A role for the mitotic proteins Bub3 and BuGZ in transcriptional regulation of catalase-3 expression

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Abstract

The spindle assembly checkpoint factors Bub3 and BuGZ play critical roles in mitotic process, but little is known about their roles in other cellular processes in eukaryotes. In aerobic organisms, transcriptional regulation of catalase genes in response to developmental or environmental stimuli is necessary for redox homeostasis. Here, we demonstrate that Bub3 and BuGZ negatively regulate cat-3 transcription in the model filamentous fungus Neurospora crassa. The absence of Bub3 caused a significant decrease in BuGZ protein levels. Our data indicate that BuGZ and Bub3 interact directly via the GLEBS domain of BuGZ. Despite loss of the interaction, the amount of BuGZ mutant protein negatively correlated with the cat-3 expression level, indicating that BuGZ amount rather than Bub3-BuGZ interaction determines cat-3 transcription level. Further experiments demonstrated that BuGZ binds directly to the cat-3 gene and responses to cat-3 overexpression induced by oxidative stresses. However, the zinc finger domains of BuGZ have no effects on DNA binding, although mutations of these highly conserved domains lead to loss of cat-3 repression. The deposition of BuGZ along cat-3 chromatin hindered the recruitment of transcription activators GCN4/CPC1 and NC2 complex, thereby preventing the assembly of the transcriptional machinery. Taken together, our results establish a mechanism for how mitotic proteins Bub3 and BuGZ functions in transcriptional regulation in a eukaryotic organism.

Author summary

The spindle assembly checkpoint is a key node in cell cycle during mitosis or meiosis which is responsible for proper chromosome segregation, and Bub3 along with its recently identified chaperon BuGZ are typical components in this biological process. Here, we discover a novel function of this couple of proteins in transcriptional regulation of catalase genes through extensive genetic screening in filamentous fungus Neurospora crassa. To our surprise, the regulatory relationship between Bub3 and BuGZ in fungi is quite...
different from that in higher eukaryotes, which may be attributed to the discrepancy that occur over long evolutionary stages. Moreover, the Bub3-BuGZ interaction which is essential for the checkpoint function shows insignificance in gene regulation. In contrast, the amount of BuGZ proved to be the key factor. In addition to DNA binding capacity, BuGZ is able to respond to oxidative stress and specifically inhibit gene overexpression. Besides, the conserved zinc finger domains of BuGZ also play vital roles in transcriptional repression beyond its classical DNA binding function. Our results not only expand the mechanism of catalase transcriptional regulation for maintaining redox homeostasis, but also indicate the multifunctionality of traditional mitotic proteins in life activity.

Introduction

Reactive oxygen species (ROS) are byproducts of oxygen consumption and cellular metabolism in aerobic organisms [1,2]. ROS are a heterogeneous group of highly reactive ions and molecules derived from oxygen, including superoxide anion, hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals, and singlet oxygen, which have inherent chemical properties that confer reactivity to different biological targets [3,4]. ROS can be deleterious or beneficial [5,6]. Excess toxic ROS can cause oxidation damage to macromolecules such as DNA, lipids, and proteins and underlie a wide spectrum of human disorders [7–10]. It is crucial that excess ROS be removed to maintain redox homeostasis [11,12]. Detoxification of ROS is achieved by enzymatic antioxidants such as superoxide dismutase, catalase, peroxiredoxin, glutathione peroxidase, and glutathione reductase [13,14]. Catalases are necessary for conversion of H$_2$O$_2$ to innocuous dioxygen and water and are highly conserved and precisely regulated at multiple levels in aerobionts to achieve redox homeostasis [15].

The filamentous fungus Neurospora crassa has three monofunctional catalases, CAT-1, CAT-3 and CAT-4, and one catalase peroxidase, CAT-2 [16]. CAT-1 and CAT-3 are large-subunit catalases that are responsible for most catalase activities. The activities of CAT-1 and CAT-3 are differentially regulated during the asexual life cycle [16,17]. CAT-1 is associated with non-growing cells and accumulates in asexual spores (conidia), whereas CAT-3 activity increases during exponential growth and is induced under different stress conditions [16–20]. CAT-3 is present in growing mycelia, where it mediates resistance to oxidative stress; its function cannot be replaced by other catalases [20]. Our previous studies have identified several transcriptional regulators of cat-3 expression. For example, GCN4/CPC1, a multifunctional regulator, coordinates with histone acetyltransferase GCN5 to activate cat-3 expression under oxidative stress [21], and NC2 complex activates cat-3 transcription by promoting INO80C-mediated H2A.Z removal on chromatin [22]. However, the regulatory mechanism of cat-3 transcriptional repression process is almost unknown.

The spindle assembly checkpoint (SAC), also known as the mitotic checkpoint, is a cell-cycle checkpoint that maintains genome stability by preventing the separation of the duplicated chromosomes until each chromosome is properly attached to the spindle via its kinetochore [23–27]. The molecular components of this pathway were first identified in S. cerevisiae and subsequently in higher eukaryotic organisms. The main components of the SAC are Bub and Mad family members [28–31]. The Bub proteins are components of the mitotic checkpoint complex, which inhibits the activity of anaphase-promoting complex/cyclosome (APC/C) and establishes correct kinetochore-microtubule attachments [32]. As a pioneering factor that recruits checkpoint proteins to the kinetochore, Bub3 interacts with Bub1 or BubR1 through their highly conserved GLE-2-binding sequence (GLEBS) domains; the interaction
with Bub3 is critical for their loading on the kinetochore [26,33]. Recently, BuGZ (also known as ZNF207), a novel GLEBS-containing protein that interacts with Bub3 was identified [34,35]. BuGZ is required for kinetochore loading of Bub3 and is necessary for proper chromosome alignment and mitotic progression [34–36]. A phase transition of BuGZ promotes microtubule polymerization and spindle apparatus assembly, which is critical for activation of the mitotic kinase AurA [37,38]. In addition, Bub3 and BuGZ are also implicated in the occurrence of cancer [39–42].

