repair activity of W303α (WT), JDY91 (*bre5Δ*), JDY518 (*bul1Δ*), JDY520 (*bul1ΔAbre5Δ*), and JDY19 (*yku80Δ*) strains transformed with supercoiled or EcoRI-digested pRS416. (D) Telomeric silencing assay performed on AEY1017 (WT), JDY228 (*bre5Δ*) or JDY517 (*bre5Δ bul1Δ*) strains transformed with either pRS415 (–) or JD449 (pBRE5) plasmids.

strain (Fig. 4B), suggesting that the loss of the ubiquitin ligase activity rescues the defect created by the loss of the ubiquitin protease activity. To determine whether this is specific to phleomycin resistance or whether this is applicable to the role of the Bre5/Ubp3 complex in NHEJ as well, we tested these strains using the plasmid repair assay as described above. We found that deletion of *BUL1* had no significant effect on repair activity in this assay when compared with the wild-type strain (Fig. 4C). Significantly, deletion of *BUL1* in the *bre5Δ* strain results in the restoration of NHEJ levels to approximately wild-type levels (Fig. 4C). These data indicate that *BUL1* and *BRE5* function antagonistically with regard to both phleomycin resistance and NHEJ activity.

One obvious possible interpretation of these results is that the two complexes globally regulate TFIID activity via the ubiquitination status of Taf1 and/or Taf5, and in the absence of either complex, this leads to misregulation of genes important for NHEJ activity and/or phleomycin resistance. In order to get indications about the mechanisms whereby *bre5Δ* mutations affect DNA damage tolerance, we analyzed transcript profiles of *bre5Δ* and *bul1Δ* mutants, untreated or treated with phleomycin, using DNA arrays. As the sensitivities to DNA damaging agents of these two mutants oppose each other, we first looked for opposing effects on transcript levels. It was clear, however, that the two transcript profiles were similar overall. The correlation coefficient was 0.34 (untreated *bre5Δ* versus *bul1Δ*); this is greater than the similarity between phleomycin-treated and untreated *bre5* ($r = 0.11$). Nevertheless, there were some cases of opposing regulation. Genes with a role in Golgi vesicle transport (*APL6*, *COP1*, *GGA2*, *SEC23*, *SEC24*, *SFB3*, *SFT1*) were down-regulated in *bre5Δ*, but not in *bul1Δ*, mutants. Similarly, genes required for amino acid synthesis (*CIT2*, *GLN1*, *TRP2*, *ASN2*, *BAT2*, *ALD3*) were repressed in phleomycin-treated *bre5Δ* mutants but conversely up-regulated in *bul1Δ* mutants. Consistent with the opposing roles of Bre5 and Bul1 in ubiquitin metabolism, *DSK2*, encoding a ubiquitin-like polyubiquitin-binding protein, was robustly up-regulated in *bre5Δ* but down-regulated in *bul1Δ* mutants. Interestingly, Dsk2 forms a complex with Rad23, which binds to damaged DNA with Rad4 and these proteins are involved in targeting some proteins for degradation. The misregulation of this gene may therefore contribute to the opposing effects of Bre5 and Bul1 in DNA damage responses.

Looking for additional differences that might explain the sensitivity of *bre5Δ* mutants to DNA damaging agents, we did not find changes of genes required for DNA repair or chromatin organization of a magnitude that warrant specific conclusions. However, we did note that transcripts encoding proteins involved in cell wall biogenesis (*YLR194C*, *CAP2*, *CIS3*, *ECM27*, *GFA1*, *HSP150*, *SLA2*, *PIR3*, *CWP1*, *YPS3*, *SLT2*, *PST1*) were down-regulated in phleomycin-treated *bre5Δ* cells.

