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The Yeast GATA Factor Gat1 Occupies a Central Position in Nitrogen Catabolite Repression-Sensitive Gene Activation▽

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Saccharomyces cerevisiae **cells are able to adapt their metabolism according to the quality of the nitrogen sources available in the environment. Nitrogen catabolite repression (NCR) restrains the yeast's capacity to use poor nitrogen sources when rich ones are available. NCR-sensitive expression is modulated by the synchronized action of four DNA-binding GATA factors. Although the first identified GATA factor, Gln3, was considered the major activator of NCR-sensitive gene expression, our work positions Gat1 as a key factor for the integrated control of NCR in yeast for the following reasons: (i) Gat1 appeared to be the limiting factor for NCR gene expression, (ii)** ***GAT1*** **expression was regulated by the four GATA factors in response to nitrogen availability, (iii) the two negative GATA factors Dal80 and Gzf3 interfered with Gat1 binding to DNA, and (iv) Gln3 binding to some NCR promoters required Gat1. Our study also provides mechanistic insights into the mode of action of the two negative GATA factors. Gzf3 interfered with Gat1 by nuclear sequestration and by competition at its own promoter. Dal80-dependent repression of NCR-sensitive gene expression occurred at three possible levels: Dal80 represses** ***GAT1*** **expression, it competes with Gat1 for binding, and it directly represses NCR gene transcription.**

All living cells monitor their environment to ensure that sufficient nutrients are available to complete a full cell cycle. Adaptation of growth and metabolism to the nitrogen supply is a major issue for all organisms, is altered in cancer cells (17), and has been particularly well documented in the eukaryotic model organism *Saccharomyces cerevisiae*.

Yeast cell division is inhibited by nitrogen starvation, and in contrast, sporulation and hyphal growth are hampered by rich nitrogen sources. Metabolic reprogramming in the transition from nitrogen-starved to nitrogen-rich yeast cultures occurs at two main levels: (i) posttranslationally, affecting the activity of amino acid permeases through control of their internalization and degradation in the vacuole (reviewed in references 21 and 27); and (ii) transcriptionally, to restrain the cells' capacity to synthesize enzymes and permeases required for using nonpreferred nitrogen sources (proline, allantoin, and GABA) when readily usable nitrogen sources (glutamine and asparagine) are available. The latter repressive effect is called *n*itrogen *c*atabolite *r*epression (NCR). Expression of NCR-sensitive genes is coordinated by the prion-like Ure2 protein and four DNA-binding proteins possessing homologous GATA-type zinc fingers: two activators (Gln3 and Gat1/Nil1) and two repressors (Dal80/Uga43 and Gzf3/Deh1/Nil2) (see references 9, 23, 26, and 27 and references therein). In the presence of good nitrogen sources, the GATA activators are sequestered to the cytoplasm by Ure2, whereas upon depletion of the repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes is activated by Gln3, Gat1, or both (10, 11, 29, 31, 36).

Besides the action of the two GATA activators, modulation of NCR gene expression is performed by the GATA repressors Dal80 and Gzf3. Whereas the two activators possess a homologous asparagine-rich region located approximately 200 amino acid residues from the N terminus (36), the two repressors share a C-terminal leucine zipper domain responsible for the ability of these proteins to homo- and heterodimerize (37). Both Dal80 and Gzf3 bind in vitro to GATAA-containing promoter fragments (8). Whereas binding of Dal80 requires two GATAAG sequences separated by 15 to 30 bp (14), Gzf3 can antagonize the transcription of a *lacZ* reporter gene controlled by a single GATAAG site (30). Since the zinc finger regions of Dal80 and Gzf3 are highly homologous to those of the GATA activators, it was assumed that they repress by competing with Gat1 or Gln3 for the GATA sites located in the promoters of NCR genes (2, 8, 15, 30, 34). Nevertheless, the two GATA repressors play very different roles in the modulation of NCR-sensitive gene expression. Dal80, whose expression is NCR regulated (13), limits the activation of NCR-sensitive gene expression in cells grown on a nonpreferred source of nitrogen (16). In contrast, Gzf3 seems to inhibit gene expression specifically under conditions of nitrogen repression (34).

Recent advances in the understanding of the mechanisms regulating Gln3 and Gat1 function came from studies of the immunosuppressant drug rapamycin and its target kinases Tor1 and Tor2, since Gln3 and Gat1 respond similarly to rapamycin inhibition of Tor1 and Tor2, to nitrogen deprivation, and to growth on a nonpreferred nitrogen source (4, 6, 22). According to the model, a nitrogen shortage, similarly to rapamycin treatment, would inactivate Tor1 and Tor2, leading to dephosphorylation of Gln3 and Gat1, causing them to dissociate from Ure2 and enter the nucleus to activate NCR-sensitive transcription. It was proposed that the Sit4 phos-

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TABLE 1. Strains used in this work

Strain	Pertinent genotype[a]	Parent	Complete genotype	Primer
25T0b	WT		*MAT*α *ura3 his3 trp1*	
25T0b(a)	WT	25T0b	*MAT***a** *ura3 his3 trp1*	
FV022	*gln3*Δ	25T0b	*MAT*α *ura3 his3 trp1 gln3*::*kanMX*	GLN3-L1-L4
FV023	*gat1*Δ	25T0b	*MAT*α *ura3 his3 trp1 gat1*::*natMX*	GAT1-L1-L4
FV024	*gln3*Δ *gat1*Δ	FV023	*MAT*α *ura3 his3 trp1 gln3*::*kanMX gat1*::*natMX*	GAT1-L1-L4
FV034	*GAT1*-MYC^{13}	25T0b	*MAT*α *ura3 his3 trp1 GAT1*-MYC^{13}[*HIS3*]	GAT1-TAG-F/R
FV036	*GLN3*-MYC^{13}	25T0b	*MAT*α *ura3 his3 trp1 GLN3*-MYC^{13}[*HIS3*]	GLN3-TAG-F/R
FV041	*gat1*Δ *GLN3*-MYC^{13}	FV023	*MAT*α *ura3 his3 trp1 gat1*::*natMX GLN3*-MYC^{13}[*HIS3*]	GLN3-TAG-F/R
FV078	*DAL80*-MYC^{13}	25T0b	*MAT*α *ura3 his3 trp1 DAL80*-MYC^{13}[*HIS3*]	DAL80-TAG-F/R
FV079	*GZF3*-MYC^{13}	25T0b	*MAT*α *ura3 his3 trp1 GZF3*-MYC^{13}[*HIS3*]	GZF3-TAG-F/R
FV080	*dal80*Δ	25T0b(a)	*MAT***a** *ura3 his3 trp1 dal80*::*kanMX*	DAL80-L1-L4
FV081	*dal80*Δ *GAT1*-MYC^{13}	FV034	*MAT*α *ura3 his3 trp1 dal80*::*kanMX GAT1*-MYC^{13}[*HIS3*]	DAL80-L1-L4
FV082	*dal80*Δ *GLN3*-MYC^{13}	FV036	*MAT*α *ura3 his3 trp1 dal80*::*kanMX GLN3*-MYC^{13}[*HIS3*]	DAL80-L1-L4
FV083	*gzf3*Δ	25T0b(a)	*MAT***a** *ura3 his3 trp1 gzf3*::*kanMX*	GZF3-L1-L4
FV084	*gzf3*Δ *GAT1*-MYC^{13}	FV034	*MAT*α *ura3 his3 trp1 gzf3*::*kanMX GAT1*-MYC^{13}[*HIS3*]	GZF3-L1-L4
FV098	*gat1*Δ *dal80*Δ *GLN3*-MYC^{13}	FV082	*MAT*α *ura3 his3 trp1 gat1*::*natMX dal80*::*kanMX GLN3*-MYC^{13}[*HIS3*]	GAT1-L1-L4
FV104	*dal80*Δ *GZF3*-MYC^{13}	FV079	*MAT*α *ura3 his3 trp1 dal80*::*kanMX GZF3*-MYC^{13}[*HIS3*]	DAL80-L1-L4
FV106	*gat1*Δ *GZF3*-MYC^{13}	FV079	*MAT*α *ura3 his3 trp1 gat1*::*kanMX GZF3*-MYC^{13}[*HIS3*]	GAT1-L1-L4
FV108	*gln3*Δ *DAL80*-MYC^{13}	FV078	*MAT*α *ura3 his3 trp1 gln3*::*kanMX DAL80*-MYC^{13}[*HIS3*]	GLN3-L1-L4
FV109	*gat1*Δ *DAL80*-MYC^{13}	FV078	*MAT*α *ura3 his3 trp1 gat1*::*kanMX DAL80*-MYC^{13}[*HIS3*]	GAT1-L1-L4
FV111	*gat1*Δ *dal80*Δ	FV080	*MAT***a** *ura3 his3 trp1 gat1*::*natMX dal80*::*kanMX*	GAT1-L1-L4
FV113	*gln3*Δ *gzf3*Δ	FV083	*MAT***a** *ura3 his3 trp1 gln3*::*natMX gzf3*::*kanMX*	GLN3-L1-L4
FV114	*gat1*Δ *gzf3*Δ	FV083	*MAT***a** *ura3 his3 trp1 gat1*::*natMX gzf3*::*kanMX*	GAT1-L1-L4
FV135	*GZF3*-ΔLZ-MYC^{13}	25T0b	*MAT*α *ura3 his3 trp1 GLN3*-ΔLZ-MYC^{13}[*HIS3*]	GZF3ΔLZ-TAG-F/GZF3-TAG-R
FV170	P$_{GAL1}$-*GAT1*-MYC^{13}	FV034	*MAT*α *ura3 his3 trp1* [*TRP1*]P$_{GAL1}$-*GAT1*-MYC^{13}[*HIS3*]	PGAL1-GAT1-F/R
FV172	P$_{GAL1}$-*GAT1*-MYC^{13}	FV084	*MAT*α *ura3 his3 trp1 gzf3*::*kanMX* [*TRP1*]P$_{GAL1}$-*GAT1*-MYC^{13}[*HIS3*]	PGAL1-GAT1-F/R
FV190	P$_{GAL1}$-*GAT1*-MYC^{13}	FV170	*MAT*α *ura3 his3 trp1 dal80*::*kanMX* [*TRP1*]P$_{GAL1}$-*GAT1*-MYC^{13}[*HIS3*]	DAL80-L1-L4
FV222	*GAT1*-TAP	25T0b	*MAT*α *ura3 his3 trp1 GAT1*-TAP[*HIS3*]	GAT1-L1-L4
FV231	*GAT1*-TAP *GZF3*-MYC^{13}	FV222	*MAT*α *ura3 his3 trp1 GAT1*-TAP[*HIS3*] *GZF3*-MYC^{13}[KanMX]	GZF3-TAG-F/R
FV232	*GAT1*-TAP *GZF3*-ΔLZ-MYC^{13}	FV222	*MAT*α *ura3 his3 trp1 GAT1*-TAP[*HIS3*] *GLN3*-ΔLZ-MYC^{13}[KanMX]	GZF3ΔLZ-TAG-F/GZF3-TAG-R
03740c	*gln3*Δ *GAT1*-MYC^{13}	FV022a × FV034	*ura3 his3 trp1 gln3*::*kanMX GAT1*-MYC^{13}[*HIS3*]	