Bub3 and BuGZ are also known to function in other biological processes together or separately. For example, in the interphase nucleus, BuGZ and Bub3 interact with the splicing machinery and are required for pre-mRNA splicing and inhibition of R-loop-mediated DNA damage to human cells [43,44]. Moreover, Bub3 is involved in the process of telomere DNA replication, DNA methylation modification, and gene transcription inhibition [45–47].

Here, we investigated the functions of Bub3 and BuGZ in N. crassa. We found that Bub3 represses cat-3 transcription by maintaining the stability of BuGZ. BuGZ specifically binds to the cat-3 gene and shows increased recruitment in response to overexpressed cat-3 stimulated by oxidative stresses. The conserved N-terminal zinc finger domains of BuGZ are crucial for transcriptional repression but useless for DNA binding. Deletion of BuGZ led to increased recruitment of transcription activators GCN4/CPC1 and NC2 complex to regulatory regions of the cat-3 gene. Taken together, our results reveal how mitotic proteins Bub3 and BuGZ function in the regulation of intracellular redox homeostasis by controlling cat-3 expression. Our work also establishes the mechanism for the role of Bub3 and BuGZ in transcriptional regulation.

Results

*bub3* mutants resist H$_2$O$_2$-induced ROS stress and have elevated *cat-3* expression

To identify the regulatory factors involved in coping with oxidative stress, we examined the H$_2$O$_2$ sensitivity of available single gene deletion mutants of N. crassa. The strain with a *bub3* deletion (*bub3*KO, NCU09744) exhibited significant enhanced resistance to H$_2$O$_2$ compared with the wild-type (WT) strain (S1A and S1B Fig). Amino acid sequence alignment showed that the Bub3 is highly conserved among eukaryotes (S2A Fig). To confirm the function of Bub3 in H$_2$O$_2$ resistance, a plasmid carrying the sequence encoding Myc-Bub3 driven by a promoter inducible by quinic acid (QA) was introduced into the *bub3*KO strain. The ectopic expression of Myc-Bub3 and addition of QA restored wild-type levels of growth and the sensitivity to H$_2$O$_2$ (Fig 1A and 1B), indicating that the observed H$_2$O$_2$-resistant phenotype of the *bub3*KO strain was due to loss of Bub3. It should be noted that the rescue phenotype was only seen when the *bub3*KO was in the nonband (nbd) background which has a wild-type ras-1 gene [51]. Thus, subsequent experiments related to *bub3*KO transformants were carried out in this genetic background.

Since catalases are responsible for catalyzing the breakdown of H$_2$O$_2$ into water and oxygen in *N. crassa*, these results suggested that Bub3 regulates catalase expression in response to H$_2$O$_2$-induced oxidative stress. Bub3 is a component of kinetochore-bound SAC complex [28–31], but little is known about its transcriptional function. To determine whether Bub3
directly affects the activity of catalases, we used a zymogram in-gel assay to evaluate catalase activity in WT and bub3KO strains. In the mutant, there was a slightly enhancement of activity of CAT-1 and ~ 5-fold increase of CAT-3 activity compared to their activities in the WT strain (S1C Fig), suggesting that the H2O2-resistant phenotype observed in mutants was probably due to dramatically elevated CAT-3 activity. Western blot analyses revealed that the up-regulation of CAT-3 activity in mutants resulted from increased CAT-3 protein levels (S1D Fig), indicating that Bub3 is required for repression of CAT-3 expression. RT-qPCR analyses also showed a significant increase of cat-3 mRNA in the bub3KO strain compared to that in WT strain (S1E Fig). Moreover, ectopic expression of Myc-Bub3 reduced CAT-3 protein and cat-3 mRNA expression in the bub3KO strain to the WT levels (Fig 1C–1E). Taken together, these results indicate that Bub3 is a negative regulator of cat-3 expression.

Fig 1. Bub3 functions to suppress cat-3 gene expression. (A) Mycelial growth of WT, bub3KO, and bub3KO,Bub3 (nbd) strains on plates with addition of 1x10⁻³ M QA and 0, 10, or 20 mM H2O2. (B) Quantitation of growth relative to WT of bub3KO and bub3KO, Bub3 (nbd) strains under conditions described in panel A. (C) In-gel catalase activity assay of protein extracts from WT, bub3KO, and bub3KO,Bub3 strains. (D) The level of CAT-3 protein in WT, bub3KO, and bub3KO,Bub3 strains determined by western blot analyses. The membranes stained by Coomassie blue served as the loading control. (E) Levels of cat-3 mRNA in bub3KO and bub3KO,Bub3 strains relative to that in the WT strain as determined by RT-qPCR analyses. Cultures were inoculated at the center of petri dishes (9 cm in diameter) and grown at 25°C under constant light. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. *P < 0.05, **P < 0.01, and ***P < 0.001.

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BuGZ functions as a negative regulator of cat-3 gene expression

In higher eukaryotes, the interaction between Bub3 and its chaperone BuGZ contributes to the stabilization and kinetochore loading of Bub3, to achieve proper chromosome alignment and mitotic progression during cell division [34–36, 39, 52, 53]. Although BuGZ homolog is conserved in filamentous fungi (S2B Fig), they are not found in budding and fission yeasts [35]. In N. crassa, deletion of bugz (NCU06145, previously known as all development altered-21) [54] resulted in abnormalities in mycelial growth, hyphal formation, and conidial development that were more severe than those of the bub3KO strain (Fig 2A). Like the bub3KO strain, the bugzKO strain had an H2O2-resistant phenotype (Fig 2B and 2C). Moreover, the activity and the levels of cat-3 mRNA and protein were also significantly increased in the bugzKO strain (Fig 2D–2F). These data indicate that BuGZ, like Bub3, is required for suppression of cat-3 transcription.