To characterize the effects of *bre5Δ* and *bul1Δ* mutations on gene expression on a broader scale, we also compared our obtained expression profiles with a large dataset ("compendium") of approximately 300 array experiments representing *S. cerevisiae* systematic deletion mutants and inhibitor treatments [19]. Cluster analysis of the compendium together with our data from *bre5Δ* and *bul1Δ* mutants clearly placed expression profiles from the latter two closely together. This confirms our conclusion that the overall similarities between the expression profiles of these two mutants are far greater than the differences. The *bul1Δ* mutant present in the compendium clustered in proximity with our expression data from *bre5Δ* and *bul1Δ* mutants as expected, validating the approach. Using a variety of clustering conditions, we observed two groups of experiments that robustly clustered together with profiles from the *bre5Δ* and *bul1Δ* mutants. The first group includes the cell wall inhibitors Calcofluor White and nikkomycin, as well as *erp4Δ*, *rvs161Δ*, and *mnn1Δ*, all indicative of perturbations of vesicle transport and the plasma membrane and cell wall. The second group comprises the histone deacetylase complex mutants *rpd3Δ* and *sin3Δ* (which are involved in both transcriptional silencing at telomeres and general gene regulation) as well as *sir1Δ*, *sir2Δ*, *sir4Δ*, and *zds1Δ* (which are involved in transcriptional silencing at telomeres).

Because of this, and despite the genetic data (Fig. 2E) suggesting that the Bre5/Ubp3 complex does not work with Sir4 to mediate NHEJ, we further investigated the possibility that the roles of the Bre5/Ubp3 complex in NHEJ and in telomeric silencing are mechanistically related. Using a strain with a reporter gene inserted into telomere VII [29], we found that the *bre5Δ* mutation results in increased silencing when compared with the wild-type control as previously reported for *ubp3Δ* [30], and this effect can be complemented by the introduction of a plasmid with the wild-type *BRE5* gene expressed under the control of its own promoter (Fig. 4D).