[a] WT, wild type.

phatase would play a central role in the control of Gln3 phosphorylation and consequently of its subcellular localization (3, 5). However, more recently, we demonstrated that the extent of the Sit4 requirement for Gln3 nuclear localization was both nitrogen source and strain dependent (39). Moreover, our previous study allowed us to conclude that Tor pathway regulation of Gat1 differs markedly from that of Gln3: a yet-unexplored Sit4- and Ure2-independent branch is involved in Gat1-dependent Tor control of NCR (20). Moreover, no evidence demonstrating nitrogen-regulated Gat1 phosphorylation has been published in the literature so far (3, 24).

In order to more thoroughly investigate the involvement of Gat1 in NCR, we undertook a detailed analysis of the requirements for in vivo Gat1 binding to its DNA targets. Our work also aimed at determining the mechanisms involved in the antagonism between Gat1 and Gzf3 or Dal80. Our study provides a wealth of data confirming that *GAT1* expression is NCR sensitive and regulated by the four GATA factors, including Gat1 itself. For the first time, DNA binding of Gzf3 and Dal80 could be demonstrated in vivo. Chromatin immunoprecipitation (ChIP) analyses confirmed the competition between Gat1 and Dal80 at the *UGA4* promoter. Our ChIP analyses also confirmed the competition between Gat1 and Gzf3 under conditions of nitrogen repression, but we could only demonstrate it at the *GAT1* promoter. Since Gzf3 binding to the *GAP1* promoter was Gat1 dependent and increased in proline-grown cells, it is unlikely that Gzf3 acts by competing with Gat1 for binding to the *GAP1* promoter. The interaction between Gzf3 and Gat1 led us to propose that Gzf3 prevents Gat1 from DNA binding by nuclear sequestration.

MATERIALS AND METHODS

Strains and culture conditions. *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. Growth conditions were identical to those described by Scherens et al. (31). Yeast cells (4-ml cultures for quantitative real-time PCR [qRT-PCR]; 100-ml cultures for ChIP experiments) were grown to mid-log phase (A_{660} = 0.6) in yeast nitrogen base minimal medium containing the indicated nitrogen source at a final concentration of 0.1%. Appropriate supplements (50 μg/ml leucine, 25 μg/ml uracil, histidine, and tryptophan) were added to the medium as necessary to cover auxotrophic requirements.

Strain construction. All strains constructed in this study are isogenic to the FY1679 wild-type strain (listed in Table 1). Deletion strains, involving insertion of KanMX and NatMX cassettes, were constructed using the long flanking homology strategy of Wach (41) as described in reference 39 using primers listed in Table 2. Chromosomal *DAL80*, *GAT1*, *GLN3*, and *GZF3* gene products were tagged at their C termini with 13 copies of the Myc epitope (Myc^{13}) as described by Longtine et al. (25), using primers described in Table 2 or in reference 20. FV222 was constructed by replacing the wild-type *GAT1* allele by *GAT1*-TAP,

TABLE 2. Primers used in this work

Name[a]	Sequence (5′ to 3′)
Oligos for deletion strain construction	
Dal80-L1	TGAGTTCCAGACGCTGTT
Dal80-L2	GGGGATCCGTCGACCTGCAGCTCTCTTATATATAATATGATATAATATAATGTAATATAG
Dal80-L3	AACGAGCTCGAATTCATCGATGAGAAAGGATGTAGCGTTCATGACAGTATAAATATAACAG
Dal80-L4	GCAAACTTGCCCCATTGA
Gzf3-L1	ACCGGTTTGGTCAAGGTT
Gzf3-L2	GGGGATCCGTCGACCTGCAGCAGTACAAATACCCGAAATGATATTCGTATATCTAGTATTATAGC
Gzf3-L3	AACGAGCTCGAATTCATCGATGATAGATCTTTTTTTCAAAAAAATCAGCATCATTTTTCA
Gzf3-L4	TGCCAATGCTGGCATGAT
Oligos for tagged strain construction	
Dal80-TAG-F	ACTACACTCCAGAGCTTCAGTACAATCTCACCCACAACATCGGATCCCCGGGTTAATTAA
Dal80-TAG-R	CCTGTTATATTTATACTGTCATGAACGCTACATCCTTTCTTATCAGAATTCGAGCTCGTTTAAAC
Gzf3-TAG-F	CGTTTGCAAAGGACAGTAAAACAAGAAGGGAATAAAGGAGGACGGATCCCCGGGTTAATTAA
GZF3ΔLZ-TAG-F	ATTGCACAAAGAAGAAGAAATTATAAAGCTCAAAACTAGACGGATCCCCGGGTTAATTAA
Gzf3-TAG-R	TTTGAAAAATGATGCTGATTTTTTTGAAAAAAAGATCTAAGAATTCGAGCTCGTTTAAAC
Oligos for qChIP	
DAL5-U1	GTTCATTAGTCGCCTACAGC
DAL5-U2	CAGAGCCCCGCATATTTTGA
GAP1-P1	GACCTCATGCAGCAAAGTCA
GAP1-P2	CCGGTTGCTCCAGAAGATAA
GAT1-P3	TGGGTGCATGACTGATTGGT
GAT1-P4	GTGGTCGGACGCAATGATTT
UGA4-P1	ATCGCTTATCGTGCGCCAAA
UGA4-P2	ACCGGTATTGGACCATCGAA
Oligos for qRT-PCR	
DAL80-O2	TCAAGCTGATAGGCCTTGGT
DAL80-O3	CCCACGTGCCAGAATTGTTT
GAP1-O3	TCAGGGTCCAAATGGCAAGA
GAP1-O4	CAATGGAGTTTGGGCAGTGA
GAT1-O1	ACCTCGATGACACCGATACT
GAT1-O2	AGCAGGTCTGAGTGCACAAT
GZF3-O1	TTATGGCATCGCAGGCTACA
GZF3-O2	TTGCTCGGCAGATACTGCTT
TBP1-O1	TATAACCCCAAGCGTTTTGC
TBP1-O2	GCCAGCTTTGAGTCATCCTC
UGA4-O1	AACTGCTTGCAGAGATCGGT
UGA4-O2	CAGATGCAATGGAGGGCAAT

[a] Oligos, oligonucleotides.

which was obtained by PCR amplification of a *GAT1*-TAP-tagged strain using GAT1L1 and GAT1L4 primers (20). Functionality of the tagged constructs was validated using qRT-PCR to compare NCR regulation in tagged versus untagged strains (data not shown).