Considering that there are other enzymes important for H2O2 detoxification such as the peroxiredoxins, which may be regulated by Bub3 or BuGZ and involved in H2O2-resistance, we further created the bub3KO cat-3KO and bugzKO cat-3KO double mutants. It is obvious that they both exhibit a H2O2-sensitive phenotype similar to that of cat-3KO strain (S3 Fig), confirming that the H2O2-resistant phenotype in bub3KO or bugzKO mutants was predominantly due to dramatically elevated CAT-3 activity.

The function of BuGZ in H2O2 resistance through regulation of cat-3 transcription was confirmed genetically. Ectopic expression of Myc-BuGZ in the bugzKO strain resulted in WT-level growth and the sensitivity to H2O2 (S4A and S4B Fig). Ectopic expression of Myc-BuGZ in the bugzKO strain also efficiently suppressed cat-3 expression to the WT level (S4C–S4E Fig). To confirm the relationship between BuGZ and Bub3 in regulation of cat-3 expression, we generated the bugzKO bub3KO double mutant. The activity and protein levels of CAT-3 in the double mutant were the same within experimental error as in the single mutants (Fig 2G and 2H), which indicates that BuGZ and Bub3 function in the same pathway to regulate cat-3 expression.

The level of BuGZ protein, but not the Bub3-BuGZ interaction, is responsible for repressing cat-3 expression

To determine how BuGZ and Bub3 coordinate to regulate the expression of cat-3, we generated polyclonal Bub3- and BuGZ-specific antibodies, which specifically recognized the endogenous protein based on predicted molecular weight in the WT strain but not in the knock-out strains (S5 Fig). To our surprise, the western blot results showed that although the level of Bub3 was slightly increased in the bugzKO strains, the lack of Bub3 led to considerable reduction in levels of BuGZ compared to that present in the WT strain (Fig 3A). This is quite different from the situation in higher organisms, in which BuGZ is required for Bub3 stability [34,35]. Since the deletion of bub3 did not cause the down-regulation of bugz expression (Fig 3B), these results suggest that Bub3 stabilizes BuGZ protein at post-transcriptional level in N. crassa.

Previous studies have shown that the GLEBS domain of BuGZ forms a series of salt bridges with the WD40 domains of Bub3; two conserved glutamate residues in the GLEBS domain are key to the interaction with Bub3 [34,55]. The GLEBS domain of BuGZ in N. crassa shares high homology with the same region in higher eukaryotes with the glutamate residues conserved (Fig 3C). To test the importance of these glutamates to the interaction with Bub3, we generated qa-2 promoter driven Myc-tagged BuGZ constructs with the GLEBS domain deleted or the conserved glutamates mutated and introduced into the bugzKO strain. A co-immunoprecipitation assay verified that all mutant BuGZ proteins had lost the ability to interact with Bub3 (Fig 3D). In cells with an EE to AA mutation or with an EE deletion, levels of the Myc-tagged
mutant BuGZ was considerably reduced compared to the Myc-tagged wild-type BuGZ; however, deletion of the entire GLEBS domain resulted in higher levels of BuGZ (Fig 3D). We next generated a series of strains with deletion of different segments inside the GLEBS domain of BuGZ (Fig 3C). The interaction between Myc-BuGZΔG1 and Bub3 was weakened compared to that between the Myc-tagged wild-type BuGZ and Bub3, while the lack of all other segments

Fig 2. BuGZ and Bub3 function in the same pathway to repress cat-3 expression. (A) Aerial hyphae and conidia of WT, bugzKO, and bub3KO strains on slants. Cultures were grown at 25˚C under constant light after 1 day at 30˚C. (B) Mycelial growth of WT and bugzKO strains on plates with 0, 10, or 20 mM H2O2. (C) Quantitation of growth of bugzKO strain relative to WT under conditions described in panel B. (D) In-gel catalase activity assays of protein extracts from WT and bugzKO strains. The membranes stained by Coomassie blue served as the loading control. (E) Quantification of/cat-3 mRNA in bugzKO strain relative to WT. (G) In-gel catalase activity assays of protein extracts from WT, bugzKO, bub3KO, and bugzKO/bub3KO strains. (H) Western blot analysis of CAT-3 protein in WT, bugzKO, bub3KO, and bugzKO/bub3KO strains. The membranes stained by Coomassie blue served as the loading control. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. * P < 0.05, ** P < 0.01, and *** P < 0.001.

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BuGZ stability does not depend on the Bub3-BuGZ interaction. (A) Western blot for BuGZ and Bub3 proteins in WT, bugzKO, and bub3KO strains. The membranes stained by Coomassie blue served as the loading control. (B) RT-qPCR quantification of bugz mRNA in bub3KO strain relative to WT. (C) Amino acid sequence alignment of the conserved GLEBS domain of BuGZ from Neurospora crassa, Homo sapiens, Mus musculus, Xenopus laevis, and Danio rerio. The conserved EE residues are indicated by a black box, and G1–G5 segments are marked. (D) Co-immunoprecipitation assays analyzed by western blot to evaluate the interaction between Myc-BuGZ, Myc-BuGZΔGLEBS, Myc-BuGZΔEE, or Myc-BuGZΔEE and endogenous Bub3. (E) Co-immunoprecipitation assays analyzed by western blot to evaluate the interaction between Myc-BuGZ, Myc-BuGZΔGLEBS, Myc-BuGZΔG1–ΔG5, or Myc-BuGZΔEE and endogenous Bub3. (F) Western blot analyses of the levels of BuGZ protein in WT, bugzKO, bugzΔBuGZ, BuGZ, bugzΔBuGZΔ.
BuGZ<sup>GLEBS</sup>, bugz<sup>KO</sup>, BuGZ<sup>AG1Δ-G5Δ</sup>, and bugz<sup>KO</sup>, BuGZ<sup>AGE</sup> strains. The membranes stained by Coomassie blue served as the loading control. A non-specific protein band is marked by an asterisk. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. *P < 0.05, **P < 0.01, and ***P < 0.001.