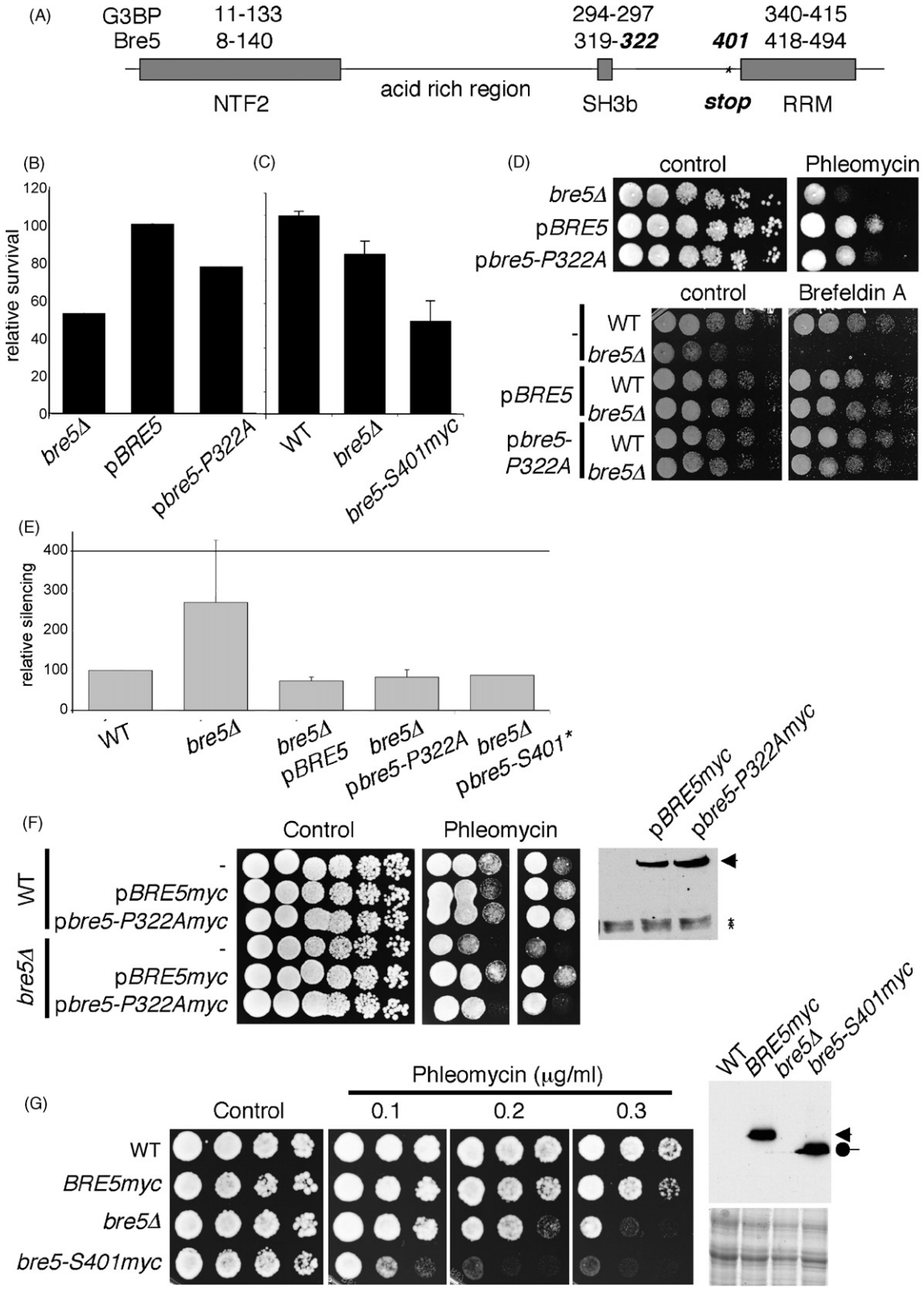
We then tested whether the disruption of *BUL1* in the mutant is able to rescue the silencing defect of the *bre5Δ* mutant strain as it does for the mutant strain NHEJ activity and phleomycin sensitivity, and importantly, we find that it does not (Fig. 4C and D). Taken together with our finding that *bre5Δ* and *sir4Δ* mutations show an additive effect in NHEJ assays when combined, these data suggest that the NHEJ activity of Bre5/Ubp3 is not due to an effect on Sir4 activity or on silencing.

BRE5 was originally identified in a screen for mutant strains with hypersensitivity to Brefeldin A [32], which, although it disrupts the Golgi apparatus, does not appear to result in the generation of DNA lesions. We also investigated the relationship between *BRE5* and *BUL1* in cellular resistance to Brefeldin A. Again, in contrast to the results obtained in the NHEJ and phleomycin sensitivity assays, the deletion of *BUL1* in the *bre5Δ* mutant background does not rescue the hypersensitivity of the *bre5Δ* mutant strain to Brefeldin A (Fig. 4B, lower panels). Bul1 and Bre5 are therefore not acting antagonistically with regard to the global regulation of transcription, telomeric silencing, or cellular resistance to Brefeldin A. This relationship between the two genes is specific to DNA damage responses. Together, these data suggest that there are specific targets important for DNA damage responses whose ubiquitination status is antagonistically regulated by the two enzyme complexes.

3.5. *Separation of BRE5 functions*

In addition to the N-terminal NFT_2-like domain of Bre5, which has been demonstrated to be important for mediating the interaction with Ubp3, Bre5 also contains a putative SH3 bind-

ing domain (PxxP) in its central region and an RNA recognition motif (RRM) in the C-terminus of the protein (Fig. 5A). We decided to investigate whether either of these conserved regions is important for mediating DNA damage responses. We created a plasmid in which the second proline of the SH3 binding region has been mutated to an alanine residue (*bre5-P322A*) and tested this construct for its ability to complement the *bre5Δ* mutation in the strain lacking the silent mating type cassettes for repairing an HO endonuclease-induced DSB. We found that this mutant construct had a modest, but statisti-



