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Repression of G_1/S Transcription Is Mediated via Interaction of the GTB Motifs of Nrm1 and Whi5 with Swi6

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In *Saccharomyces cerevisiae*, G_1/S transcription factors MBF and SBF regulate a large family of genes important for entry to the cell cycle and DNA replication and repair. Their regulation is crucial for cell viability, and it is conserved throughout evolution. MBF and SBF consist of a common component, Swi6, and a DNA-specific binding protein, Mbp1 and Swi4, respectively. Transcriptional repressors bind to and regulate the activity of both transcription factors. Whi5 binds to SBF and represses its activity at the beginning of the G_1 phase to prevent early activation. Nrm1 binds to MBF to repress transcription as cells progress through S phase. Here, we describe a protein motif, the GTB motif (for $\underline{G}_1$/S $\underline{t}$ranscription factor $\underline{b}$inding), in Nrm1 and Whi5 that is required to bind to the transcription factors. We also identify a region of the carboxy terminus of Swi6 that is required for Nrm1 and Whi5 binding to their target transcription factors and show that mutation of this region overrides the repression of MBF- and SBF-regulated genes by Nrm1 and Whi5. Finally, we show that the GTB motif is the core of a functional module that is necessary and sufficient for targeting of the transcription factors by their cognate repressors.

The cell cycle is driven by sequential waves of gene expression coupled with regulated protein degradation. G_1/S transcription is activated at the beginning of a new cell cycle leading to the expression of around 300 genes in the budding yeast *Saccharomyces cerevisiae*, including the many genes responsible for morphogenesis, DNA replication, and DNA repair (1, 2). The G_1/S family of genes is controlled by two transcription factors, SBF and MBF, each comprised of a common component, Swi6, and a sequence-specific DNA binding protein, Swi4 and Mbp1, respectively. SBF acts primarily as a transcriptional activator that regulates many genes involved in morphogenesis and the timing of cell cycle commitment. In contrast, MBF acts largely as a transcriptional repressor that controls the expression of many genes involved in DNA replication and repair (2, 3–5).

The G_1/S transcription factors depend upon transcriptional repressors to confer timely expression of their target genes. The proper regulation of SBF targets requires the transcriptional repressor Whi5 (6, 7), which, when bound to SBF during early G_1 phase, represses its activity. Cln3/cyclin-dependent kinase (CDK) phosphorylates Whi5, promoting dissociation from SBF and export from the nucleus leading to transcriptional activation of its targets. As cells progress through S phase, accumulation of Clb/CDK activity phosphorylates SBF, releasing it from the promoters and thereby repressing transcription of its target genes (8). Although the mechanism of activation of MBF remains obscure, it is activated coordinately with SBF in a manner dependent upon Cln/CDK. However, inactivation of MBF-dependent transcription requires binding of Nrm1, a transcriptional corepressor encoded by an MBF target that, when expressed, represses MBF-dependent transcription via a negative feedback loop (4). The distinct mechanisms for repression of SBF and MBF enable cells to differentially regulate their target genes at the end of the G_1 phase. For example, MBF target genes are expressed in response to genotoxic stress, whereas SBF targets are repressed. The effector protein kinase of the *S. cerevisiae* S-phase checkpoint, Rad53, phosphorylates Nrm1 and prevents its binding to the promoters, allowing the sustained expression of MBF target genes during DNA replication stress, a response required to maintain genomic stability and cell viability (4, 9, 10).

In the distantly related fission yeast, *Schizosaccharomyces pombe*, regulation of G_1/S transcription relies entirely on MBF, which is comprised of two copies of the Swi6 homolog, Cdc10, along with Res1 and Res2 (11). In fission yeast, SpNrm1 binds to and inhibits MBF-dependent transcription as cells progress through S phase (4), and, as in the budding yeast, that regulation is antagonized by genotoxic stress (12). Despite poor conservation of the G_1/S transcription factors and their transcriptional repressors at the level of amino acid sequence between yeast and humans, there is striking conservation of the regulatory pathways by which these factors exert their effects. In mammalian cells, the G_1/S transcriptional activator E2F1-E2F3, which exhibits no sequence homology to SBF components, is regulated by the retinoblastoma tumor suppressor and transcriptional repressor Rb in a manner analogous to the regulation of SBF by Whi5 in budding yeast. Rb binds to E2F until accumulation of G_1 cyclin/CDK activity phosphorylates it and releases it from promoters, allowing G_1/S transcription to be activated (13). The high degree of conser-

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TABLE 1 List of yeast strains used in this study

Yeast strain	Genotype	Reference or source
CWY231	15Daub; *MAT***a** *ade1 leu2-3,112 his2 trp1-1 ura3Δns bar1Δ*	16
CWY758	*SWI4*-3×HA-*KAN*[r]	K. Flick and C. Wittenberg, unpublished data
CWY874	*mbp1*::*URA3*	17
CWY1415	*whi5*::*KAN*[r]	7
CWY1446	*WHI5*-13×Myc-*KAN*[r]	7
CWY1520	*MBP1*-TAP-*KAN*[r]	4
CWY1521	*SWI4*-TAP::*KAN*[r]	This study
CWY1559	*NRM1*-13×Myc-*URA3*	4
CWY1561	*NRM1*-13×Myc-*URA3 MBP1*-TAP-*KAN*[r]	4
CWY1624	*swi4*::*KAN*[r]	4
CWY1628	*NRM1*-13×Myc-*URA3 MBP1*-TAP-*KAN*[r] *swi6*::*TRP1*	4
CWY1733	*whi5*::*KAN*[r] *URA3*::*WHI5*^{WIQ}-13×Myc-*HIS2*	This study
CWY1739	*whi5*::*KAN*[r] *URA3*::*WHI5*^{WIQ}-13×Myc-*TRP1 SWI6*-3×HA-*LEU2*	This study
CWY1740	*whi5*::*KAN*[r] *URA3*::*WHI5*^{WIQ}-13×Myc-*HIS2 SWI4*-3×HA-*TRP1*	This study
CWY1744	*whi5*::*KAN*[r] *URA3*::*WHI5*-13×Myc-*HIS2 SWI4*-3×HA-*TRP1*	This study
CWY1745	*whi5*::*KAN*[r] *URA3*::*WHI5*-13×Myc-*HIS2 SWI6*-3×HA-*LEU2*	This study
CWY1763	*SRL3*-13×Myc-*TRP1*	This study
CWY1821	*TRP1*::*GAL-NRM1ΔN*-13×Myc-*URA3*	4
CWY1868	*WHI5*-13×Myc-*KAN*[r] *swi6*::*URA3*	This study
CWY1989	*nrm1*::*TRP1* YIplac211-*nrm1*ΔGTB-13×Myc-*KAN*[r]	This study
CWY1993	*NRM1*-13×Myc-*URA3 SWI6*-3×HA-*LEU2*	This study
CWY1994	*nrm1*::*TRP1* YIplac211-*nrm1*ΔGTB-13×Myc-*KAN*[r] *SWI6*-3×HA-*LEU2*	This study
CWY2344	*NRM1*-13×Myc-*KI URA3 MBP1*-TAP-*KAN*[r] *SWI6*ΔC-3×HA-*HIS2*	This study
CWY2345	*TRP1*::*GAL-NRM1*ΔN-13×Myc-*URA3 SWI6*ΔC-3×HA-*HIS2*	This study
CWY2361	*WHI5*-13×Myc-*KAN*[r] *SWI6*ΔC-3×HA-*URA3*	This study
CWY2369	*SWI6*ΔC-3×HA-*URA3*	This study
CWY2370	*SWI6*-3×HA-*URA3*	This study
CWY2381	*SWI6*ΔC-*URA3*	This study
CWY2425	*WHI5*-13×Myc-*KAN*[r] *SWI6*ΔC-*URA3*	This study

vation of the G_1/S transcriptional regulatory pathway indicates that it is crucial for maintaining fitness in these organisms. Indeed, mutation of the G_1/S transcription factors and their regulatory proteins, such as Rb, is a hallmark of cancer (14).

Here, we show that the transcriptional repressors Whi5 and Nrm1 both interact with the carboxy terminus of Swi6 to inhibit transcription. Deletion of the Swi6 carboxy terminus renders cells insensitive to both Nrm1 and Whi5. We also identify a motif shared by the transcriptional repressors that is necessary and sufficient for their interaction with the Swi6 carboxy terminus. This motif, the GTB ($\underline{G}_1$/S $\underline{t}$ranscription factor $\underline{b}$inding) motif, is conserved among fungi and represents the core of a transferable domain that is sufficient to confer the specificity of Nrm1 and Whi5 for MBF and SBF, respectively. These findings indicate that, in addition to conservation of the pathways for G_1/S transcription regulation, the structural basis for the interaction between the repressors and their target transcription factors is conserved.