Western blots. Total protein extracts were obtained from 10-ml cultures using trichloroacetic acid precipitation as described in reference 40. One-twentieth of the total extracts was loaded on a 4 to 12% NuPage gel (Invitrogen), run for 75 min according to the manufacturer's instructions, and transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare) for 1 h in an XCell II blot module (Invitrogen) according to the manufacturer's protocol. Membranes were incubated with mouse monoclonal anti-Myc antibodies (9E10; Santa Cruz) (1:800) using an ECL Advance Western blotting detection kit, and Myc-tagged proteins were visualized using a chemiluminescence camera (Chemi-Smart from Vilbert-Lourmat). Band intensities were quantified using the Bio-1D algorithm and normalized with the intensity of two different bands in the corresponding Ponceau-stained lane.

Coimmunoprecipitation. Cultures (100 ml) were harvested, washed once in 50 mM Tris, pH 8, and resuspended in 2 ml of buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 1 mM phenylmetyhlsulfonyl fluoride, and complete protease inhibitor cocktail tablets [Roche]). Lysis was performed by shaking with 425- to 600-μm acid-washed glass beads (Sigma) on an IKA Vibrax VXR orbital shaker at maximum speed for 30 min at 4°C. Cell debris and glass beads were removed by centrifugation. Immunoprecipitation of TAP-tagged proteins was performed by incubating 500 μl of total cell extracts with 50 μl of prewashed (three times in 0.1% phosphate-buffered saline–bovine serum albumin) Dynabeads PAN mouse immunoglobulin G (Invitrogen) and 50 μl of 1% phosphate-buffered saline–bovine serum albumin during 2 h of orbital shaking (800 rpm) at 30°C. Immune complexes were washed three times in lysis buffer, eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer, and loaded on SDS-polyacrylamide gel for anti-Myc Western blotting.

Indirect immunofluorescence (IF) microscopy. Gat1-Myc[13], Gln3-Myc[13], and Gzf3-Myc[13] subcellular localization was performed similarly to the Gln3-Myc[13] localization described in reference 18.

qRT-PCR. Total RNA was extracted from 4-ml cultures as described previously (32). cDNA was generated from 100 to 500 ng of total RNA using a RevertAid H Minus first-strand cDNA synthesis kit with oligo$(dT)_{18}$ primers from Fermentas using the manufacturer's recommended protocol. cDNAs were subsequently quantified by RT-PCR using the Maxima SYBR green qPCR master mix from Fermentas as described in reference 20. Sequences of the primers for qPCR are provided in Table 2.

ChIP. Cell extracts, immunoprecipitations, and DNA quantifications were conducted as described in reference 20, using the Maxima SYBR green qPCR master mix from Fermentas. Sequences of the primers for qPCR are provided in Table 2. Immunoprecipitation (IP) and input (IN) values obtained for the unbound control (DAL5U) were subtracted from the corresponding IP/IN values obtained for the *GAP1*, *GAT1*, and *UGA4* promoters. The mean represents at least two replicate immunoprecipitations performed on a minimum of two independent cultures.

RESULTS

Gat1 binding to the promoters of NCR genes reflects its abundance. We previously used ChIP to demonstrate induced binding of Gat1-Myc[13] to the promoters of well-characterized NCR-sensitive, permease-encoding genes (*DAL5* and *GAP1*) upon rapamycin treatment (19, 20). Using the same technique, we analyzed the in vivo binding of Gat1-Myc[13] to the *GAP1* promoter (P_{GAP1}) upon growth on a nonpreferred nitrogen source like proline in a wild-type strain of the FY genetic background (FV034). In vivo binding of Gat1-Myc[13] to P_{GAP1} also occurred when cells were grown on proline (Fig. 1A). We performed similar ChIP experiments in Gat1-Myc[13] strains lacking the other NCR transcriptional activator Gln3 or the Dal80 repressor (in strains 03740c and FV081, respectively). Gat1-Myc[13] binding to P_{GAP1} was significantly reduced in proline-grown *gln3*Δ cells and increased in proline-grown *dal80*Δ cells (Fig. 1A). Given that *GAT1* expression is regulated by the nitrogen source (7), that Gln3 and Dal80 can bind GATA sequences upstream of *GAT1* in vitro (7), and that a *GAT1-lacZ* fusion responds to deletions of *GLN3* and *DAL80* (8, 16, 30), we hypothesized that the influence of Gln3 and Dal80 on Gat1 binding to DNA might result from their impact on *GAT1* expression. Therefore, we analyzed the expression of the native