cally significant, defect in survival when compared with the strain complemented by a plasmid bearing the wild-type *BRE5* gene (Fig. 5B). We additionally tested this mutant allele in cellular resistance to phleomycin and Brefeldin A, and found that, while the *bre5*-P322A mutant construct was hypersensitive to phleomycin, it was indistinguishable from wild-type when challenged with Brefeldin A (Fig. 5D). We examined the behaviour of this mutant construct in the telomeric silencing assay, and found that there was no significant difference from the wild-type *BRE5* plasmid (Fig. 5E). Together, these data suggest that the putative SH3 binding motif is important for DNA damage responses, but not for telomeric silencing or Brefeldin A resistance. To rule out the possibility of the *bre5*-P322A mutant being partially defective in NHEJ due to protein instability, we tagged WT *BRE5* and the *bre5*-P322A mutant with Myc epitopes and re-tested their phenotypes in parallel with quantifying tagged protein levels. While the Myc-tagged proteins displayed the same phenotypes, WT and mutant Bre5 are present at comparable levels in the cell (Fig. 5F).

To investigate the role of the RRM in DNA damage responses, we created a truncation mutation in which the entire domain is deleted (*bre5*-S401*; Fig. 5A). The *bre5*-S401* construct, like the *bre5*-P322A mutant strain, was indistinguishable from the wild-type construct in the telomeric silencing assay (Fig. 5E). In contrast, the deletion of BRE5's C-terminal domain significantly impaired both its ability to repair an HO-induced DNA break in the strain lacking HML and HMR (Fig. 5C) and its resistance to DNA damage (Fig. 5G). As with the *bre5*-P322A mutation, the expression levels of the mutant protein were comparable to WT (Fig. 5G).

Previously, it was demonstrated that the deletion of *BRE5* is synthetic lethal in combination with a *sfd3* null mutation [8]. Interestingly, a construct lacking the RRM domain was able to complement this lethality, indicating that this domain was not essential for its function in this regard. Taken together with our results, it suggests that both the putative SH3 domain and the RRM are specifically important for mediating DNA damage responses.

4. Discussion

Consistent with previous reports, we find that Bre5 and Ubp3 form a complex [8,15,22,25], and that this is stable even under relatively stringent conditions (Fig. 1A). In addition, we find that *BRE5* and *UBP3* are working on the same pathway to mediate phleomycin resistance and NHEJ. While a wild-type *UBP3* gene can complement the *ubp3Δ* mutant phenotypes, a construct with a point mutation in the catalytic domain of the enzyme cannot. Together, these data suggest that the Bre5/Ubp3 complex is functioning in DNA damage responses, and that its ability to do so is dependent on the ubiquitin protease activity of the complex.

The sensitivity of *bre5Δ* and *ubp3Δ* mutants to phleomycin could be a consequence of the alterations in cell wall integrity in these strains. However, we also find a defect in two different NHEJ assays that would not be predicted to be affected by altered cell wall integrity. These data suggest that the Bre5/Ubp3 ubiquitin protease complex functions in facilitating the NHEJ DNA DSB repair pathway, and that this is likely to contribute, at least in part, to the hypersensitivity of *bre5Δ* and *ubp3Δ* mutants to the DNA DSB inducing agent phleomycin.

We found that a population of Bre5 is associated with chromatin, but found that global chromatin structure was unaffected in the *bre5Δ* mutant, suggesting that the mechanism by which Bre5 mediates cellular resistance to phleomycin is not due to a protective architectural chromatin role. Moreover, our data suggest that the role of Bre5 and Ubp3 in mediating NHEJ is not due to misregulation of transcriptional silencing. Given the reported association of the Bre5/Ubp3 complex with TFIID, we investigated the genetic relationship with the Rsp5/Bul1 ubiquitin ligase complex, which was also found to be associated with TFIID and appeared to antagonistically regulate the ubiquitination status of the TFIID subunits Taf1 and Taf5 [2]. In doing so, we found that these complexes function antagonistically with regard to both phleomycin sensitivity and NHEJ activity, but not in Brefeldin A resistance or telomeric silencing.