MATERIALS AND METHODS

Strains. All yeast strains used in this study were derived from 15Daub (*MAT***a** *ade1 leu2-3,112 his2 trp1-1 ura3Δns bar1Δ*). The yeast strains used are presented in Table 1. Generation of a *SWI6* mutant encoding a protein that lacks the last 50 amino acids of the C terminus (*SWI6*ΔC), *nrm1* encoding a protein with a deletion of the GTB motif (*nrm1*ΔGTB), and all the tagged proteins and deletion mutants was performed using the PCR method described by Longtine et al. (15). Whi5^{WIQ}, consisting of Whi5 with a mutation of R185, A189, and K192 to W185, I189, and Q192, respectively, was constructed via site-directed mutagenesis with a QuikChange XL site-directed mutagenesis kit (Agilent Technologies), followed by DNA sequencing to confirm the generation of mutations.

Antibodies. The antibodies against budding yeast Swi4 and Swi6 were prepared by D. Lee and N. F. Lowndes and characterized by M. R. Harris, D. Lee, S. Farmer, N. F. Lowndes, and R. A. M. de Bruin (submitted for publication).

Cell synchronization and cell size experiments. Mating pheromone arrest synchrony experiments were carried out as described previously (4). For cell size experiments, cells were grown exponentially in appropriate medium, consisting of yeast extract, peptone, and dextrose (YEPD) or YEP-galactose, and size was determined using a Coulter Z2 (Beckman) cell counter.

Coimmunoprecipitation. Immunoprecipitations were carried out using lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100) with protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin). Immunoprecipitated proteins were resolved in 10% SDS-PAGE gels, and antibodies anti-Myc, antihemagglutinin (HA), anti-Swi6, anti-Swi4, and peroxidase-antiperoxidase (PAP) soluble complex were used to detect the tagged proteins appropriately.

Pulldowns. Glutathione *S*-transferase (GST)–GTB (Nrm1), GST-GTB (Whi5), and GST tag alone were expressed in *Escherichia coli* BL21(DE3) RIL and purified by using glutathione-Sepharose beads. Beads were incubated with extracts from the appropriate yeast strains in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100) with protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin) for 2 h at 4°C. Next, beads were washed four times with the same lysis buffer and resolved by 10% SDS-PAGE. Appropriate antibodies were used to detect the tagged proteins.

Real-time PCR and RT-PCR. Total RNA was isolated using an RNeasy Kit (Qiagen). iQ SYBR green Supermix (Bio-Rad) was used for quantitative PCR on chromatin immunoprecipitation (ChIP) samples, and an iScript OneStep RT-PCR kit with SYBR green (Bio-Rad) was used for reverse transcription-PCR (RT-PCR). Reactions were run on a Chromo-4

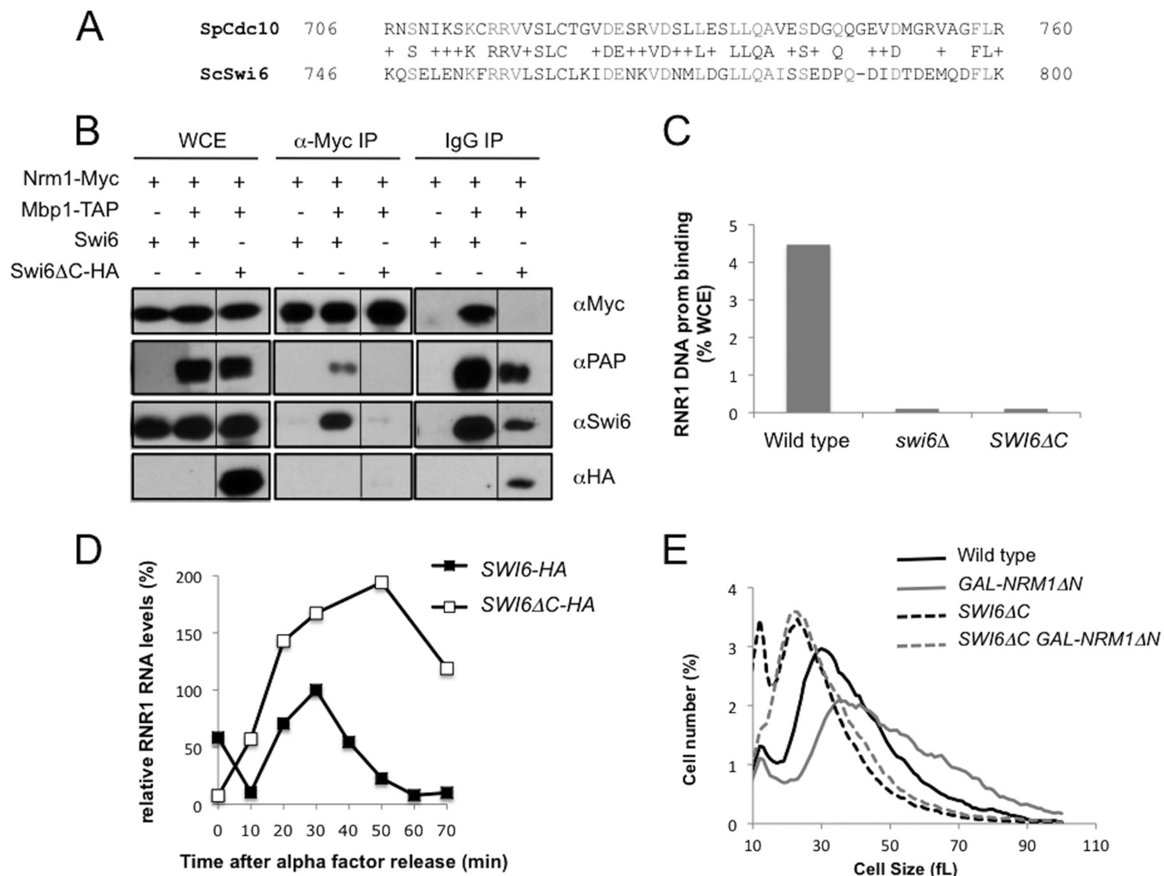

FIG 1 C terminus of Swi6 is required for Nrm1 binding to MBF. (A) Alignment of the last 50 residues of *S. cerevisiae* (Sc) Swi6 and *S. pombe* (Sp) Cdc10. (B) Nrm1 does not interact with MBF when the C terminus of Swi6 is deleted. Immunoblot showing whole-cell extracts (WCE), anti-Myc, and IgG immunoprecipitation (IP) of Mbp1-TAP or Nrm1-Myc in a wild-type or *SWI6*ΔC background. All immunoblots were derived from samples run on a single SDS-polyacrylamide gel. (C) C terminus of Swi6 is required for Nrm1 binding to MBF-dependent promoters. Quantitation of Nrm1-Myc binding to *RNR1* promoter (prom) DNA by chromatin immunoprecipitation (ChIP) in wild-type, *swi6*Δ, and *SWI6*ΔC cells growing exponentially. (D) MBF-regulated transcription is induced in *SWI6*ΔC cells. Wild-type and *SWI6*ΔC cells exponentially growing in rich medium were synchronized in G_1 by α-factor and subsequently released into rich medium. MBF-regulated gene *RNR1* RNA from arrested cells (0 min) and from cells released from the arrest for the indicated interval was quantitated by RT-qPCR, and results are shown as a percentage of the maximal RNA level in wild-type cells. (E) Overexpression of a stable form of Nrm1 does not increase cell size in *SWI6*Δ*C* cells. Cell size was determined for wild-type, *GAL-NRM1*ΔN, *SWI6*ΔC, and *SWI6*ΔC *GAL-NRM1*ΔN cells growing exponentially in galactose-containing medium to induce Nrm1ΔN overexpression. α, anti.

qPCR (quantitative PCR) I system (MJ Research) under standard PCR and RT-PCR conditions. Data were analyzed by using MJ Opticon Monitor, version 3.0, analysis software.

ChIP analysis. Chromatin immunoprecipitation was performed as described previously (12).

RESULTS

The C terminus of Swi6 is required for Nrm1 binding to MBF. The interaction between Nrm1 and MBF in *Schizosaccharomyces pombe* requires the last 61 residues of the carboxy terminus of Cdc10 (12). Since Nrm1 regulation of MBF in *S. pombe* and *S. cerevisiae* is conserved (4), we determined whether the sequences required for the interaction between Nrm1 and its target transcription factor are also conserved. SpCdc10 and ScSwi6, its functional homolog in *S. cerevisiae*, are poorly conserved in terms of sequence. The alignment of the two proteins shows that only the carboxy-terminal half of the protein is conserved, with 30% identity and 49% similarity. Interestingly, the conservation is highest within the last 50 residues, exhibiting 45% identity and 72% similarity (Fig. 1A), suggesting that the carboxy terminus of ScSwi6 and that of SpCdc10 might have similar functions.