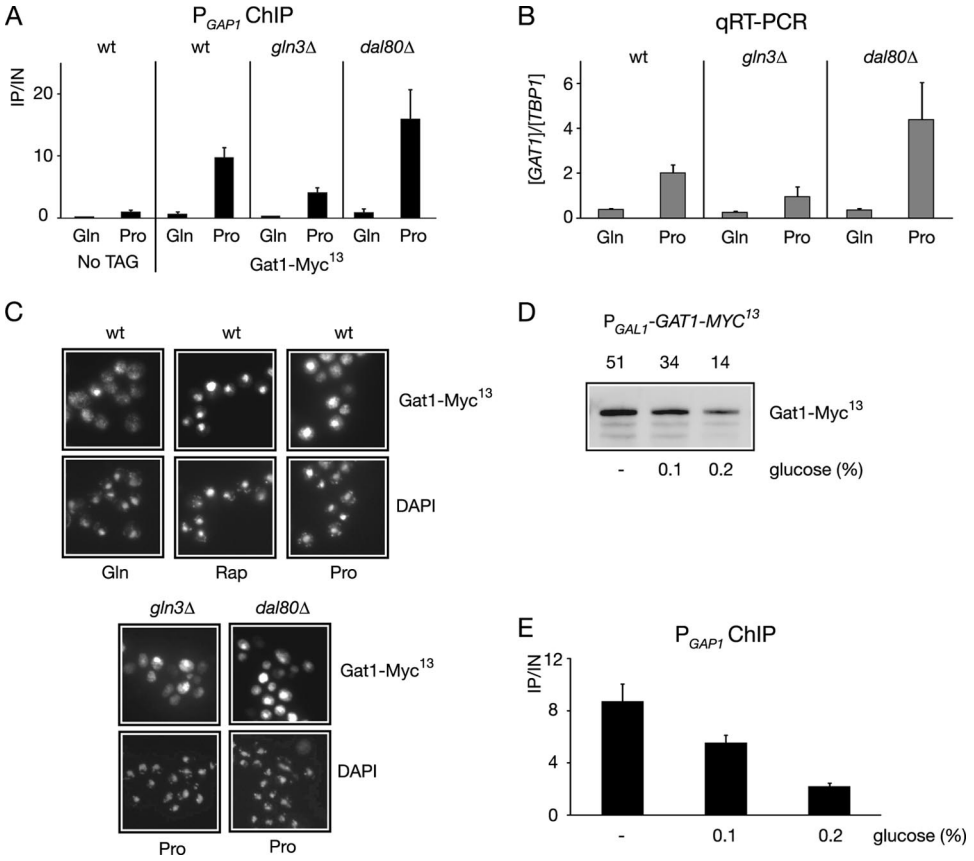

FIG. 1. Gat1 binding to the *GAP1* promoter reflects its abundance. (A) Effects of *gln3*Δ and *dal80*Δ on Gat1-Myc^{13} binding to P_{GAP1}. Untagged wild-type (wt), *GAT1*-MYC^{13} wild-type, *gln3*Δ, and *dal80*Δ cells were grown in glutamine or proline. ChIP was performed using antibodies against c-*myc*. qPCR of IP and IN fractions was performed with primers specific for the *GAP1* promoter (GAP1P; see Table 2 for primer list) and for a region 2.5 kb upstream of the *DAL5* open reading frame as a control (DAL5U). For each immunoprecipitation, IP/IN values were calculated as follows: $([GAP1P]^{IP}/[GAP1P]^{IN} - [DAL5U]^{IP}/[DAL5U]^{IN})$. Histograms represent the averages of two immunoprecipitations performed on at least two experiments from independent cultures. Error bars indicate standard errors. (B) Effects of *gln3*Δ and *dal80*Δ on *GAT1* expression. Total RNA was isolated from glutamine- or proline-grown wild-type, *gln3*Δ, and *dal80*Δ cells. *GAT1* mRNA levels were quantified by qRT-PCR using primers specific for the open reading frame of *GAT1* (GAT1O). *GAT1* values were normalized with *TBP1*. Values represent the averages of at least three experiments from independent cultures. Error bars indicate standard errors. (C) Effects of *gln3*Δ and *dal80*Δ on Gat1-Myc^{13} subcellular localization. *GAT1*-MYC^{13} wild-type cells, grown in glutamine with or without rapamycin (0.2 μg/ml) or in proline, and proline-grown *GAT1*-MYC^{13} *gln3*Δ and *dal80*Δ cells were processed for indirect IF. Gat1-Myc^{13} and DNA were detected with anti-*myc* and DAPI (4′,6′-diamidino-2-phenylindole), respectively. (D and E) Artificially changing the Gat1 concentration proportionally changes its binding levels. A P_{GAL1}-*GAT1*-MYC^{13} strain was grown in 1% galactose and proline with or without 0.1% or 0.2% glucose. (D) Effects of increasing amounts of glucose on P_{GAL1}-*GAT1*-MYC^{13} expression. Gat1-Myc^{13} quantification values of anti-*myc* Western blotting are indicated above the figure. (E) Effects of decreasing amounts of Gat1-Myc^{13} on its in vivo binding to P_{GAP1}. ChIP was conducted as described for panel A.

GAT1 gene at its own locus using qRT-PCR (Fig. 1B). As expected, *GAT1* expression was derepressed on proline in a wild-type strain (25T0b). This derepression was reduced when *GLN3* was deleted (in strain FV022) and increased upon *DAL80* deletion (in strain FV080). Since Gat1 localization, like that of Gln3, was also regulated by the nitrogen supply (3, 7, 24), we verified the subcellular localization of a Gat1-Myc^{13} protein expressed from its native chromosomal locus by indirect IF in wild-type strains and in strains lacking either Gln3 or Dal80 (Fig. 1C). On glutamine, Gat1 was mostly cytoplasmic but, unlike Gln3, it was not excluded from the nucleus. Moreover, its localization was less nuclear in proline-grown cells than in rapamycin-treated, glutamine-grown cells. Deleting Gln3 and Dal80 only affected the expression levels of Gat1-Myc^{13}, as expected from our qRT-PCR data, and consequently it respectively decreased and increased the amount of nuclear Gat1-Myc^{13}. Together, these data indicate that Gat1 in vivo binding to its DNA target in proline-grown cells parallels its expression level. To confirm this observation, we performed similar ChIP experiments using a strain in which Gat1-Myc^{13} was expressed from the *GAL1* promoter (strain FV170). Adding glucose to the proline/galactose-containing medium reduced the Gat1-Myc^{13} expression levels (Fig. 1D) and concomitantly lowered its binding to P_{GAP1} (Fig. 1E).

Gat1 conditions Gln3 binding to NCR promoters. The presence of Gat1 at NCR promoters obviously generates transcriptional activation of NCR genes, since Gat1 is one of the transcriptional activators of NCR genes, but it may also favor the recruitment of other GATA factors or stabilize activating complexes. Indeed, in our previous study, we provided evidence in favor of Gat1 participating in the promotion of Gln3 binding upon rapamycin treatment (20). In order to study the influence

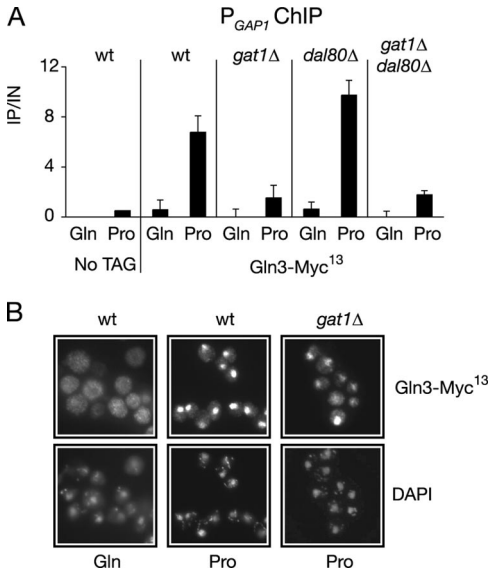

FIG. 2. Gat1 conditions Gln3 binding to P_{GAP1}. (A) Effects of *gat1*Δ and *dal80*Δ on Gln3-Myc^{13} binding to P_{GAP1}. Glutamine- or proline-grown, untagged wild-type (wt), *GLN3*-MYC^{13} wild-type, *gat1*Δ, *dal80*Δ, and *gat1*Δ *dal80*Δ cells were analyzed by ChIP as described for Fig. 1A. (B) Effects of *gat1*Δ on Gln3-Myc^{13} subcellular localization. *GLN3*-MYC^{13} glutamine- or proline-grown wild-type and proline-grown *gat1*Δ cells were processed for indirect IF. Gln3-Myc^{13} and DNA were detected with anti-*myc* and DAPI (4′,6′-diamidino-2-phenylindole), respectively.

of Gat1 on Gln3 binding to NCR-sensitive promoters when cells are grown on proline, we performed ChIP analysis of Gln3-Myc^{13} binding to P_{GAP1} in wild-type (FV036), *gat1*Δ (FV041), *dal80*Δ (FV082), and *gat1*Δ *dal80*Δ (FV098) strains grown on glutamine- and proline-containing media. As was the case upon rapamycin treatment (19, 20), in vivo binding of Gln3-Myc^{13} to P_{GAP1} was significantly enhanced in wild-type cells grown on proline versus that with glutamine (Fig. 2A). Deleting *GAT1* drastically decreased the amount of Gln3-Myc^{13} bound to P_{GAP1} on proline (Fig. 2A), although the absence of Gat1 does not influence *GLN3* expression (28; also data not shown) and had no effect on Gln3-Myc^{13} subcellular localization on proline (Fig. 2B). Deleting *DAL80* moderately increased the binding of Gln3-Myc^{13} to P_{GAP1} on proline (Fig. 2A). Given the low Gln3-Myc^{13} binding observed in a proline-grown strain lacking both *DAL80* and *GAT1* (Fig. 2A), we assume that the increase of Gln3-Myc^{13} binding to P_{GAP1} in proline-grown *dal80*Δ cells is caused by the increase of Gat1 binding in these cells. Parallelism between the binding efficiency of Gat1-Myc^{13} (Fig. 1A) and that of Gln3-Myc^{13} (Fig. 2A) strongly suggests interdependence between the two GATA activators for DNA binding.