Fig. 5 – Separation of function mutations in *BRE5*. (A) Cartoon of domains found in human G3BP and *S. cerevisiae* Bre5. Amino-acid residues delimiting each domain are indicated. The residues that were mutated (P322A and S401stop) are italicized. (B and C) Relative survival of strains containing the inducible HO endonuclease compared with isogenic strains lacking GAL-HO. (B) *bre5Δ* mutant strains (JDY281/JDY279) transformed with plasmids pRS415 (empty vector; *bre5Δ*), JD449 (*bre5Δ* p*BRE5*) or JD450 (*bre5Δ* p*bre5-P322A*). (C) WT (JKM115), *bre5Δ* mutant (JDY279) or the truncated *bre5* mutant (JDY603; *bre5-S401*myc*) transformed with pGAL-HO were grown in inducing (galactose) or uninducing (lactate) media and their relative survival after HO endonuclease expression was calculated. (D) Upper panels: five-fold serial dilutions of JDY91 (*bre5Δ*) transformed with pRS416 (empty vector; *bre5Δ*), JD447 (p*BRE5*) or JD448 (p*bre5-P322A*) were grown on media with or without 0.5 μg/ml phleomycin. Lower panels: W303α (WT) and JDY91 (*bre5Δ*) strains were transformed with pRS416 (empty vector; -), JD447 (p*BRE5*) or JD448 (p*bre5-P322A*) and assayed on media with or without Brefeldin A. (E) Telomeric silencing assay performed on AEY1017 strain transformed with pRS415 (empty vector; WT) and JDY228 (*bre5Δ*) strain transformed with either pRS415 (empty vector; *bre5Δ*), JD449 (*bre5Δ* p*BRE5*), JD450 (*bre5Δ* p*bre5-P322A*) or JD453 (*bre5Δ* p*bre5-S401). (F) W303α (WT) and JDY91 (*bre5Δ*) strains were transformed with pRS416 (empty vector; -), JD522 (p*BRE5*-Myc) or JD556 (p*bre5-P322A*) and assayed on media with or without phleomycin. Total protein extracts were prepared from the transformed JDY91 (*bre5Δ*) strains and analyzed by Western blot with α-Myc antibodies (final panel). The arrow indicates Bre5-Myc bands and * indicates a nonspecific band. (G) W303α (WT), JDY102 (*BRE5*-myc), JDY91 (*bre5Δ*), and JDY624 (bre5-S401*-myc) strains were assayed on media with or without phleomycin. Total protein extracts were prepared from these strains and analyzed by Western blot with α-Myc antibodies (final upper panel) or stained with Coomassie (final lower panel). The arrow indicates Bre5-Myc and the circle indicates the truncated protein.**

Interestingly, however, when we investigated whether these two complexes are mediating these effects through regulation of the general transcription factor TFIID, our microarray data clearly showed that the *bre5Δ* and *bul1Δ* mutant strain profiles are more similar to each other than different.

It is possible that Bre5 and Bul1 regulate DNA damage responses through the regulation of the ubiquitination status of the TFIID subunits Taf1 and Taf5 in a local manner. That is, the DNA damage responsive behaviour of Bre5/Ubp3 and Rsp5/Bul1 may be limited to particular regions of the genome. These may correspond to specific promoters, such as *DSK2*, which we found to be antagonistically regulated by Bre5 and Bul1, or to sites of DNA damage. In this regard, it is interesting to note that Taf1 targets histone H3 K14 for acetylation, and mutation of this site in H3 leads to defects in NHEJ [33]. Alternatively, however, the lack of opposing effects on global transcription may indicate that the antagonistic roles played by the two ubiquitin-regulating complexes are not mediated through their association with and/or regulation of TFIID.