To study the function of the C terminus of Swi6, we generated a Swi6 mutant that lacks the last 50 amino acids, which we refer to as Swi6ΔC. The effect of that mutant on the interaction between Swi6 and its binding partners was evaluated by coimmunoprecipitation in cells expressing either wild-type Swi6 or Swi6ΔC along with tandem affinity purification (TAP)-tagged Mbp1 and Myc-tagged Nrm1 (Fig. 1B). Immunoprecipitation of Nrm1-Myc also precipitates Mbp1-TAP, and vice versa. However, when the carboxy terminus of Swi6 was deleted, the interaction between Nrm1-Myc and Mbp1-TAP was no longer detected. Importantly, Mbp1 still binds to Swi6ΔC, indicating that the deletion of the C terminus affects Nrm1 binding but not the formation of the MBF complex.

To establish whether the effect of the Swi6 carboxy terminus deletion on the interaction between Nrm1 and MBF is also reflected in their interaction when MBF is bound to DNA, we performed chromatin immunoprecipitation (ChIP) of Nrm1 in cells

expressing wild-type *SWI6*, *swi6*Δ, and *SWI6*ΔC during late G_1 phase. Cells were synchronized during G_1 phase by treatment with the mating pheromone α-factor and then released into the cell cycle for 60 min, the interval when Nrm1 binds to the MBF-dependent promoters. Binding of Nrm1 to the MBF target was abrogated in the *SWI6*ΔC mutant, similar to the *swi6*Δ mutants, confirming the importance of the carboxy terminus of Swi6 for Nrm1 binding (Fig. 1C).

Transcription of MBF target genes is repressed as cells progress from G_1 into S phase as a consequence of Nrm1 binding to MBF at promoters although, in the absence of Nrm1, those genes are repressed after a delay by Clb/CDK (4, 8). Thus, we expect *SWI6*ΔC cells to exhibit a delay in repression of MBF targets as cells progress into S phase. To test this hypothesis, we analyzed expression of the MBF target gene *RNR1* in wild-type and *SWI6*ΔC cells synchronized in G_1 with α-factor and released into the cell cycle. As shown in Fig. 1D, *SWI6*ΔC cells exhibit sustained *RNR1* expression, indicating that in the absence of the C terminus of Swi6, Nrm1 can no longer bind to MBF and repress its targets.

To determine whether the effect of *SWI6*ΔC on Nrm1 binding and gene expression was detectable in terms of cellular phenotype, we evaluated whether overexpression of a stabilized form of Nrm1 (Nrm1ΔN) in the *SWI6*ΔC background leads to the increase in cell size as a result of the cell cycle delay during G_1 phase that was previously associated with the *NRM1*ΔN mutant (4). Whereas wild-type cells expressing Nrm1ΔN from the *GAL1* promoter are significantly larger than wild-type cells, the overexpression of Nrm1ΔN has no effect in cells lacking the C terminus of Swi6 (Fig. 1E), consistent with a failure of Nrm1 to repress MBF-regulated transcription.

Together, these observations indicate that the last 50 amino acids of Swi6 are crucial for Nrm1 binding to MBF and for its function as a repressor of MBF-regulated transcription.

Whi5 binds to SBF through the C terminus of Swi6. In addition to its role in the formation of MBF, Swi6 also participates in the formation of the SBF transcription factor along with the DNA binding protein Swi4. SBF is bound by the transcriptional repressor Whi5 at the beginning of G_1 phase, leading to the repression of SBF targets. Deletion of Whi5 derepresses transcription during early G_1 phase and promotes premature bud formation and progression through G_1/S transition, leading to the production of smaller daughter cells (6, 7, 18). We noticed during our analysis of the effect of GAL-*NRM1* on the *SWI6*ΔC mutant that the cells are smaller than wild-type cells, at least when grown on galactose (Fig. 1E). That phenotype is consistent with a loss of function of Whi5, suggesting that the carboxy terminus of Swi6 might also be required for the interaction between Whi5 and SBF. To test this possibility, wild-type and *SWI6*ΔC cells were grown in rich glucose medium, and cell size was analyzed (Fig. 2A). The *SWI6*ΔC mutant cells are smaller than wild-type cells, consistent with a role for the carboxy terminus of Swi6 in the interaction between Whi5 and SBF.

To determine whether deletion of the last 50 residues of Swi6 abrogates Whi5 binding, we evaluated that interaction in *SWI6*ΔC mutants expressing Myc-tagged Whi5 from the endogenous locus. Whereas Whi5 coimmunoprecipitates with Swi6 and Swi4 in wild-type cells, neither Swi6 nor Swi4 coimmunoprecipitates with Whi5 in *SWI6*ΔC mutants (Fig. 2B). However, Swi4 still binds to Swi6ΔC, demonstrating that, similar to the situation with Nrm1 and MBF, the carboxy terminus of Swi6 is important for its interaction with the transcriptional repressor Whi5 but not for the formation of the SBF complex (Fig. 2B).

To determine whether the loss of interaction between Whi5 and SBF observed by coimmunoprecipitation also holds for SBF bound to chromatin, ChIP analysis of Myc-tagged Whi5 was performed in wild-type cells and *swi6*Δ and *SWI6*ΔC mutants. In wild-type cells arrested during G_1 by α-factor, Whi5 binds to the promoters of the SBF-dependent genes, *CLN2* and *SVS1*, but not to the MBF-dependent gene promoter *RNR1* (Fig. 2C). However, that binding is completely abrogated when the carboxy terminus of Swi6 is deleted, indicating that this region of Swi6 is required for binding between Whi5 and SBF at promoters.

Consistent with the role of Whi5 as a transcriptional repressor, SBF-dependent transcription is activated prematurely during the cell cycle in the absence of Whi5 (7). To determine whether the deletion of the C terminus of Swi6 recapitulates that behavior, we evaluated the expression of the SBF-dependent transcript *CLN2* during the cell cycle in wild-type and *SWI6*ΔC mutant cells synchronized by α-factor arrest and released. *CLN2* expression is activated prematurely in *SWI6*ΔC mutants, consistent with the requirement for the carboxy terminus of Swi6 for Whi5 binding to SBF and for its function as a transcriptional repressor (Fig. 2D). Importantly, the robust periodic activity of G_1/S genes in the *SWI6*ΔC mutant demonstrates that, despite its incapacity to bind to the transcriptional repressors, Swi6ΔC is competent to form a fully functional G_1/S transcription factor.

Nrm1 and Whi5 share a region of homology designated the GTB motif. The 50 carboxy-terminal amino acids of Swi6 are required for binding of the transcriptional repressors Nrm1 and Whi5 to MBF and SBF, respectively (Fig. 1 and 2). This suggested that Nrm1 and Whi5 might share a structural motif required for that interaction. Alignment of Nrm1 and Whi5 homologs in *S. cerevisiae* and *S. pombe* reveals a conserved region of 11 residues contained within a predicted alpha-helical domain that has the consensus motif LXXRLXXAXXK (Fig. 3A). That motif defines a protein family in *S. cerevisiae* that includes Whi5, Nrm1, and Srl3, a CDK-binding protein of unknown function (19). Interestingly, a protein that appears to behave as a functional homolog of both Nrm1 and Whi5 was recently identified in *Candida albicans* based upon the presence of that motif, which appears to be extensively conserved among fungal species (20). The motif has also been identified via bioinformatics approaches and has been named the "Whi5-like" motif (21). We refer to this as the $\underline{G}_1$/S $\underline{t}$ranscription factor $\underline{b}$inding (GTB) motif, based upon its function in Nrm1 and Whi5 (see below).