Gat1 is the target of Dal80-mediated repression on proline. Our data and previous observations that Gat1 expression is tightly regulated and that this regulation influences its in vivo DNA binding, affecting gene expression directly (as a transcriptional activator) and indirectly (as a modulator of Gln3 binding), led us to hypothesize that Gat1 could occupy a central position in NCR-sensitive gene activation. In this context, it was of high interest to define how Dal80 could interfere with Gat1-mediated transactivation. We therefore analyzed, by

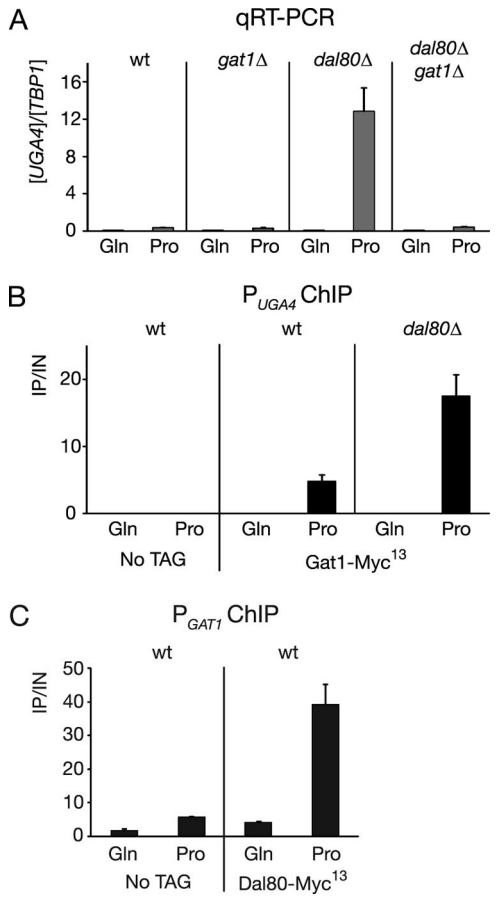

FIG. 3. Dal80 regulates *UGA4* expression through the control of *GAT1* transcription. (A) *gat1*Δ is epistatic to *dal80*Δ for *UGA4* expression. mRNA levels of *UGA4* were determined in glutamine- and proline-grown wild-type (wt), *gat1*Δ, *dal80*Δ, and *gat1*Δ *dal80*Δ strains as described for Fig. 1B using qRT-PCR with UGAO primers. (B) Dal80 affects Gat1-Myc^{13} binding to P_{UGA4}. Glutamine- and proline-grown untagged wild-type, *GAT1*-MYC^{13} wild-type, and *dal80*Δ strains were subjected to ChIP analysis as described for Fig. 1A using UGA4P primers. (C) Dal80 binds to P_{GAT1}. Glutamine- and proline-grown untagged and *DAL80*-MYC^{13} wild-type cells were subjected to ChIP analysis as described for Fig. 1A using GAT1P primers.

qRT-PCR, the expression of a gene that is particularly sensitive to Dal80-mediated repression: *UGA4* (38). *UGA4* expression was analyzed in a wild-type strain and in cells lacking *GAT1*, *DAL80*, or both (FV111). In proline-grown, wild-type cells, *UGA4* activation was very low (Fig. 3A). Deleting *DAL80* increased *UGA4* expression 40-fold (Fig. 3A), and *gat1*Δ was epistatic to *dal80*Δ, since the expression level in a *gat1*Δ *dal80*Δ strain was similar to that in a *gat1*Δ strain. To determine whether Dal80 interfered with Gat1 DNA binding, we performed ChIP experiments in wild-type and *dal80*Δ Gat1-Myc^{13} cells (Fig. 3B). Gat1-Myc^{13} binding to P_{UGA4} was induced in proline-grown, wild-type cells, compared to levels in glutamine-grown cells, even if *UGA4* was only weakly expressed in the wild-type strain (Fig. 3A and B). Moreover, deleting *DAL80* led to an increase in Gat1-Myc^{13} binding to P_{UGA4} on proline compared to the level in the wild type. The rise in Gat1-Myc^{13} DNA binding could be at least partially associated with an increase in *GAT1* expression levels, since the *GAT1*

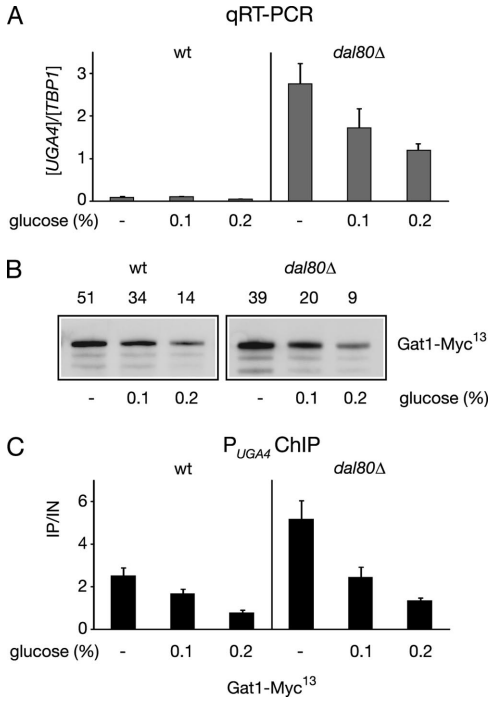

FIG. 4. Dal80 downregulates *UGA4* expression through multiple mechanisms. Wild-type (wt) and *dal80*Δ P_{GAL1}-*GAT1*-MYC^{13} strains were grown in 1% galactose and proline with or without 0.1% or 0.2% glucose. (A) Effects of *dal80*Δ on *UGA4* expression in a P_{GAL1}-*GAT1*-MYC^{13} strain. mRNA levels of *UGA4* were determined as described for Fig. 3A. (B) Effects of increasing amounts of glucose on P_{GAL1}-*GAT1*-MYC^{13} expression. Gat1-Myc^{13} quantification values of anti-*myc* Western blotting are indicated above the figure. (C) Effects of *dal80*Δ on Gat1-Myc^{13} binding to P_{UGA4} in a P_{GAL1}-*GAT1*-MYC^{13} strain. ChIP analysis was conducted as described for Fig. 3B.

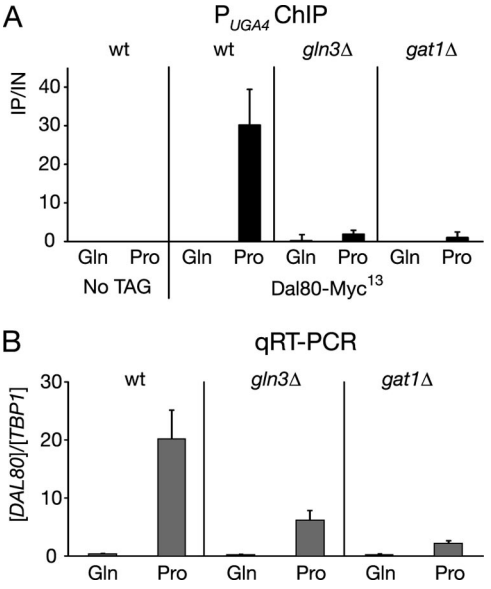

FIG. 5. Dal80-Myc^{13} binding to P_{UGA4} parallels its expression level. (A) Effect of *gat1*Δ and *gln3*Δ on Dal80-Myc^{13} binding to P_{UGA4}. Glutamine- or proline-grown untagged wild-type (wt), *DAL80*-MYC^{13} wild-type, *gln3*Δ, and *gat1*Δ cells were subjected to ChIP analysis as described for Fig. 3B. (B) Effect of *gln3*Δ and *gat1*Δ on *DAL80* expression. mRNA levels of *DAL80* were determined in glutamine- and proline-grown wild-type, *gln3*Δ, and *gat1*Δ cells as described for Fig. 1B using qRT-PCR with DAL80O primers.