One possible subset of targets for antagonistic regulation by these two complexes include more specific transcriptional regulators that are known to be ubiquitinated. For example, we noted up-regulation of gene products for amino acid synthesis in *bul1Δ* mutants and a corresponding down-regulation in *bre5Δ* mutants. Several global regulators of amino acid synthesis are known to be ubiquitinated, including the amino acid permease Gap1 [36]. The protein products of several genes down-regulated in *bre5* mutants are required for or are targets of ER to Golgi vesicular transport. This could be due to decreased deubiquitination of Sec23, a known target of Ubp3 [8]. Vesicular transport is needed for biogenesis of the cell wall and plasma membrane. This could thus underlie the observed down-regulation of genes required for cell wall biogenesis in *bre5Δ* mutants after phleomycin treatment, which was further corroborated by the clustering of the expression profile of these mutants with profiles from cells treated with cell-wall damaging agents or mutants with known cell wall deficiencies. It is possible that the increased permeability of *bre5Δ* mutants due to defects in the cell wall or plasma membrane could lead to increased sensitivity to certain DNA-damaging agents. Our observation that *bre5Δ* mutants are more sensitive to phleomycin than *yku80Δ* mutants (Fig. 1B), even though the NHEJ defect is more pronounced in the latter mutant (Fig. 1C), would be in line with this interpretation. Genes with a more direct role in DNA damage repair and tolerance could be misregulated in *bre5Δ* mutants, but our array studies have not unequivocally singled them out.

In addition to the regulation of transcription factors, the Bre5/Ubp3 ubiquitin protease complex could target proteins more directly involved in DNA damage responses. One possibility in this regard is the core histones. An increase in ubiquitination levels of human core histones has recently been found to occur in response to DNA damage [6,39], and it is therefore likely that deubiquitination might also be involved. Yeast H2B K123 ubiquitination/deubiquitination is important for DNA damage responses [14,16], and, while we do not find a link between H2B ubiquitination and the Bre5/Ubp3 complex (data not shown), it is plausible however that, as in humans, other histone residues might be ubiquitinated and those in turn can be target for Ubp3/Bre5 deubiquitination. Another potential DNA damage specific target of this complex is the Ddr48 protein, which was identified in a large-scale proteomic approach as a Ubp3 interacting factor [25]. The transcription of the gene encoding Ddr48 is up-regulated after DNA damage [28], suggesting that it may be important for either phleomycin resistance and/or NHEJ activity. Importantly, it is clear that there are targets of the Bre5/Ubp3 ubiquitin protease complex and the Rsp5/Bul1 ubiquitin ligase complex that are specific to DNA damage responses, and it will be of great interest to identify these.

We also identified two separation of function mutations in *BRE5* that are defective for both phleomycin resistance and NHEJ activity, but function normally in other assays such as telomeric silencing and Brefeldin A resistance. One of these mutations, the truncation of the RRM domain, was also found previously to function normally in complementing the synthetic lethality of a *sfd3Δ/bre5Δ* mutation. Together, these data further solidify the conclusion that there are specific functions of the Bre5/Ubp3 complex in DNA damage responses. Since both mutations lead to a loss of a putative binding domain (although we note that in neither case have these domains been directly demonstrated to act as binding domains), these mutations may lead to a decreased affinity of the complex deubiquitination targets important for phleomycin resistance and/or NHEJ activity, and may therefore be useful in the future in identifying these targets.

Human homologues of both Bre5 and Ubp3 exist; G3BP and USP10, and these have been shown to interact [35]. While the majority of studies on G3BP have not addressed the role of the USP10 ubiquitin protease, it is reasonable to speculate that it, like Ubp3, is involved in many, if not all, of the cellular functions of G3BP. Both G3BP and USP10 have been found to be overexpressed in human cancers [3,17,34], raising the possibility that they are up-regulated in response to changes in genomic stability. In light of our studies, it will be interesting to investigate whether the G3BP/USP10 ubiquitin protease complex is also involved in mediating DNA damage responses.

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