The GTB motif is required for the function of Nrm1 and Whi5 as transcriptional repressors. To determine whether this motif fulfills a functional role in the regulation of the G_1 transcription factors by their transcriptional repressors, we mutated the motifs and evaluated the effect on that interaction. First, we deleted the GTB motif in Nrm1 and assessed the ability of the mutant, which we designated *nrm1*ΔGTB, to bind to Swi6. Whereas wild-type Nrm1 coimmunoprecipitates with Swi6, coimmunoprecipitation of Myc-tagged Nrm1ΔGTB with Swi6 was not detected (Fig. 3B), indicating that the GTB motif is required for Nrm1 interaction with Swi6. To establish whether this mutation also affected the interaction of Nrm1 with MBF bound to promoters, ChIP was performed using wild-type cells and *nrm1*ΔGTB mutants synchronized in G_1 phase by α-factor arrest and released for 60 min, the time of peak binding of wild-type Nrm1 to pro-

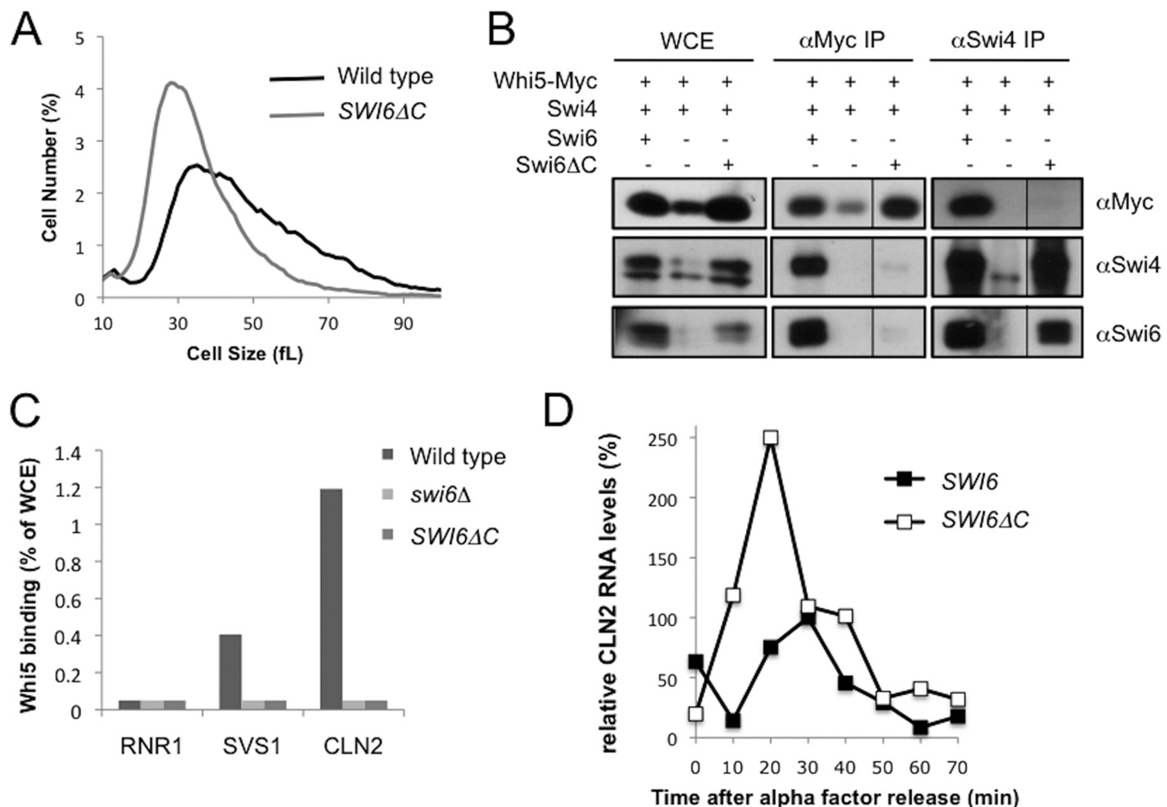

FIG 2 C terminus of *Swi6* is required for *Whi5* binding to SBF. (A) *SWI6*ΔC cells are smaller than wild-type cells. Cell size was determined for wild-type, *SWI6*ΔC, and *whi5*Δ cells growing exponentially in rich medium. (B) C terminus of Swi6 is required for Whi5 interaction with SBF. Immunoblot showing whole-cell extracts (WCE), anti-Myc, and anti-Swi4 immunoprecipitation (IP) of Swi4 or Nrm1-Myc in a wild-type or *SWI6*ΔC background. All immunoblots were derived from samples run on a single SDS-polyacrylamide gel. (C) Deletion of the C terminus of Swi6 abrogates Whi5 binding to SBF-dependent promoters. Quantitation of Whi5-Myc binding to *RNR1* (MBF dependent), *CLN2*, and *SVS1* (SBF dependent) promoter DNA by chromatin immunoprecipitation (ChIP) in wild-type, *swi6*Δ, and *SWI6*ΔC cells growing exponentially. (D) SBF-regulated transcription is induced earlier in *SWI6*ΔC cells. Wild-type and *SWI6*ΔC cells growing exponentially in rich medium were synchronized by α-factor and subsequently released into rich medium. *CLN2* RNA from arrested cells (0 min) and cells released from the arrest for the indicated interval was quantitated by RT-qPCR, and results are shown as a percentage of the maximal RNA level in wild-type cells.

moters. In contrast to binding with wild-type Nrm1, binding to MBF-regulated promoters was lost when the GTB motif of Nrm1 was deleted (Fig. 3C), indicating that this region is, indeed, necessary for Nrm1 binding to MBF at promoters. Finally, we evaluated the effect of the ΔGTB mutation upon the ability of Nrm1 to repress MBF-regulated transcription. Wild-type and *nrm1*ΔGTB cells were synchronized during G_1 phase and released into the cell cycle, and the expression of MBF and SBF target genes was analyzed. Whereas *CLN2*, an SBF target gene, is not affected by the *nrm1*ΔGTB mutation, *RNR1* expression is hyper-induced, and the duration of expression is extended in the mutant (Fig. 3D). This is similar to the effect of deletion of the NRM1 gene (4), indicating that the GTB motif is required for Nrm1 function as a transcriptional repressor.

We then asked whether the same motif in Whi5 was relevant for its repression of SBF-regulated gene expression during early G_1 phase. Three of the highly conserved residues of the GTB motif of Whi5, R185, A189, and K192, were mutated to W185, I189, and Q192, respectively, to generate the *whi5*^{WIQ} mutant. Myc-tagged Whi5 or Whi5^{WIQ} was introduced into strains expressing either HA-tagged Swi4 or Swi6, and the interaction between these proteins was evaluated by coimmunoprecipitation (Fig. 4A). Whereas wild-type Whi5 coimmunoprecipitates with both Swi4 and Swi6, as previously reported (6, 7), neither component of SBF was observed in immunoprecipitates of Whi5^{WIQ}, consistent with a role for the GTB motif of Whi5 in its interaction with Swi6. To determine whether this defect was also reflected in the binding of Whi5 to SBF at its target promoters, we performed ChIP with wild-type Myc-tagged Whi5 or Whi5^{WIQ} in cells arrested in G_1 phase by α-factor, the interval in which Whi5 binding to promoters is maximal, and released for 40 min, the interval of peak SBF expression (Fig. 4B). Whereas binding of wild-type Whi5 to the SBF-dependent promoters *CLN2* and *SVS1* was observed, binding of Whi5^{WIQ} was not detected in the G_1-arrested cells, consistent with a requirement of the GTB motif for Whi5 binding to SBF at target promoters. Finally, we examined the effect of the *whi5*^{WIQ} mutant on SBF-dependent transcription during G_1 phase. Expression of G_1 genes was analyzed in wild-type cells and *whi5*^{WIQ} mutants synchronized with α-factor and released, as described previously. Whereas the expression of *RNR1*, an MBF-regulated target, was not significantly affected by the *whi5*^{WIQ} mutant (Fig. 4C, left panel), expression of *CLN2*, an SBF-regulated gene, was prematurely induced in the mutant strain (Fig. 4C, right panel), consistent with an essential role of the GTB motif for Whi5 function. To