transcript level is higher in proline-grown *dal80*Δ cells than in wild-type cells (Fig. 1B). Consistently, Dal80-Myc^{13} binding to P_{GAT1} was induced in proline-grown, wild-type cells (Fig. 3C). In order to evaluate the direct action of Dal80 at NCR-sensitive promoters without interference caused by the NCR-sensitive, Dal80-regulated expression of *GAT1*, we placed *GAT1* under the control of the *GAL1* promoter, thereby rendering *GAT1* expression no longer NCR sensitive but glucose repressible. We quantified the expression of *UGA4* in the P_{GAL1}-*GAT1*-MYC^{13} strain carrying either the wild-type *DAL80* or a *dal80*Δ (FV190) allele and cultivated it in the presence of galactose and proline with increasing amounts of glucose. *UGA4* transcript levels remained low in wild-type P_{GAL1}-*GAT1*-MYC^{13} cells grown in the presence of proline and galactose, even when no glucose was added to the culture medium (Fig. 4A). Deletion of *DAL80* generated higher *UGA4* transcript levels for every concentration of glucose tested (Fig. 4A), suggesting that *DAL80* can act directly at P_{UGA4}. We then checked if, in these two P_{GAL1}-*GAT1*-MYC^{13} strains, Gat1-Myc^{13} binding to P_{UGA4} was affected by Dal80. Binding of Gat1-Myc^{13} to P_{UGA4} followed its expression level (Fig. 4B and C) and was moderately increased in *dal80*Δ cells compared to the level in the wild type (Fig. 4C), indicating that Dal80 interferes with Gat1 binding to DNA, but only very weakly compared to the expression increase caused by the absence of Dal80. In other words, at equal binding levels of Gat1-Myc^{13} (compare 1% Gal for the wild type and 1% Gal plus 0.1% Glu for the *dal80*Δ strain [Fig. 4C]), we noticed a huge difference in *UGA4* transcript levels (compare 1% Gal for the wild type and 1% Gal plus 0.1% Glu for the *dal80*Δ strain [Fig. 4A]), suggesting that Dal80 has an additional role in *UGA4* repression, interfering with transcriptional activation. The last step in demonstrating in vivo competition between Dal80 and Gat1 for binding to NCR-sensitive promoters was to analyze the profile of Dal80 binding to P_{UGA4}. Therefore, we performed ChIP analysis of wild-type (FV078), *gln3*Δ (FV108), and *gat1*Δ (FV109) strains expressing Dal80-Myc^{13} to determine its binding at the promoter of *UGA4* (Fig. 5A). In proline-grown, wild-type cells, Dal80-Myc^{13} binding to P_{UGA4} was induced. Reducing Dal80 expression, by growing cells on glutamine or in GATA activator mutants (Fig. 5B), resulted in a corresponding decrease of Dal80-Myc^{13} binding (Fig. 5A), suggesting that Dal80-Myc^{13} binding to promoters in vivo is solely controlled by its expression level.

Gat1 is the target of Gzf3-mediated repression on glutamine. Aiming at the elucidation of the mechanisms regulating Gat1 activity on glutamine, we quantified the expression of *GAP1*—a well-characterized target of Gzf3-mediated repression (34)—using qRT-PCR in a wild-type strain and in cells lacking *GAT1*, *GLN3*, or *GZF3*, either in single units (FV023, FV022, and FV083) or in pairwise combinations (*gat1*Δ *gln3*Δ, FV024; *gat1*Δ *gzf3*Δ, FV114; and *gln3*Δ *gzf3*Δ, FV113). Detailed analysis of Fig. 6 generates the following observations: (i) expression of *GAP1* in proline-grown cells was abolished only when the two activators were lacking (*gat1*Δ *gln3*Δ), suggesting that both Gln3 and Gat1 are involved in *GAP1* trans-

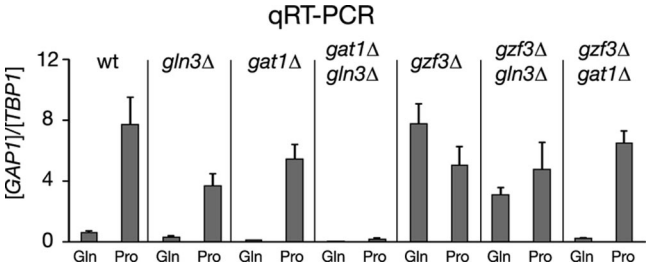

FIG. 6. Gat1 is the target of Gzf3-mediated repression on glutamine. *GAP1* mRNA levels were analyzed in glutamine- and proline-grown wild-type (wt), *gln3*Δ, *gat1*Δ, *gat1*Δ *gln3*Δ, *gzf3*Δ, *gln3*Δ *gzf3*Δ, and *gat1*Δ *gzf3*Δ strains as described for Fig. 1B using qRT-PCR with GAP1O primers.

activation; (ii) maximal expression of *GAP1* in proline-grown cells required the presence of both Gln3 and Gat1; and (iii) *GAP1* expression in glutamine-grown cells lacking *GZF3* reached the wild-type derepressed level regardless of the presence of Gln3 (*gln3*Δ *gzf3*Δ) but depending on Gat1 (*gat1*Δ *gzf3*Δ). These results are consistent with earlier reports suggesting that the negative effect of *GZF3* on glutamine occurs through interference with Gat1-mediated activation (30, 34).

Gat1 binding and *GAT1* expression are downregulated by Gzf3 on glutamine. We next investigated the impact of a *GZF3* deletion on Gat1 DNA binding. Glutamine- and proline-grown *GAT1*-MYC^{13} wild-type and *gzf3*Δ (FV084) cells were subjected to ChIP analysis for the presence of Gat1-Myc^{13} at P_{GAP1}. Whereas deleting *GZF3* had no impact on Gat1-Myc^{13} binding in proline-grown cells, it moderately increased in glutamine-grown *gzf3*Δ cells versus the level in the wild type (Fig. 7A). Consistent with the increased Gat1-Myc^{13} binding to P_{GAP1} in glutamine-grown *gzf3*Δ cells, the observed *GAT1* expression levels were higher than those in wild-type cells grown in the same conditions (Fig. 7B). As expected, Gat1-Myc^{13} binding remained proline inducible, given the fact that *GAT1* expression (Fig. 7B) and Gat1 localization (data not shown) were still regulated by the nitrogen source. The elevated *GAP1* expression level reached in *gzf3*Δ cells (comparable to the wild-type, derepressed level; Fig. 6), despite rather low Gat1 binding, is explained by Dal80-Myc^{13} binding in glutamine-grown *gzf3*Δ cells that is correspondingly lower than that in proline-grown, wild-type cells (data not shown). Next, we analyzed the binding of Gat1-Myc^{13} and Gzf3-Myc^{13} (FV079) at P_{GAT1}. In glutamine-grown, wild-type cells, Gat1-Myc^{13} bound its own promoter but, as expected, in a rather small amount compared to the level it reached on proline (Fig. 7C). These results corroborate previous data indicating that *GAT1* is autoregulated (30), though the opposite has also been reported (8). Deleting *GZF3* moderately increased Gat1-Myc^{13} binding at P_{GAT1}. In addition, Gzf3-Myc^{13} significantly bound P_{GAT1} on glutamine (Fig. 7D), which is in agreement with previous hypotheses that Gzf3 downregulates *GAT1* expression, possibly by preventing its autoactivation (30) through competition for binding to the same GATA sequences in P_{GAT1}, as it was presumed to occur at other NCR-sensitive promoters (34).

Gat1 binding to NCR promoters is counteracted by Gzf3. In order to determine whether Gzf3 controls NCR gene expression only through *GAT1* expression, we quantified *GAP1* expression by qRT-PCR in P_{GAL1}-*GAT1*-MYC^{13} strains carrying either a wild-type or a deleted allele of *GZF3* (FV172) and cultivated in the presence of galactose and glutamine with increasing amounts of glucose (Fig. 8A). In both wild-type and *gzf3*Δ strains, reducing *GAT1* expression (Fig. 8B) led to a decrease in *GAP1* expression. Interestingly, at equal Gat1 concentrations in wild-type and *gzf3*Δ strains (Fig. 8B), *GAP1*

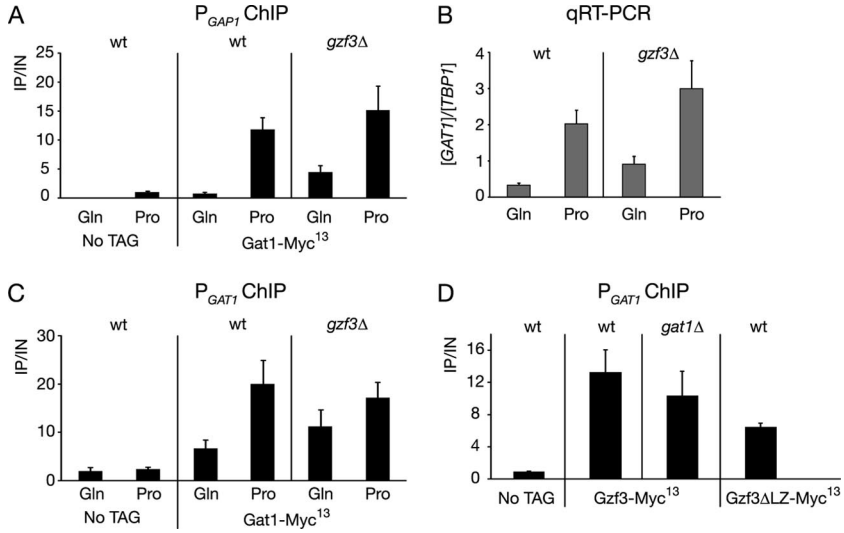

FIG. 7. Gzf3 affects Gat1 binding to P_{GAP1} through downregulation of *GAT1* expression by competing with Gat1 for binding at P_{GAT1}. (A) Effects of *gzf3*Δ on Gat1-Myc^{13} binding to P_{GAP1}. Glutamine- and proline-grown, untagged wild-type (wt), *GAT1*-MYC^{13} wild-type, and *gzf3*Δ strains were analyzed by ChIP as described for Fig. 1A. (B) Effects of *gzf3*Δ on *GAT1* expression. *GAT1* mRNA levels were analyzed in glutamine- and proline-grown wild-type and *gzf3*Δ strains as described for Fig. 1B. (C) Effects of *gzf3*Δ on Gat1-Myc^{13} binding to P_{GAT1}. Glutamine- and proline-grown, untagged wild-type, *GAT1*-MYC^{13} wild-type, and *gzf3*Δ strains were analyzed by ChIP as described for Fig. 3C. (D) Binding of Gzf3-Myc^{13} to P_{GAT1}. Glutamine-grown, untagged wild-type, *GZF3*-MYC^{13} wild-type, *gat1*Δ, and *GZF3*-ΔLZ-MYC^{13} wild-type strains were analyzed by ChIP as described for panel C.