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resulted in complete loss of interaction between the two proteins (Fig 3E). Except for the high BuGZ level in the strain lacking the entire GLEBS domain, the amount of BuGZ protein varied among the strains with deficiency of Bub3-BuGZ interaction (Fig 3E and 3F). These data suggest that the interaction between BuGZ and Bub3 is not a determinant in maintaining BuGZ protein stability.

To explore the effects of the Bub3-BuGZ interaction and BuGZ protein stability on regulation of cat-3 expression, we evaluated the H<sub>2</sub>O<sub>2</sub> sensitivity and cat-3 expression in the bugz<sup>KO</sup> strains that express Myc-tagged BuGZ with various deletions in the GLEBS domain. Phenotypic examination and molecular data showed that the mutants with reduced BuGZ protein levels (BuGZ<sup>AG2</sup>, BuGZ<sup>AG3</sup>, BuGZ<sup>AGE</sup>) had significantly enhanced resistance to H<sub>2</sub>O<sub>2</sub> and displayed elevated cat-3 expression compared to the WT strain. In contrast, strains with unchanged (BuGZ<sup>AG1</sup>, BuGZ<sup>AG4</sup>) or up-regulated (BuGZ<sup>AGLEBS</sup>, BuGZ<sup>AGD</sup>) BuGZ levels had H<sub>2</sub>O<sub>2</sub> sensitivity and cat-3 expression comparable to the WT strain (Fig 4A-4E). Importantly, ectopic expression of Myc-BuGZ or of Myc-BuGZ<sup>GLEBS</sup> in the bub3<sup>KO</sup> strain resulted in WT levels of cat-3 expression (Figs 4F and S6). These results strongly suggest that the level of BuGZ, but not the interaction between BuGZ and Bub3, is important for cat-3 repression.

**BuGZ binds to the cat-3 region specifically for presuppression and its association with chromatin is independent of the zinc finger structure**

There are two adjacent C2H2-type zinc finger domains in the N-terminal region of BuGZ with very high homology to the same region of the protein in eukaryotes (S2B Fig). This feature suggests that BuGZ homologs may have the ability to bind DNA. To investigate whether BuGZ regulates cat-3 transcription by directly binding to the cat-3 gene, we performed ChIP assays using anti-BuGZ antibody and a set of oligonucleotide primer pairs spanning the cat-3 locus and its upstream heterochromatin region (Fig 5A). Using the bugz<sup>KO</sup> strain as a negative control, we found that BuGZ-specific association with chromatin was enriched extensively at the cat-3 locus in the WT strain. Moreover, in the absence of Bub3, recruitment of BuGZ to cat-3 locus remained but was significantly reduced compared with that in the WT strain (Fig 5B), which is consistent with the reduced level of BuGZ in the bub3<sup>KO</sup> strain.

In *N. crassa*, hyr1 (NCU09534) is the peroxiredoxin coding gene and prx1 (NCU06031) is another mitochondrial peroxiredoxin coding gene. RT-qPCR analyses showed that BuGZ could negatively regulated prx1 gene but has no effect on hyr1 expression. In contrast, Bub3 negatively regulated hyr1 but not prx1 expression (S7A Fig), indicating the difference of downstream targets between BuGZ and Bub3. Based on this, we detected the recruitment of BuGZ on the promoter region of hyr1 or prx1 for negative or positive binding controls. As we expected, BuGZ binds to the prx1 promoter significantly, while hardly associates with the hyr1 promoter as well as a telomeric heterochromatin region (4235800-4255303) on the right arm of *N. crassa* chromosome VII used for another negative control region (Fig 5B). These results proved the specificity of BuGZ binding on chromatin.

Previous studies have shown that oxidative stresses can dramatically induce cat-3 expression [56]. To further examine whether the binding of BuGZ to cat-3 responds to oxidative stresses, we treated the WT strain with H<sub>2</sub>O<sub>2</sub> or menadione (MD) for 2 hours and measured the recruitment of BuGZ to cat-3 locus. Along with the cat-3 overexpression under oxidative stimulation (S7B Fig), ChIP data revealed that the binding of BuGZ at cat-3 promoter and TSS...
Fig 4. BuGZ protein level determines extent of cat-3 transcription inhibition. (A) Mycelial growth of WT, bugz\textsuperscript{K0}, bugz\textsuperscript{K0},BuGZ, bugz\textsuperscript{K0},BuGZ\textsuperscript{GLEBS}, bugz\textsuperscript{K0},BuGZ\textsuperscript{G1\textendash}G5, and bugz\textsuperscript{K0},BuGZ\textsuperscript{EE} strains on plates with 0, 10, or 20 mM H\textsubscript{2}O\textsubscript{2}. (B) Quantitation of growth of strains described in panel A relative to WT. (C) In-gel analyses of catalases activities in extracts from WT, bugz\textsuperscript{K0}, bugz\textsuperscript{K0},BuGZ, bugz\textsuperscript{K0},BuGZ\textsuperscript{GLEBS}, bugz\textsuperscript{K0},BuGZ\textsuperscript{G1\textendash}G5, and bugz\textsuperscript{K0},BuGZ\textsuperscript{EE} strains. (D) Western blot analysis of CAT-3 protein in WT, bugz\textsuperscript{K0}, bugz\textsuperscript{K0},BuGZ, bugz\textsuperscript{K0},BuGZ\textsuperscript{GLEBS}, bugz\textsuperscript{K0},BuGZ\textsuperscript{G1\textendash}G5, and bugz\textsuperscript{K0},BuGZ\textsuperscript{EE} strains. The membranes stained by
Coomassie blue served as the loading control. (E) RT-qPCR analyses of cat-3 mRNA in bugzKO, bugzKO,BuGZ, bugzKO,BuGZAGLERS, bugzKO,BuGZAGLERSΔGLEBS, bugzKO,BuGZAGLERSΔGLEBSΔ, and bugzKO,BuGZAGLERSΔGLEBSΔG1ΔG5 strains relative to levels in the WT strain. (F) RT-qPCR analyses of cat-3 mRNA in bub3KO, bub3KO,BuGZ, and bub3KO,BuGZΔGLEBS strains relative to levels in the WT strain. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. *P < 0.05, **P < 0.01, and ***P<0.001.