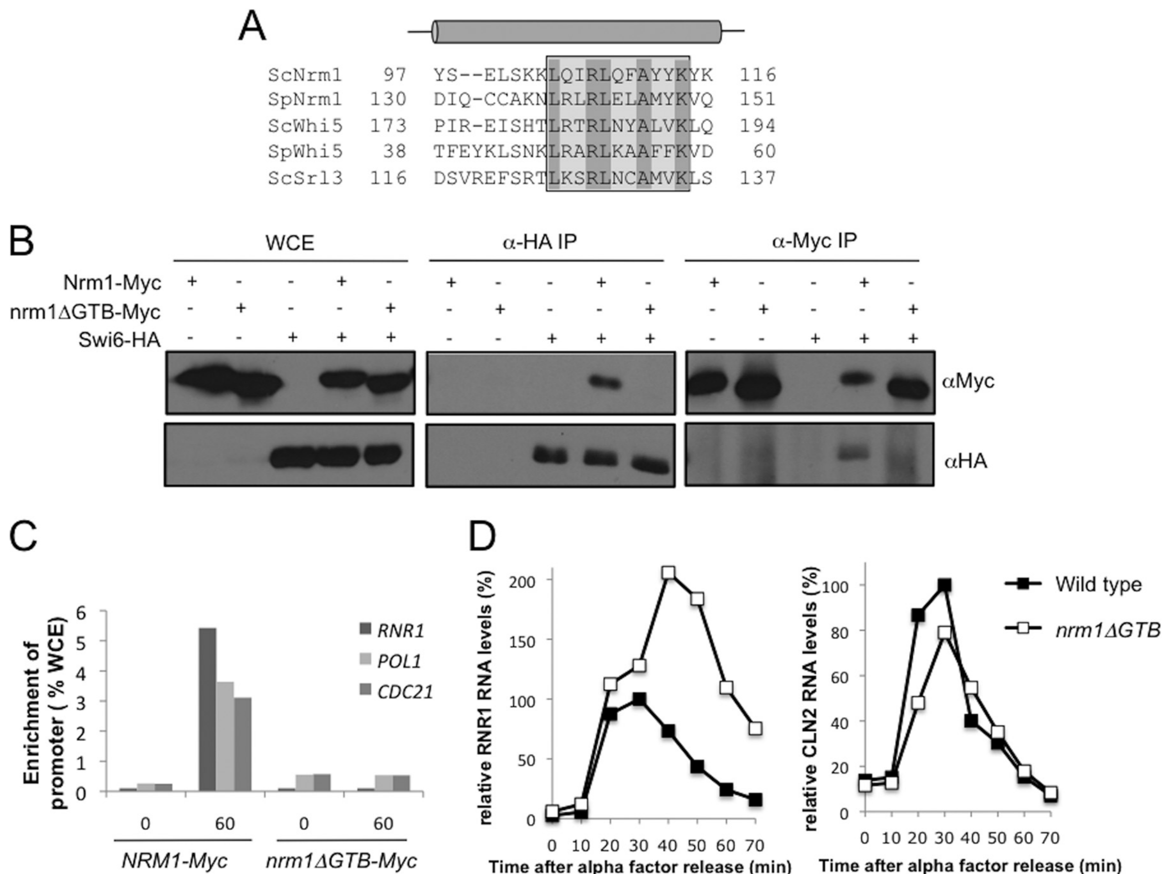

FIG 3 GTB motif is required for *Nrm1* to bind and repress MBF. (A) Alignment of GTB motifs from *S. cerevisiae* (Sc) and *S. pombe* (Sp) Whi5 and Nrm1 homologs and *S. cerevisiae* Srl3. The shaded box denotes the sequences that comprise the "core GTB"; the darker shading denotes amino acids that are conserved between the sequences. (B) *nrm1*ΔGTB does not interact with *Swi6.* Immunoblot showing whole-cell extracts (WCE), anti-HA, and anti-Myc immunoprecipitation of Swi6 or Nrm1-Myc in a wild-type or *nrm1*ΔGTB background. (C) Deletion of the GTB motif in Nrm1 abrogates binding to MBF-dependent promoters. Quantitation of Nrm1-Myc and Nrm1ΔGTB-Myc binding to *RNR1*, *POL1*, and *CDC21* promoter DNA by chromatin immunoprecipitation (ChIP) in cells arrested in G_1 by α-factor (0) and released into rich medium for 60 min. (D) MBF-regulated transcription is induced in *nrm1*ΔGTB cells. Wild-type and *nrm1*ΔGTB cells were grown in rich medium, synchronized by α-factor, and subsequently released into rich medium. *RNR1* and *CLN2* RNAs from arrested cells (0 min) and from cells released from the arrest for the indicated interval were quantitated by RT-qPCR, and results are shown as percentages of the maximal RNA level in wild-type cells.

establish whether the loss of binding of Whi5^{WIQ} and the associated premature transcriptional activation of SBF target genes have a phenotypic consequence, we evaluated the effect of the mutation on cell size, a characteristic affected by the loss of Whi5 function (Fig. 4D) (7, 18). As predicted, *whi5*^{WIQ} cells growing exponentially in rich medium, like *whi5*Δ cells, are significantly smaller than wild-type cells, confirming that the GTB motif is crucial for the established roles of Whi5.

The GTB motif-containing protein Srl3 binds to SBF-regulated promoters in hydroxyurea-treated cells. The GTB motif is conserved throughout evolution and is needed for both the Nrm1 and Whi5 transcriptional repressors to bind to their target transcription factors and inhibit their activity. Together, these observations suggest that the GTB motif might act as a functional module that proteins use to interact with G_1/S transcription factors. Thus, identifying GTB motif-containing proteins could provide a new way to discover unknown regulators of the G_1/S transcription in *S. cerevisiae* as well as the functional analogs of Nrm1/Whi5 in distantly related species. To test this hypothesis, we focused on Srl3, the only protein in budding yeast, other than Nrm1 and Whi5, that contains a GTB motif. Srl3 was originally identified in a high-throughput screen as a suppressor of Rad53 lethality, but its function remains unknown (22). Binding of Myc-tagged Srl3 to either SBF- or MBF-dependent promoters could not be detected by ChIP in cells synchronized during G_1 phase with α-factor and released into the cell cycle (Fig. 4E and data not shown). However, when cells were released from α-factor arrest in the presence of hydroxyurea (HU), a genotoxic agent that generates DNA replication stress and activates a Rad53-dependent checkpoint signal, Srl3 binding to the *CLN2* promoter was observed (Fig. 4E), further supporting the idea that the GTB motif acts as a functional module that allows proteins to interact with G_1/S transcription factors. The basis for Srl3 binding to the *CLN2* promoter in response to HU and its biological consequences remain to be established.

The GTB motif is sufficient for both binding and specificity of the G_1/S transcriptional repressors. To further pursue the hypothesis that the GTB motif is a functional module, we wanted to determine whether the isolated motif was sufficient to bind to Swi6. To address this question, a plasmid was constructed in which the DNA encoding 27 residues of Nrm1 that constitute a

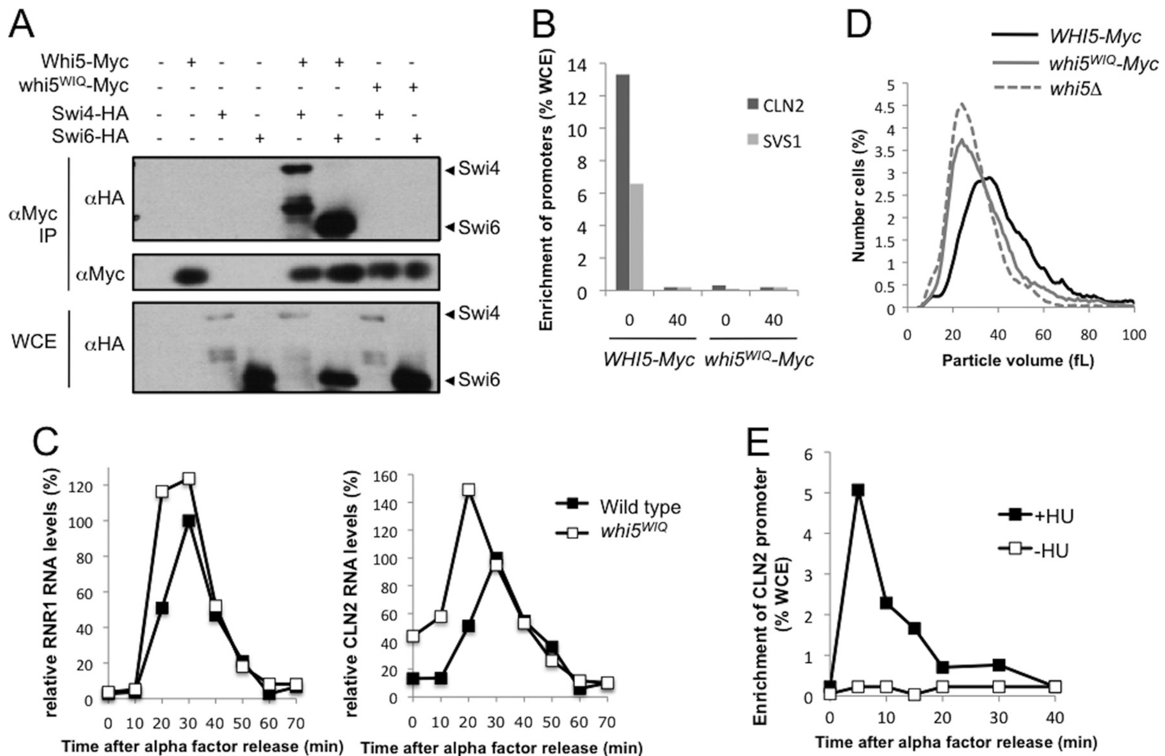

FIG 4 GTB motif is required for Whi5 to bind and repress SBF. (A) *whi5*^{WIQ} does not interact with *Swi6.* Immunoblot showing whole-cell extracts (WCE) and anti-Myc immunoprecipitations of Whi5-Myc or Whi5^{WIQ}-Myc in wild-type, *SWI4*-HA, and *SWI6*-HA cells. In the anti-HA blot, Swi6 and Swi4 protein bands are indicated by arrowheads. The faster-migrating bands in the Swi4 lane correspond to partial degradation products. (B) Mutation of the GTB motif in Whi5 abrogates binding to SBF-dependent promoters. Quantitation was performed of Whi5-Myc and Whi5^{WIQ}-Myc binding to *CLN2* and *SVS1* promoter DNA by chromatin immunoprecipitation (ChIP) in cells arrested in G_1 by α-factor (0) and released into rich medium for 40 min. (C) SBF-regulated transcription is induced earlier in *whi5*^{WIQ} cells. Wild-type and *whi5*^{WIQ} cells were grown in rich medium, synchronized by α-factor, and subsequently released into rich medium. *RNR1* and *CLN2* RNAs from arrested cells (0 min) and from cells released from the arrest for the indicated interval were quantitated by RT-qPCR, and results are shown as a percentage of the maximal RNA level in wild-type cells. (D) *whi5*^{WIQ} cells are smaller than wild-type cells. Cell size was determined for wild-type, *whi5*^{WIQ}, and *whi5*Δ cells growing exponentially in rich medium. (E) Srl3 binds to CLN2 promoter in the presence of hydroxyurea (HU). Quantitation of Srl3-Myc binding to *CLN2* promoter DNA by ChIP in cells arrested in G_1 by α-factor (0) and released into rich medium with or without 0.2 M HU for the indicated times.