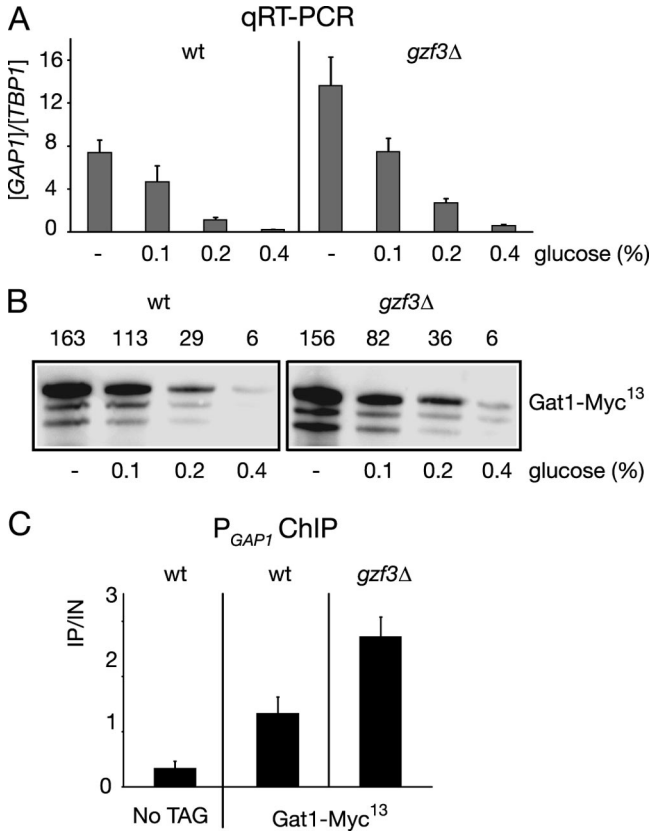

FIG. 8. Gzf3 counteracts Gat1 binding to P_{GAP1}. Untagged wild-type (wt), P_{GAL1}-*GAT1*-MYC^{13} wild-type, and *gzf3*Δ cells were grown in 1% galactose and glutamine with or without 0.1%, 0.2%, or 0.4% glucose. (A) Effects of *gzf3*Δ on *GAP1* expression in a P_{GAL1}-*GAT1*-MYC^{13} strain. *GAP1* mRNA levels were analyzed as described for Fig. 6. (B) Effects of increasing amounts of glucose on P_{GAL1}-*GAT1*-MYC^{13} expression. Gat1-Myc^{13} quantification values of anti-*myc* Western blotting are indicated above the figure. (C) Effect of *gzf3*Δ on Gat1-Myc^{13} binding to P_{GAP1} in a P_{GAL1}-*GAT1*-MYC^{13} strain. Cells were grown in galactose plus glutamine. ChIP analysis was performed as described for Fig. 1A.

expression was always higher in the absence of Gzf3 (Fig. 8A), suggesting a negative role for Gzf3 in *GAP1* expression in addition to its control of *GAT1* expression. ChIP analysis was conducted to assess the binding of constitutively expressed Gat1-Myc^{13} in wild-type and *gzf3*Δ strains expressing P_{GAL1}-Gat1-Myc^{13} grown on galactose and glutamine. Deleting *GZF3* increased the in vivo binding of Gat1-Myc^{13} to P_{GAP1} (Fig. 8C), explaining the raised *GAP1* expression levels. All together, our results suggest that besides affecting *GAT1* expression, Gzf3 affects *GAP1* expression directly by reducing Gat1 binding at P_{GAP1}.

Gat1 is sequestered by Gzf3 in the nucleus. The next step in testing the competition between Gat1 and Gzf3 for DNA binding was to analyze the binding of Gzf3 itself to P_{GAP1} in vivo. A *GZF3*-MYC^{13} wild-type strain was grown on glutamine and shifted to proline for 10, 20, and 60 min or grown on proline and shifted to glutamine for 10 and 60 min, cross-linked, and subjected to ChIP analysis (Fig. 9A). Surprisingly, Gzf3-Myc^{13} was weakly associated with P_{GAP1} in glutamine-grown cells and was massively recruited only after 20 min of the shift to proline. Consistently, Gzf3-Myc^{13} bound P_{GAP1} on proline and was removed by glutamine addition. These unexpected observations strongly suggested that the current NCR model based on Gat1-Gzf3 competition for DNA binding to the same GATA sites required revision. To start, we tried to explain the NCR-regulated DNA-binding capacity of Gzf3-Myc^{13}. First, we analyzed the expression of *GZF3* in a wild-type strain grown on glutamine or on proline (Fig. 9B), but there was no significant influence of the nitrogen supply on *GZF3* expression, in contrast to what was previously observed (30, 34). Second, we studied the subcellular distribution of Gzf3-Myc^{13} in a wild-type strain grown in glutamine or in proline (Fig. 9C), but there was no marked effect of the nitrogen source on Gzf3-Myc^{13} localization: Gzf3-Myc^{13} was constitutively found in the nucleus. Third, the unexpected presence of Gzf3-Myc^{13} at P_{GAP1} on proline could result from it being carried along with the other GATA factors, whose binding is induced in the same conditions (Fig. 1A and 2A; also data not shown). Among these, Dal80 was the most likely candidate, since Gzf3 was previously shown to form heterodimers with Dal80 through their leucine zipper domains (37). We therefore tested the ChIP binding of Gzf3-Myc^{13} in a *dal80*Δ mutant strain (FV104; Fig. 9A). Deleting *DAL80* reduced the binding of Gzf3-Myc^{13} in proline-grown cells, suggesting that proline-induced binding of Gzf3 could partially result from Dal80-mediated recruitment. However, since Gzf3-Myc^{13} binding to P_{GAP1} remained inducible on proline, we next assayed its binding in a *gat1*Δ mutant strain (FV106; Fig. 9A). Proline-induced binding of Gzf3-Myc^{13} to P_{GAP1} was completely abolished when *GAT1* was deleted. The weak binding of Gzf3-Myc^{13} observed on glutamine was also lost in the *gat1*Δ strain (Fig. 9A). Given that the literature about *GZF3* expression was contradictory (8, 34), we assayed *GZF3* expression by qRT-PCR and showed that it was not affected by the deletion of *GAT1* (Fig. 9B). Moreover, Gzf3 subcellular localization was not modified in the absence of Gat1 (Fig. 9C). It is worth noting that binding of Gzf3-Myc^{13} to P_{GAT1} occurred in glutamine-grown cells and was not Gat1 dependent (Fig. 7D), suggesting promoter specificities conditioning Gzf3 binding; this could explain the observed Gat1-Gzf3 competition at this particular promoter.

In sum, the complete lack of Gzf3 binding in *gat1*Δ cells likely results from the joint absence of Gat1 and Dal80, the latter being poorly induced and not significantly recruited to NCR-sensitive promoters in proline-grown *gat1*Δ cells (Fig. 5B and A, respectively). On the other hand, the reduction in Gzf3 binding in *dal80*Δ cells is moderated due to the increased *GAT1* expression and Gat1 binding in these mutant cells (Fig. 1B and A, respectively).