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Fig 5. BuGZ binds to the cat-3 gene region specifically and its recruitment responses to cat-3 overexpression caused by oxidative stress. (A) Schematic depicting the location of cat-3 (NCU00355) gene downstream of a 5-kb AT-rich heterochromatin region on linkage group III of Neurospora genome. Site of primer binding are indicated under the schematic. Primer H binds in a heterochromatin region; P in the promoter; T near the transcription start site; O1, O2, and O3 in the open reading frame (ORF). (B) Quantification of recruitment of BuGZ to the cat-3 locus as well as control regions in WT and bub3KO strains. The bugzKO strain was used as a negative control. The prx1 gene promoter region (prx1 P) was used as a positive binding control for BuGZ, while the hylr1 gene promoter (hylr1 P) and a telomeric heterochromatin region on the right arm of chromosome VII (VIIR T) were used as negative control groups. (C, D) ChIP analysis of the binding of BuGZ after (C) H2O2 or (D) menadione (MD) treatment for 2 hours at cat-3 locus. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. *P < 0.05, **P < 0.01, and ***P<0.001.

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Increased recruitment of transcription activators in the absence of BuGZ facilitates assembly of transcription machinery on the cat-3 chromatin

We previously identified CPC1/GCN4 and NC2 complex as activators of cat-3 transcription that directly bind to the promoter or transcription start site (TSS) of the cat-3 gene [21,22]. As our data showed that BuGZ is recruited to the cat-3 gene region (Fig 5B), we wondered whether its transcriptional repression of cat-3 results from blocking the recruitment of activators through steric hindrance. To test this hypothesis, we evaluated the occupancy of CPC1 and two subunits of NC2 complex (NC2α, NC2β) at the cat-3 locus in bugzKO strains by ChIP using anti-CPC1, anti-NC2α, and anti-NC2β antibodies, respectively. As expected, the CPC1 and NC2 complex were detected at the cat-3 promoter (P) or the transcription initiation region (T) in the WT strains, but the lack of BuGZ promoted further accumulation of these factors on the cat-3 regulatory regions (Fig 7A–7C).

Consistent with the elevated binding of CPC1 and NC2 at the cat-3 locus in the absence of BuGZ, the recruitment of the general transcription factor TFIIB was also increased at the 5’ end of cat-3 gene in the bugzKO strain compared to the WT strain (Fig 7D), indicating more efficient assembly of the pre-initiation complex of the transcriptional machinery due to the loss of BuGZ. The level of Rpb1, which is the large subunit of RNA polymerase II (RNAPII), was also dramatically increased from the TSS throughout the open reading frame of cat-3 gene in the bugzKO strain compared to the WT strain (Fig 7E). In addition, the efficiency of RNAPII elongation, as indicated by the binding level of Ser2 phosphorylated RNAPII, was also increased inside the cat-3 gene body in the bugzKO strain compared to the WT strain (Fig 7F). These results indicate that in the absence of BuGZ, assembly of the transcriptional machinery on the cat-3 gene and progression of its transcription are enhanced. Taken together, our data suggest that BuGZ chromatin binding at the cat-3 gene locus interferes with binding of transcription activators to repress its expression.
Fig 6. The intact zinc finger domains of BuGZ are essential for repressing cat-3 expression. (A) Schematic of full-length BuGZ and mutants with deletions or alanine substitutions (indicated by gray diamonds) in zinc finger domains. The position of zinc finger 1 (red rectangle), zinc finger 2 (blue rectangle), GLEBS domain (yellow rectangle) and zinc ion binding sites (light purple diamonds) are indicated. (B) Mycelial growth of WT, bugzKO, bugzKO,BuGZ and transformants with various mutations in zinc finger domains on plates with 0, 10, or 20 mM H2O2. (C) Quantitation of growth rate of strains described in panel B relative to WT. (D) In-gel catalase activity assay of protein extracts from WT, bugzKO, bugzKO,BuGZ and transformants with various mutations in zinc finger domains.
(E) Western blot for CAT-3 protein in WT, bugz<sup>KO</sup>, bugz<sup>KO</sup>,BuGZ and transformants with various mutations in zinc finger domains. The membranes stained by Coomassie blue served as the loading control. (F) RT-qPCR quantification of cat-3 mRNA in bugz<sup>KO</sup>, bugz<sup>KO</sup>,BuGZ and transformants with various mutations in zinc finger domains relative to WT. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. *P < 0.05, **P < 0.01, and ***P < 0.001.

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Fig 7. BuGZ antagonizes the recruitment of transcription activators and RNAPII to the cat-3 gene. ChIP analyses of binding of (A) CPC1, (B) NC2α, (C) NC2β, (D) TFIIB, (E) RPB-1, and (F) RNAPII phosphorylated at Ser2 at the cat-3 locus in WT and bugz<sup>KO</sup> strains.

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Discussion

Catalases are indispensable enzymatic antioxidants that maintain redox homeostasis in aerobic organisms. Transcription of the genes encoding these enzymes is under strict control to maintain redox homeostasis. We previously identified several transcription activators of cat-3 expression in \textit{N. crassa}, including GCN4/CPC1 and NC2 complex [21,22]. In this study, we sought to determine the mechanism responsible for the transcriptional repression of these genes. We found that deletion of the gene encoding for the mitotic proteins Bub3 or BuGZ leads to robust cat-3 gene activation and strong resistance to H$_2$O$_2$ treatment in \textit{N. crassa}. By constructing \textit{bub3}$^{KO}$\textit{bugz}$^{KO}$ double mutants, we discovered that Bub3 and BuGZ function in the same pathway to repress cat-3 transcription. Interestingly, although there is a strong interaction between Bub3 and BuGZ, which is important for Bub3 stability and mitotic progression in higher eukaryotes [34, 35], our data showed that the Bub3-BuGZ interaction in \textit{N. crassa} was neither required for the stability of either protein nor for the regulation of cat-3 expression. However, the significantly reduced BuGZ levels in \textit{bub3}$^{KO}$ mutants resulted in overexpression of cat-3, and ectopic expression of BuGZ or BuGZ$^{\Delta GLEBS}$ both down-regulated cat-3 transcription in the absence of Bub3, confirming BuGZ as the downstream one for cat-3 repression. Unlike Bub3 which lacks DNA binding ability, BuGZ can directly bind to the cat-3 gene but independent of its highly conserved zinc finger domains. More importantly, BuGZ could respond to the oxidative stress signals for repressing the overexpression of cat-3 specifically. ChIP assays revealed that loss of BuGZ makes the local chromatin at cat-3 more accessible to transcriptional activators which results in more efficient assembly of preinitiation complex and high elongation rate of transcription. Taken together, our study uncovered the original relationship between Bub3 and BuGZ in \textit{N. crassa} that is crucial to transcriptional repression of cat-3 gene, and established the mechanism for how these mitotic proteins could directly regulate gene transcription.