predicted α-helix containing the conserved GTB motif (Fig. 5A) was appended to that encoding the carboxy terminus of glutathione *S*-transferase (GST) for expression in *E. coli*. The GST-GTB motif fusion protein was subsequently isolated from bacterial extracts using glutathione-Sepharose beads, and the beads were incubated with yeast extracts from cells expressing either wild-type Swi6 or Swi6ΔC. The GST-GTB motif fusion, but not GST alone, bound to wild-type Swi6 in yeast extracts (Fig. 5B), demonstrating that the GTB motif itself was sufficient for Swi6 binding. Furthermore, the same fusion protein failed to bind to Swi6ΔC, indicating that binding of Swi6 by the GTB motif retained the same requirements for binding as the intact Nrm1 protein.

Nrm1 binds specifically to MBF (4), in a manner that depends upon the GTB motif. Consequently, it was of interest to determine whether the GTB motif itself was sufficient to confer specificity for MBF or whether additional specificity was conferred by Nrm1 sequences outside that motif. Consequently, we evaluated whether the Nrm1 GTB motif can discriminate between Swi6 bound to Mbp1 and Swi6 bound to Swi4. Glutathione-Sepharose beads containing the GST-GTB motif fusion were used to bind Swi6 from extracts expressing either TAP-tagged Mbp1 or TAP-tagged Swi4 or from an *mbp1*Δ mutant. Surprisingly, the Nrm1 GTB motif bound Swi6 with TAP-Mbp1 but not TAP-Swi4, indicating that this small protein fragment is sufficient to determine the specificity to bind MBF, but not SBF (Fig. 5C). Moreover, Swi6 binding is strongly facilitated by the presence of Mbp1, as the Nrm1 GTB motif binds only very weakly to Swi6 in extracts from the *mbp1*Δ mutant. Despite the capacity of the GTB motif to bind Swi6 in the absence of Mbp1, no binding of the intact Nrm1 protein to Swi6 has been observed in the absence of Mbp1 *in vivo* (4). Together these data show that the GTB motif from Nrm1 is sufficient not only to bind Swi6 but also to establish the specificity of Nrm1 for the MBF complex.

Our finding that the Nrm1 GTB is sufficient for both binding and specificity for MBF prompted us to ask whether a similar GTB-containing region of Whi5 could discriminate between SBF and MBF. A construct encoding GST with a 27-amino-acid fragment containing the predicted α-helix that includes the core GTB of Whi5 fused to the carboxy terminus was expressed using the same protocol as used for the GST fusion of the Nrm1 GTB and incubated with extracts from cells expressing either wild-type Swi6 or Swi6ΔC protein. However, that construct failed to bind to either the wild-type or mutant protein (data not shown). Further

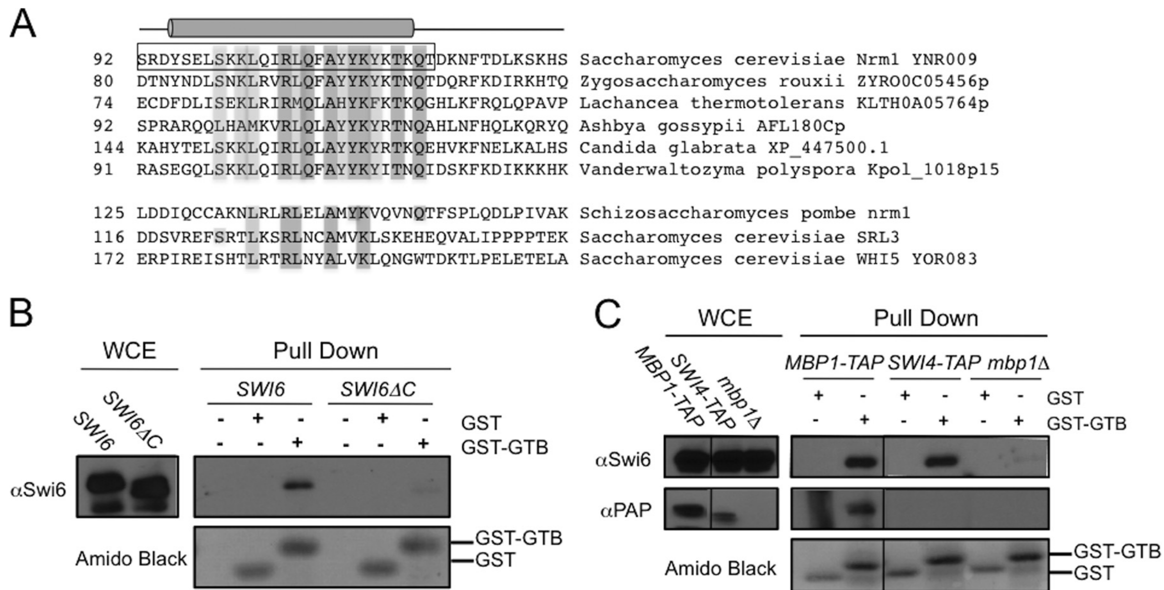

FIG 5 Nrm1 GTB motif is sufficient to bind to MBF. (A) The Nrm1 GTB motif is evolutionarily conserved among fungi. Alignment of the Nrm1 GTB motif and surrounding sequence from *Saccharomyces cerevisiae* with the predicted amino acid sequence of five other members of the phylum *Ascomycota* is shown. Amino acids that are shared by all selected species are highlighted in dark gray, and those that are shared by five of six species are highlighted in light gray. The same regions of *Schizosaccharomyces pombe* Nrm1 and *S. cerevisiae* Srl3 and Whi5 are shown below for comparison, with highlighting of the residues each has in common with the common residues of the group above. Residues highlighted in the black box were used to construct the GST-Nrm1 GTB fusion protein. (B) Nrm1 GTB motif binds to Swi6. Anti-Swi6 immunoblots showing pulldowns with glutathione-Sepharose beads bound to GST-Nrm1 GTB or GST alone (right panel) and whole-cell extracts (WCE) from wild-type or *SWI6*ΔC cell extracts. Amido black staining (lower panel) is shown as a loading control. (C) Nrm1 GTB motif has specificity for MBF. Anti-Swi6 and anti-PAP immunoblots showing pulldowns with glutathione-Sepharose beads bound to GST-Nrm1 GTB or GST alone (right panel) and whole-cell extracts (WCE) from *MBP1*-TAP and *SWI4*-TAP wild-type cells and *mbp1*Δ cell extracts. Amido black staining (lower panel) is shown as loading control.

examination of the relationship between GTB-containing proteins among fungi revealed additional conservation of sequences carboxy-terminal to the GTB-containing α-helix (Fig. 6A). Based upon the extended conserved sequence, we designed a GST fusion protein with the 40-amino-acid GTB-containing peptide containing additional Whi5 sequences at the carboxy terminus and used it in a similar regimen to that used for the smaller GST-GTB fusions (Fig. 6A). The larger fusion protein bound to glutathione-Sepharose beads efficiently pulled down wild-type Swi6 but not the mutant Swi6ΔC protein (Fig. 6B), consistent with the failure of the full-length Whi5 protein to associate with Swi6ΔC *in vivo* (Fig. 2B). Thus, the capacity of the Whi5 GTB, but not the Nrm1 GTB, to bind to Swi6 requires the presence of conserved amino acids carboxy-terminal to the core GTB motif.

We next examined the specificity of the extended Whi5 GTB for G_1/S transcription factors. Using a similar approach to that used with the Nrm1 GTB fusion, glutathione beads containing the GST fusion of the Whi5 GTB were used to evaluate SBF and MBF binding in extracts from cells expressing TAP-tagged Swi4 and Mbp1, respectively (Fig. 6C). Like the Nrm1 GTB, the Whi5 GTB was sufficient to confer specificity of binding, in this case, to Swi4 but not Mbp1. Although it bound to Swi6 in the extracts expressing Mbp1-TAP, Swi6 was not bound to Mbp1-TAP. We conclude that the isolated Whi5 GTB is sufficient for binding and specificity for SBF.

Together these data establish that the GTB behaves as an autonomous G_1/S transcription factor binding module with the capacity to discriminate between distinct family members.

DISCUSSION

Proteins consist of one or more domains or functional modules that represent units of specific structure and function. While other parts of the protein and their sequences may change considerably during evolution, these modules are constrained to preserve structure and function (23, 24). Here, we have identified a functional module that, based upon the behavior of two family members, enables proteins to bind to members of the family of transcription factors that regulate G_1/S transcription.

We have shown that both transcriptional repressors, Whi5 and Nrm1, have a conserved motif (LXXRLXXAXXK) (Fig. 5A and 6A), named the G_1/S transcription factor binding (GTB) motif based upon its function. This motif is part of a predicted α-helix that is required for the binding of the transcriptional repressors to their transcription factor targets. Either deletion of the whole GTB motif or mutation of its critical residues impairs Nrm1 and Whi5 binding to Swi6 such that they can no longer repress G_1/S transcription, indicating that the GTB motif is required for their function (Fig. 3 and 4). Moreover, the GTB motif is not only sufficient for that interaction but also sufficient to establish the specificity of binding (Fig. 5 and 6). Despite the generally poor amino acid sequence conservation among the functional analogs of the transcriptional repressors, the GTB motif is well conserved evolutionarily throughout the *Ascomycota* phylum (Fig. 5A and 6A) with homologs in some very distantly related genera, including members of the *Basidiomycetes* (data not shown). In fact, an ortholog of Nrm1/Whi5 that contains a GTB motif was recently identified in *Candida albicans* (20). Together, these observations suggest that

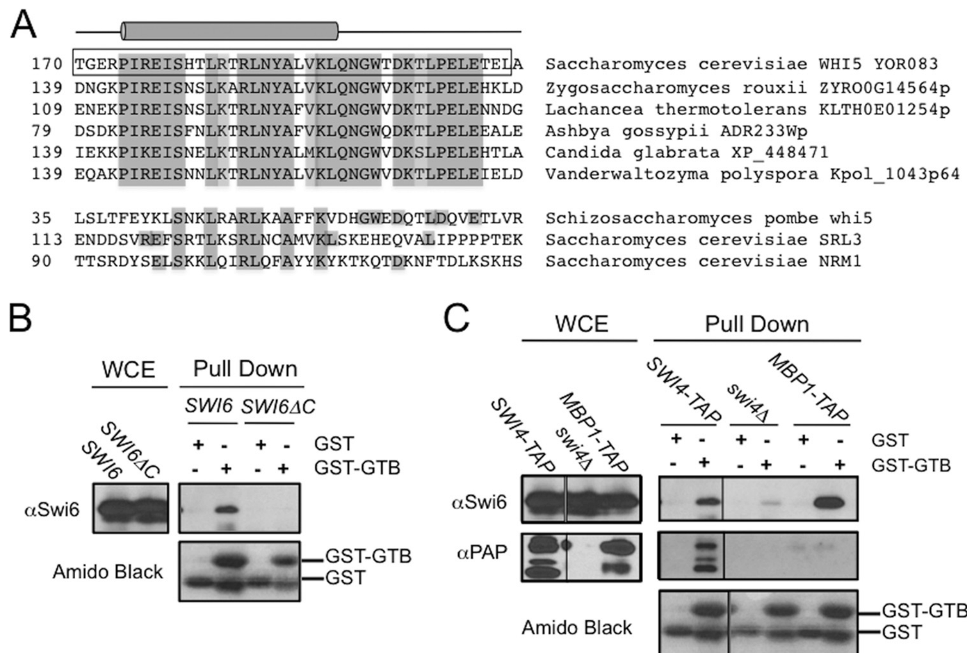

FIG 6 Whi5 GTB motif is sufficient to bind to SBF. (A) The Whi5 GTB motif is also evolutionarily conserved among fungi. Alignment of the Whi5 GTB motif with the predicted sequences of the same five species of the phylum *Ascomycota* shown in Fig. 5A. The same regions of *Schizosaccharomyces pombe* Whi5 and *Saccharomyces cerevisiae* Srl3 and Nrm1 are shown below for comparison. The criterion for highlighting is the same as described in the legend of Fig. 5A. Residues highlighted in the black box were used to construct the GST-Whi5 GTB fusion protein. (B) Whi5 GTB motif binds to Swi6. Anti-Swi6 immunoblots showing pulldowns with glutathione-Sepharose beads bound to GST-Whi5 GTB or GST alone and whole-cell extracts (WCE) from wild-type or SWI6ΔC cell extracts. Amido black staining (lower panel) is shown as a loading control. (C) Whi5 GTB motif has specificity for SBF. Anti-Swi6 and anti-PAP immunoblots showing pulldowns with glutathione-Sepharose beads bound to GST-Whi5 GTB or GST alone and whole-cell extracts (WCE) from *SWI4*-TAP, *swi4*Δ, or *MBP1*-TAP cell extracts. Amido black staining (lower panel) is shown as a loading control.

the GTB motif is a functional module that, at least based upon the behavior of Whi5 and Nrm1, is necessary and sufficient for binding to the G_1/S transcription factor complexes.

The GTB motif may be useful to identify proteins that bind G_1/S transcription factors in *S. cerevisiae* and other fungi. The identification of a likely ortholog of Nrm1/Whi5 based upon the conservation of that sequence provides one line of support for this contention. Our finding that Srl3, an *S. cerevisiae* GTB motif-containing protein of unknown function, binds to the SBF-dependent gene *CLN2* promoter also suggests a role in G_1/S gene regulation (Fig. 4E). Srl3 was identified in a screen for suppressors of Rad53 lethality, and it is found in a complex with the budding yeast cyclin-dependent kinase Cdc28 (CDK1) (19, 22, 25). Further studies will be required to determine whether Srl3 plays a role in modulating G_1/S transcription or plays some other role at the Cln2 promoter. However, given that promoter binding is observed only during replication stress, one exciting possibility is that Srl3 functions as a bridge between CDK and SBF, facilitating CDK-dependent phosphorylation of SBF under conditions of DNA replication stress. Thus, the GTB motif is a conserved functional module that may be generally useful for the identification of novel G_1/S regulatory factors.

We have also shown that the carboxy terminus of Swi6 is required for Nrm1 and Whi5 binding to MBF and SBF, respectively. The last 50 residues of Swi6 are essential for the transcriptional repressors to bind and repress G_1/S transcription but not for the formation or function of MBF and SBF complexes (Fig. 1 and 2). The C terminus of Swi6 is well conserved among its homologs in other fungi. The carboxy terminus of Cdc10, the *S. pombe* ortholog to Swi6, exhibits greater homology to the carboxy terminus of Swi6 than do other regions of the two proteins, including the well-conserved ankyrin repeats (26). The carboxy terminus of Cdc10 plays a role in regulation of MBF-dependent transcription, as shown by the elevation of G_1/S transcription throughout the cell cycle in the thermosensitive mutant Cdc10-C4, which lacks the last 61 residues (27, 28). Interestingly, Cdc10-C4 still retains the ability to bind to Res1 and Res2 in the same way that Swi6ΔC remains competent to form the MBF and SBF complexes with Mbp1 and Swi4 (29) (Fig. 1 and 2). The carboxy terminus of Swi6 has been reported to be required for its interaction with Mbp1 *in vitro* (30–32). However, we show, in agreement with Sedgwick and colleagues (33), that full-length Mbp1 interacts with Swi6 *in vivo* independent of the carboxy terminus (Fig. 1B).

Like the GTB motif, the carboxy terminus of Swi6 is extensively conserved throughout the *Ascomycota* (data not shown), while the rest of the protein sequences exhibit greater divergence. Thus, the Swi6 C terminus represents a complementary functional module to the GTB motif that has been evolutionarily conserved, presumably to maintain appropriate regulation of G_1/S transcription. That is, it appears likely that the GTB motif is conserved as an interface for the interaction of the transcriptional repressors to interact with their target transcription factors, whereas the Swi6 carboxy terminus provides a complementary module for interaction with regulators bearing the GTB motif.

A key question concerning the regulation of G_1/S transcription in *S. cerevisiae* is how transcriptional repressors achieve specificity

for their target transcription factors. Whereas some fungal species, such as *S. pombe*, have only one G_1/S transcription factor, which is homologous to MBF, in budding yeast the task of regulating G_1/S genes is distributed between two factors, SBF and MBF, that exhibit some overlap in promoter binding. The transcriptional repressors Whi5 and Nrm1 bind to SBF and MBF, respectively, and, in doing so, repress their targets (7, 12). Because the repressors are subject to distinct regulatory signals that act via different mechanisms, their binding specificity is critical in determining the nature of the transcriptional response to those different signals. Consequently, identifying the determinants of that specificity is crucial for understanding their role and regulation. We find that both repressors depend upon their GTB motif and the carboxy terminus of Swi6, the common component of SBF and MBF, for that interaction (Fig. 1 and 2).