Phylogenetic analysis previously revealed the differences in the evolutionary conservation of Bub3 and BuGZ [35]. Bub3 is highly conserved in all eukaryotes including budding and fission yeasts where it was first identified [28]; however, BuGZ orthologs are not found in yeast despite its strong conservation in higher eukaryotes [57]. Considering the evolutionary stage of fungi in the organic sphere, we assume that BuGZ function has undergone major changes. Evidence for a Bub3-BuGZ interaction in model organisms was revealed by examination of protein-protein interaction databases [58–63]. Our findings confirmed this interaction in \textit{N. crassa}, demonstrating that it is highly conserved in eukaryotes. However, the regulatory relationship between Bub3 and BuGZ in fungi is different from that in higher eukaryotes. In human cell lines and Xenopus egg extracts, the interaction is necessary for Bub3 stability and kinetochore loading during mitosis [34,35]. In \textit{N. crassa}, the deficiency of BuGZ did not affect Bub3 stability, whereas the lack of Bub3 caused a significant down-regulation of BuGZ protein levels. In \textit{N. crassa}, the mRNA levels of \textit{bugz} remain unchanged upon deletion of \textit{bub3}, which is similar to the situation in human cell lines [35]. Previous studies in HeLa cells demonstrated that only two highly conserved glutamates in GLEBS domain of BuGZ are essential for the interaction between Bub3 and BuGZ [34]. Analysis of the BuGZ mutants revealed that these glutamates are necessary for this interaction in \textit{N. crassa} as well. In cells with an EE to AA mutation or with an EE deletion, levels of the BuGZ was considerably reduced; however, deletion of different segments in the GLEBS domain results in loss of Bub3-BuGZ interaction but diversity of BuGZ protein stability. These data suggest that the Bub3-BuGZ interaction and BuGZ stability are regulated by different mechanisms.

The negative effect of BuGZ on gene transcription was previously reported. For instance, BuGZ was identified as a factors that negatively regulates human \textit{NFkB1} expression [64], and a
recent study performed in rice demonstrated that the BuGZ homolog acts as a transcriptional repressor of green revolution gene SD1/OsGA20ox2 [50]. BuGZ has also been reported to activate transcription. A systematic RNAi screen in C. elegans revealed that BuGZ-1 promotes synaptic vesicle recycling by regulating the activation of endocytosis-related genes such as rab11.1 [48]. Furthermore, a high-throughput analysis in human embryonic stem cells identified 177 direct target genes were up-regulated and 357 direct target genes were down-regulated in response to BuGZ depletion, with the strongest binding clustered around transcription start sites [49]. In this study, we demonstrated that the specifically binding of BuGZ is crucial for transcription repression of cat-3 by preventing the assembly and progression of the transcription machinery. Although the highly conserved zinc fingers are almost nonfunctional for DNA binding of BuGZ, they are essential for cat-3 repression. Given that there are no reports on the transcriptional function of BuGZ’s zinc fingers, we speculate that it may associate with some transcription-related effectors to achieve gene repression, which has been widely studied in higher organisms [65,66].

Interestingly, at the beginning we considered that high expression of cat-3 gene corresponded to low levels of BuGZ binding. However, by introducing the oxidative stresses which induced high expression of cat-3, we found that the recruitment of BuGZ to cat-3 locus further increased. Based on our data, we support a model that the cat-3 overexpression under stimulation alters the chromatin accessibility for elevated BuGZ binding, which in turn restores chromatin to a compacted state after removal of stimuli. Collectively, we conclude that BuGZ may serve as a founder protein for recruitment of chromatin modelers and antagonize activators bindings to restrict high cat-3 expression in normal growth condition. Whereas under oxidative stress, more open chromatin state leads to rapid response of prepared BuGZ for increased recruitment and effectively repression of cat-3 transcription, maintaining the ROS balance to physiological homeostasis.

Materials and methods

Strains and culture conditions

The 87–3 (bd, a) and 4200 (nbd, a) strain were used as the N. crassa wild-type strains in this study [51]. The ku70RIP (bd, a) strain, generated previously [67], was used as the recipient strain for creating the bub3 (NCU09744) and bugz (NCU06145) knock-out mutants, cat-3 KO strain and the bugzKO bub3KO, bub3KO cat-3KO or bugzKO cat-3KO double mutants. In all cases, entire open reading frames were replaced with resistance screening genes through homologous recombination according to a protocol described previously [68]. Plasmids encoding bub3 or bugz driven by the qa-2 promoter (pqa-5Myc-6His-Bub3, pqa-5Myc-6His-BuGZ) were transformed into bub3KO or bugzKO (his-3) strains, respectively, to construct bub3KO, Myc-Bub3 or bugzKO, Myc-BuGZ complementary transformants. The plasmid pqa-5Myc-6His-BuGZ was used as the template for mutagenesis, and the mutants (BuGZEE-AA, BuGZEE, BuGZAGLES, BuGZΔG1-ΔG5, BuGZΔF1, BuGZΔF2, BuGZΔF1F2, BuGZC2H2ΔA) were introduced into bugzKO strains to generate corresponding mutant transformants (bugzKO, Myc-BuGZEE-AA, bugzKO, Myc-BuGZEE, bugzKO, Myc-BuGZΔF1F2, bugzKO, Myc-BuGZΔF1F2, bugzKO, Myc-BuGZΔ1-Δ5, bugzKO, Myc-BuGZΔ1-Δ5, Myc-BuGZΔ1-Δ5). Similarly, plasmids pqa-5Myc-6His-BuGZ and pqa-5Myc-6His-BuGZAGLES were transformed into the bub3KO strain to construct bub3KO, Myc-BuGZ and bub3KO, Myc-BuGZAGLES transformants, respectively.