One of two mechanisms may explain the capacity of the transcriptional repressors to discriminate between the two transcription factors. First, Whi5 and Nrm1 may interact directly with Swi4 and Mbp1, respectively, as well as with Swi6. Alternatively, the carboxy terminus of Swi6 may assume a different conformation when bound to Swi4 or Mbp1, thereby promoting specific recognition by Whi5 and Nrm1. Although we have not addressed that question directly, we have demonstrated that the GTB motifs of Nrm1 and Whi5 are sufficient to discriminate between SBF and MBF, respectively, and that the isolated motifs bind very weakly to Swi6 in the absence of Swi4 or Mbp1 (Fig. 5C and 6C). The capacity to bind Swi6 in the absence of the other transcription factor component appears not to occur with the intact repressors, suggesting that there is a conflict between other portions of the repressor proteins and the intact transcription factor. Nevertheless, we conclude that these short polypeptide fragments containing the GTB motif not only contain the residues necessary for Swi6 binding but also have diversified sufficiently to confer specificity for a particular transcription factor.

The functional GTB motif module has been evolutionarily constrained, presumably because it forms the surface responsible for the interaction between the transcriptional repressors and their target G_1/S transcription factors. However, the remaining Whi5 and Nrm1 proteins have evolved significantly such that the repressors not only bind different transcription factors but also that the regulation of that binding is subject to distinct mechanisms of control. Whereas Whi5 binding to its target transcription factor is regulated via CDK-dependent phosphorylation, Nrm1 binding to its target is primarily regulated by the timing of its accumulation during the cell division cycle as cells progress to S phase. That is, Nrm1 is regulated primarily at the level of gene expression, whereas Whi5 is regulated posttranslationally via covalent modification. Thus, the consequence of the combined pressures of evolution and conservation have led to this exquisitely tuned system wherein two classes of G_1/S genes are coordinately expressed during the cell division cycle but subject to distinct regulatory inputs.

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REFERENCES

1. **Breeden LL.** 2003. Periodic transcription: a cycle within a cycle. Curr. Biol. **13**:R31–R38.
2. **Wittenberg C, Reed SI.** 2005. Cell cycle-dependent transcription in yeast: promoters, transcription factors, and transcriptomes. Oncogene **24**: 2746–2755.
3. **Bean JM, Siggia ED, Cross FR.** 2005. High functional overlap between MluI cell-cycle box binding factor and Swi4/6 cell-cycle box binding factor in the G1/S transcriptional program in *Saccharomyces cerevisiae*. Genetics **171**:49–61.
4. **de Bruin RA, Kalashnikova TI, Chahwan C, McDonald WH, Wohlschlegel J, Yates J, III, Russell P, Wittenberg C.** 2006. Constraining G1-specific transcription to late G_1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback. Mol. Cell **23**:483–496.
5. **Koch C, Moll T, Neuberg M, Ahorn H, Nasmyth K.** 1993. A role for the transcription factors Mbp1 and Swi4 in progression from G_1 to S phase. Science **261**:1551–1557.
6. **Costanzo M, Nishikawa JL, Tang X, Millman JS, Schub O, Breitkreuz K, Dewar D, Rupes I, Andrews B, Tyers M.** 2004. CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast. Cell **117**:899–913.
7. **de Bruin RA, McDonald WH, Kalashnikova TI, Yates J, III, Wittenberg C.** 2004. Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5. Cell **117**:887–898.
8. **Amon A, Tyers M, Futcher B, Nasmyth K.** 1993. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell **74**:993–1007.
9. **Bastos de Oliveira FM, Harris MR, Brazauskas P, de Bruin RA, Smolka MB.** 2012. Linking DNA replication checkpoint to MBF cell-cycle transcription reveals a distinct class of G1/S genes. EMBO J. **31**:1798–1810.
10. **Travesa A, Kuo D, de Bruin RA, Kalashnikova TI, Guaderrama M, Thai K, Aslanian A, Smolka MB, Yates JR, III, Ideker T, Wittenberg C.** 2012. DNA replication stress differentially regulates G1/S genes via Rad53-dependent inactivation of Nrm1. EMBO J. **31**:1811–1822.
11. **Bahler J.** 2005. Cell-cycle control of gene expression in budding and fission yeast. Annu. Rev. Genet. **39**:69–94.
12. **de Bruin RA, Kalashnikova TI, Aslanian A, Wohlschlegel J, Chahwan C, Yates JR, III, Russell P, Wittenberg C.** 2008. DNA replication checkpoint promotes G1-S transcription by inactivating the MBF repressor Nrm1. Proc. Natl. Acad. Sci. U. S. A. **105**:11230–11235.
13. **Cooper K.** 2006. Rb, *whi* it's not just for metazoans anymore. Oncogene **25**:5228–5232.
14. **Burkhart DL, Sage J.** 2008. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat. Rev. Cancer **8**:671–682.
15. **Longtine MS, McKenzie A, III, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast **14**:953–961.
16. **Hadwiger JA, Reed SI.** 1988. Invariant phosphorylation of the *Saccharomyces cerevisiae* Cdc28 protein kinase. Mol. Cell. Biol. **8**:2976–2979.
17. **Stuart D, Wittenberg C.** 1995. CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells. Genes Dev. **9**:2780–2794.
18. **Jorgensen P, Nishikawa JL, Breitkreutz BJ, Tyers M.** 2002. Systematic identification of pathways that couple cell growth and division in yeast. Science **297**:395–400.
19. **Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415**:141–147.
20. **Ofir A, Hofmann K, Weindling E, Gildor T, Barker KS, Rogers PD, Kornitzer D.** 2012. Role of a Candida albicans Nrm1/Whi5 homologue in cell cycle gene expression and DNA replication stress response. Mol. Microbiol. **84**:778–794.
21. **Marchler-Bauer A, Zheng C, Chitsaz F, Derbyshire MK, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Lu S, Marchler GH, Song JS, Thanki N, Yamashita RA, Zhang D, Bryant SH.**

2013. CDD: conserved domains and protein three-dimensional structure. Nucleic Acids Res. **41**:D348–D352.

22. **Desany BA, Alcasabas AA, Bachant JB, Elledge SJ.** 1998. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. **12**:2956–2970.
23. **Caetano-Anolles G, Wang M, Caetano-Anolles D, Mittenthal JE.** 2009. The origin, evolution and structure of the protein world. Biochem. J. **417**:621–637.
24. **Trifonov EN, Frenkel ZM.** 2009. Evolution of protein modularity. Curr. Opin. Struct. Biol. **19**:335–340.
25. **Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, Shokat KM, Morgan DO.** 2003. Targets of the cyclin-dependent kinase Cdk1. Nature **425**:859–864.
26. **Ewaskow SP, Sidorova JM, Hendle J, Emery JC, Lycan DE, Zhang KY, Breeden LL.** 1998. Mutation and modeling analysis of the *Saccharomyces cerevisiae* Swi6 ankyrin repeats. Biochemistry **37**:4437–4450.
27. **McInerny CJ, Kersey PJ, Creanor J, Fantes PA.** 1995. Positive and negative roles for cdc10 in cell cycle gene expression. Nucleic Acids Res. **23**:4761–4768.
28. **Reymond A, Simanis V.** 1993. Domains of p85cdc10 required for function of the fission yeast DSC-1 factor. Nucleic Acids Res. **21**:3615–3621.
29. **Whitehall S, Stacey P, Dawson K, Jones N.** 1999. Cell cycle-regulated transcription in fission yeast: Cdc10-Res. protein interactions during the cell cycle and domains required for regulated transcription. Mol. Biol. Cell **10**:3705–3715.
30. **Andrews BJ, Moore LA.** 1992. Interaction of the yeast Swi4 and Swi6 cell cycle regulatory proteins in vitro. Proc. Natl. Acad. Sci. U. S. A. **89**:11852–11856.
31. **Primig M, Sockanathan S, Auer H, Nasmyth K.** 1992. Anatomy of a transcription factor important for the start of the cell cycle in *Saccharomyces cerevisiae*. Nature **358**:593–597.
32. **Siegmund RF, Nasmyth KA.** 1996. The *Saccharomyces cerevisiae* start-specific transcription factor Swi4 interacts through the ankyrin repeats with the mitotic Clb2/Cdc28 kinase and through its conserved carboxy terminus with Swi6. Mol. Cell. Biol. **16**:2647–2655.
33. **Sedgwick SG, Taylor IA, Adam AC, Spanos A, Howell S, Morgan BA, Treiber MK, Kanuga N, Banks GR, Foord R, Smerdon SJ.** 1998. Structural and functional architecture of the yeast cell-cycle transcription factor swi6. J. Mol. Biol. **281**:763–775.