The solid medium for plate assays contained 1× Vogel’s salts, 0.1% glucose, 0.17% arginine, 50 ng/mL biotin, and 1.5% (w/v) agar with or without H2O2 and in the absence or presence of 1×10–3 M QA. The slant medium contained 1× Vogel’s salts, 3% sucrose, and 1.5% (w/v) agar. Liquid cultures were grown at 25°C with shaking in minimal medium (1× Vogel’s salts and 2%
glucose) for 18 h in constant light. When QA was added to liquid cultures, cultures were grown in low-glucose medium (1× Vogel’s salts, 0.1% glucose, 0.17% arginine) with 1x10⁻² M QA.

**Plate assay**

The medium for plate assays contained 1× Vogel’s salts, 0.1% glucose, 0.17% arginine, 50 ng/mL biotin, and 1.5% (w/v) agar with different concentrations of H₂O₂. When QA was added, 0.1% glucose was replaced with 1x10⁻³ M QA.

About 7-day-old conidia of specific strains were inoculated in petri dishes with 50 mL liquid medium containing 1× Vogel’s salts and 2% glucose under static culture condition at 25°C for 1–2 days. Disks of mycelium film were cut with a cork borer and placed in petri dishes. WT or mutant strains were inoculated at the center of the disks and grown under constant light at 25°C on the medium containing 0, 10, or 20 mM H₂O₂. When the WT strain almost completely covered the medium without H₂O₂, all the plates were scanned, and the average growth rate of each strain relative to that in medium without H₂O₂ (0 mM) was calculated. Each experiment was performed at least three times independently.

**Protein analyses**

Protein extraction, quantification and western blot analyses were performed as described previously [69]. Equal amounts of total protein (40 μg) of different samples were loaded into distinct lanes on prepared SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto PVDF membranes. Western blot analyses were performed using antibodies against the proteins of interest.

**In-gel assay for catalase activity**

Cell extracts from disks of mycelium film cultivated in liquid culture for 18 h were used for the in-gel catalase activity assays as described previously [70,71]. Equal amounts of total protein (25 μg) of different samples were loaded into distinct lanes on prepared 7.5% native-polyacrylamide gels. After electrophoresis, the gel was rinsed with doubly distilled H₂O and then immersed in 10 mM H₂O₂ with gentle shaking for 10 min. The gel was then transferred into a mixture of freshly prepared 1% potassium hexacyanoferrate (III) and 1% iron (III) chloride hexahydrate. With gentle shaking for 1–2 min, catalase activity was visualized as a band where H₂O₂ was decomposed by catalases.

**RNA analyses**

For the RT-qPCR, total RNA was isolated with TRIzol agent containing 38% phenol in 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 5.0), and 5% glycerol. Each RNA sample (total RNA, 5 μg) was treated with a double-strand specific DNase to remove contaminating genomic DNA and then subjected to reverse transcription with the Maxima H Minus cDNA Synthesis Master Mix containing Maxima H Minus Reverse Transcriptase and Thermo Scientific RiboLock RNase Inhibitor (Thermo Scientific M1682). Finally, the cDNA products were amplified by real-time PCR. The primers used for qPCR are listed in S1 Table. The relative values of gene expression were calculated using the $2^{-\Delta\Delta CT}$ method by comparing the cycle number of each sample with that of the untreated control [72]. The results were normalized to the expression levels of β-tubulin gene.
Generation of antibodies against Bub3 and BuGZ

GST-Bub3 (containing Bub3 amino acids M115-L263) and GST-BuGZ (containing BuGZ amino acids A191-P352) fusion proteins were expressed in *Escherichia coli* BL21 cells, and the soluble recombinant proteins were purified and used as the antigens to immunize rabbits to generate rabbit polyclonal antiserum as described previously [69].

Co-immunoprecipitation assay

Cell extracts from the mycelium cultured in liquid medium were used for co-immunoprecipitation analyses. Protein extraction and quantification were performed as described previously [69]. Extracts (total protein, 2 mg/mL) in extraction buffer (50 mM HEPES (pH7.4), 137 mM NaCl and 10% glycerol) were incubated with 2 μL of anti-c-Myc mouse monoclonal antibody (TransGen Biotech, #HT101) at 4˚C with rotation. After about 4 h, 40 μL pre-equilibrated protein G beads (GE Healthcare, #17-0885-02) were added and incubated for 2 h at 4˚C with rotation. Beads were then washed 6–8 times with ice-cold extraction buffer, and mixed with protein loading buffer followed by boiling for 10 min to elute the bound protein. Finally, the immunoprecipitated proteins were analyzed by western blotting.

ChIP analyses

ChIP assays were performed as described previously [73]. In brief, tissues were fixed with 1% formaldehyde for 15 min at 25˚C with shaking. Reactions were stopped by addition of glycine at a final concentration of 125 mM for 5 min. Cross-linked tissues were ground and resuspended in lysis buffer containing proteinase inhibitors, and chromatin was sheared by sonication to 500–1000 bp fragments. Samples of 2 mg/mL protein were used per immunoprecipitation reaction, and 10 μL was kept as the input DNA. The ChIP assay was carried out with 10 μL of antibody to BuGZ (self-prepared), 3 μL of antibody to H3 (CST, #2650), 2 μL of antibody to H2B (Abcam, #ab1790), 5 μL of antibody to CPC-1 (self-prepared), 8 μL of antibody to TFIIB (self-prepared), 5 μL of antibody to RPB-1 (self-prepared), and 7 μL of antibody to RNAPII phosphorylated at Ser2 (Abcam, #ab5095). Immunoprecipitated DNA was quantified using real-time PCR (ABI, 7500) with primer pairs listed in S2 Table. ChIP-qPCR data are presented as a percentage of input DNA.

Supporting information

*S1 Fig.* *bub3<sup>KO</sup>* mutant is resistant to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and shows elevated *cat-3* expression. (A) Mycelial growth of WT and *bub3<sup>KO</sup>* (bd) strains on plates with addition of 0, 10, or 20 mM H<sub>2</sub>O<sub>2</sub>. (B) Quantitation of growth relative to WT of *bub3<sup>KO</sup>* (bd) strain under conditions described in panel A. (C) In-gel catalase activity assay of protein extracts from WT and *bub3<sup>KO</sup>* strains. (D) The level of CAT-3 protein in WT and *bub3<sup>KO</sup>* strains determined by western blot analyses. The membranes stained by Coomassie blue served as the loading control. (E) Levels of *cat-3* mRNA in *bub3<sup>KO</sup>* strain relative to that in the WT strain as determined by RT-qPCR analyses. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed *t*-test. *P < 0.05, **P < 0.01, and ***P < 0.001. (TIF)

*S2 Fig.* Bub3 and BuGZ are highly conserved among eukaryotes. Amino acid sequence alignments of (A) Bub3 and (B) BuGZ from *Neurospora crassa*, *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. Seven WD40 repeats of Bub3, two zinc finger domains and the GLEBS domain of BuGZ are marked in black boxes. The zinc ion binding
sites of BuGZ are indicated with red asterisks.

(TIF)

S3 Fig. The H₂O₂-resistant phenotype of bub3KO and bugzKO strain was predominantly due to elevated CAT-3 activity. (A) Mycelial growth of WT, cat-3KO, bub3KO, bub3KO cat-3KO, bugzKO and bugzKO cat-3KO strains on plates with 0, 5, 10, or 20 mM H₂O₂. (B) Quantitation of growth relative to WT of cat-3KO, bub3KO, bub3KO cat-3KO, bugzKO and bugzKO cat-3KO strains under conditions described in panel A.

(TIF)

S4 Fig. BuGZ is a negative transcriptional regulator of cat-3 gene expression. (A) Mycelial growth of WT, bub3KO, and bub3KO BuGZ strains on plates with 0, 10, or 20 mM H₂O₂. (B) Quantitation of growth relative to WT of bub3KO and bub3KO BuGZ strains under conditions described in panel A. (C) In-gel catalase activity in protein extracts from WT, bub3KO, and bub3KO BuGZ strains. (D) Western blot analysis of CAT-3 protein in WT, bub3KO, and bub3KO BuGZ strains. The membranes stained by Coomassie blue served as the loading control. (E) RT-qPCR quantification of cat-3 mRNA relative to WT in bub3KO and bub3KO BuGZ strains. Error bars indicate SD (n = 3). Significance was evaluated by two-tailed t-test. *P < 0.05, **P < 0.01, and ***P < 0.001.

(TIF)

S5 Fig. Rabbit-derived polyclonal antibodies specifically recognize N. crassa Bub3 and BuGZ. Immunodetection of Bub3 or BuGZ in WT and (A) bub3KO or (B) bub3KO BuGZ strains using polyclonal antibodies that specifically recognize Bub3 or BuGZ protein, respectively. The membranes stained by Coomassie blue served as the loading control.

(TIF)

S6 Fig. Ectopic expression of BuGZ or BuGZΔGLEBS protein reduce CAT-3 expression in bub3 mutants. (A) In-gel catalase activity assay of protein extracts from WT, bub3KO, bub3KO, Bub3, bub3KO BuGZ and bub3KO BuGZΔGLEBS strains. (B) Western blot for CAT-3 protein in WT, bub3KO, bub3KO, Bub3, bub3KO BuGZ and bub3KO BuGZΔGLEBS strains. The membranes stained by Coomassie blue served as the loading control.

(TIF)

S7 Fig. BuGZ negatively regulates the peroxiredoxin encoding gene prx1 but its recruitment does not respond to MD-induced prx1 overexpression. (A) RT-qPCR quantification of peroxiredoxin encoding gene hyr1 and prx1 mRNA relative to WT in bub3KO and bub3KO strains. (B) Levels of cat-3 mRNA change in WT strain after H₂O₂ or MD treatment for 2 hours determined by RT-qPCR analyses. (C) Levels of prx1 mRNA change in WT strain after MD treatment for 2 hours determined by RT-qPCR analyses. (D) ChIP analysis of the binding of BuGZ after MD treatment for 2 hours at prx1 locus. Primer prx1 P, prx1 T, prx1 O₁, prx1 O₂ indicate the promoter, TSS and ORF regions of prx1 respectively as marked in cat-3 locus.

(TIF)

S8 Fig. Mutations in zinc finger domains do not dramatically affect the DNA binding ability of BuGZ, as well as the stability of BuGZ and Bub3-BuGZ interaction. (A, B) ChIP analysis of the binding of Myc-BuGZ with various mutations in zinc finger domains at cat-3 (A) promoter or (B) TSS region (the primer P and primer T are identical to those in Fig 5A). (C) Western blot analyses of the level of BuGZ protein in WT, bub3KO, bub3KO BuGZ and transformants with various mutations in zinc finger domains. The membranes stained by Coomassie blue served as the loading control. A non-specific protein band is marked by an asterisk. (D) Co-immunoprecipitation analyses of interactions between Myc-BuGZ with various mutations.
in zinc finger domains and endogenous Bub3.

(TIF)

S1 Table. Primers for RT-qPCR assays.
(XLSX)

S2 Table. Primers for ChIP-qPCR assays.
(XLSX)

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