To test the hypothesis that in vivo DNA binding of Gzf3 occurs as a result of its interaction with Gat1, we assayed the in vivo interaction between Gat1-TAP and Gzf3-Myc^{13} using co-immunoprecipitation. Wild-type cells expressing Gat1-TAP and Gzf3-Myc^{13} from their native loci (FV231) were grown on glutamine or proline. Gat1-TAP was immunoprecipitated using magnetic beads coated with monoclonal human anti-mouse immunoglobulin G, and the presence of Gzf3-Myc^{13} was assayed in the immunopurified fraction by Western blotting. We demonstrated an interaction between the two GATA factors in proline- and glutamine-grown cells (Fig. 9D). These results suggest that, in conditions of nitrogen repression, Gzf3 pre-

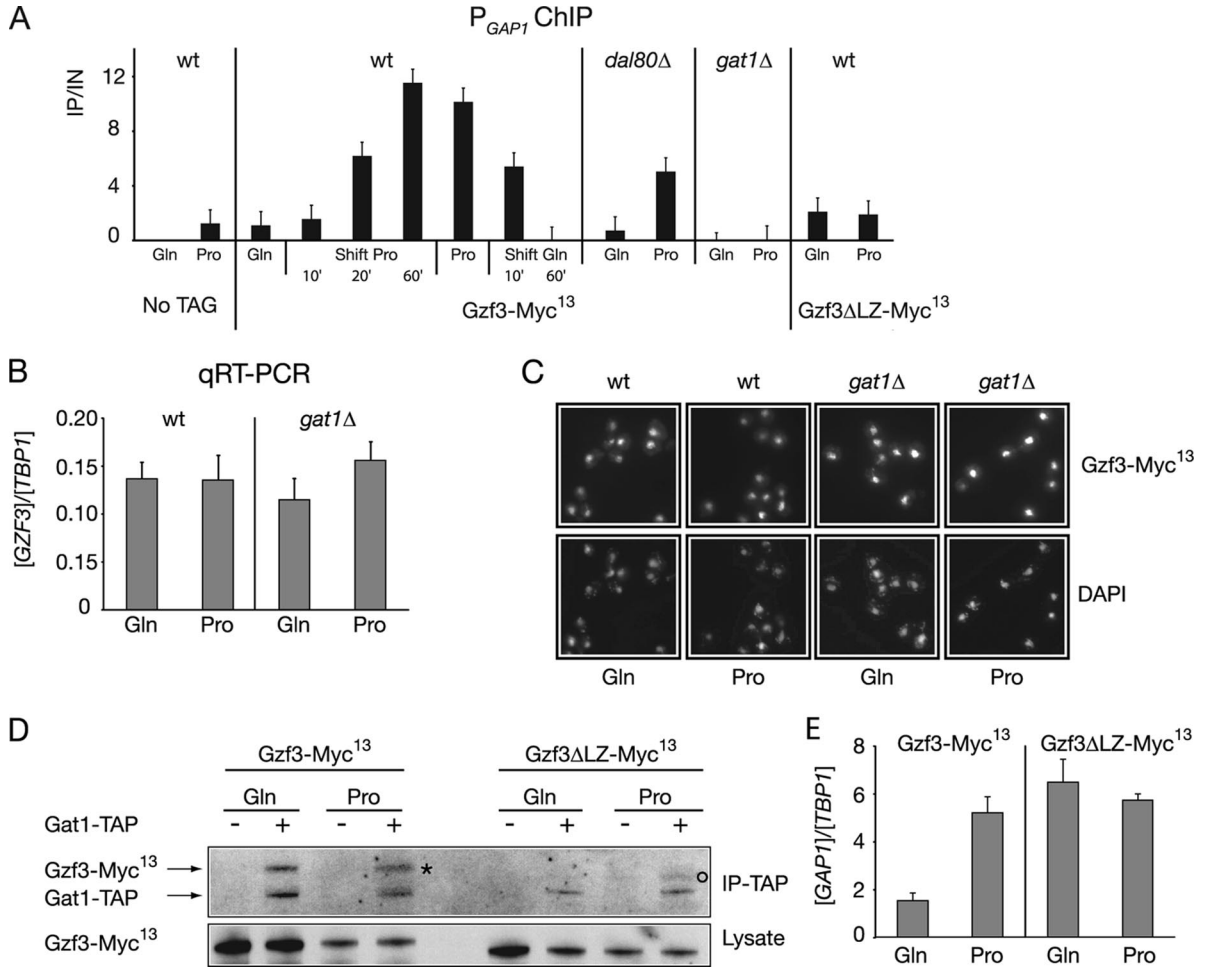

FIG. 9. Gzf3 is recruited by Dal80 and Gat1 at P_{GAP1}. (A) In vivo binding of Gzf3-Myc^{13} at P_{GAP1}. Untagged wild-type (wt), *GZF3*-MYC^{13} wild-type, *gat1*Δ, *dal80*Δ, and *GZF3*-ΔLZ-MYC^{13} wild-type strains were grown in glutamine or proline. The wild-type *GZF3*-MYC^{13} strain was grown in glutamine and shifted to proline for 10, 20, and 60 min or in proline and shifted to glutamine for 10 and 60 min. ChIP analysis was performed as described for Fig. 1A. (B) Effects of *gat1*Δ on *GZF3* expression. *GFZ3* mRNA levels were quantified in glutamine- or proline-grown wild-type and *gat1*Δ strains using qRT-PCR as described for Fig. 1B using GZF3O primers. (C) Effects of *gat1*Δ on Gzf3-Myc^{13} subcellular localization. Glutamine- or proline-grown wild-type and *gat1*Δ *GZF3*-MYC^{13} cells were processed for indirect IF. Gzf3-Myc^{13} and DNA were detected using anti-*myc* and DAPI (4′,6′-diamidino-2-phenylindole), respectively. (D) Coimmunoprecipitation of Gzf3-Myc^{13} and Gzf3-ΔLZ-Myc^{13} with Gat1-TAP. *GZF3*-MYC^{13}, *GZF3*-ΔLZ-MYC^{13}, *GAT1*-TAP *GZF3*-MYC^{13}, and *GAT1*-TAP *GZF3*-ΔLZ-MYC^{13} strains were grown in glutamine or proline. Total proteins were extracted, immunoprecipitated, and subjected to Western blot analysis. (*, Gzf3-Myc^{13}; °, Gzf3-ΔLZ-Myc^{13}). (E) Deleting the leucine zipper of Gzf3 affects its repressing ability. *GAP1* mRNA levels were quantified in glutamine- or proline-grown *GZF3*-MYC^{13} and *GZF3*-ΔLZ-MYC^{13} strains as described for Fig. 6.

vents nuclear Gat1 from binding to its target GATA sites by intranuclear sequestration. The observation that the Gzf3-Gat1 interaction is maintained in proline-grown cells explains the in vivo binding of Gzf3 to target NCR-sensitive promoters in conditions of derepression in *dal80*Δ cells, probably through Gat1-mediated recruitment.

Leucine zipper of Gzf3 is required for its recruitment to P_{GAP1} on proline and for repression on glutamine. In order to investigate the domains of Gzf3 that are important for its recruitment to NCR-sensitive promoters, we compared the binding of wild-type Gzf3-Myc^{13} to that of Gzf3-Myc^{13} devoid of its C-terminal leucine zipper domain (*GZF3*-ΔLZ-MYC^{13}; FV135), previously identified to be responsible for its heterodimerization with Dal80 (37). In glutamine-grown cells (Fig. 7D), deleting the leucine zipper of Gzf3 affected its binding to the *GAT1* promoter, which is consistent with its binding as a homodimer at this particular promoter. In proline-grown cells (Fig. 9A), Gzf3-ΔLZ-Myc^{13} binding to P_{GAP1} was severely affected, indicating that the leucine zipper region of Gzf3 was involved in the protein-protein interactions responsible for its recruitment to the *GAP1* promoter, probably through its interaction with Dal80. However, Gzf3-ΔLZ-Myc^{13} binding was not completely abolished in the way that it was in a *gat1*Δ strain, suggesting that other domains are involved in its recruitment to P_{GAP1} through Gat1, under both repressing and derepressing conditions. To test the involvement of the leucine zipper of Gzf3 in its interaction with Gat1, we performed a coimmunoprecipitation experiment with wild-type cells expressing Gat1-TAP and Gzf3-ΔLZ-Myc^{13} from their native loci (FV232) and grown on glutamine or proline. In proline-

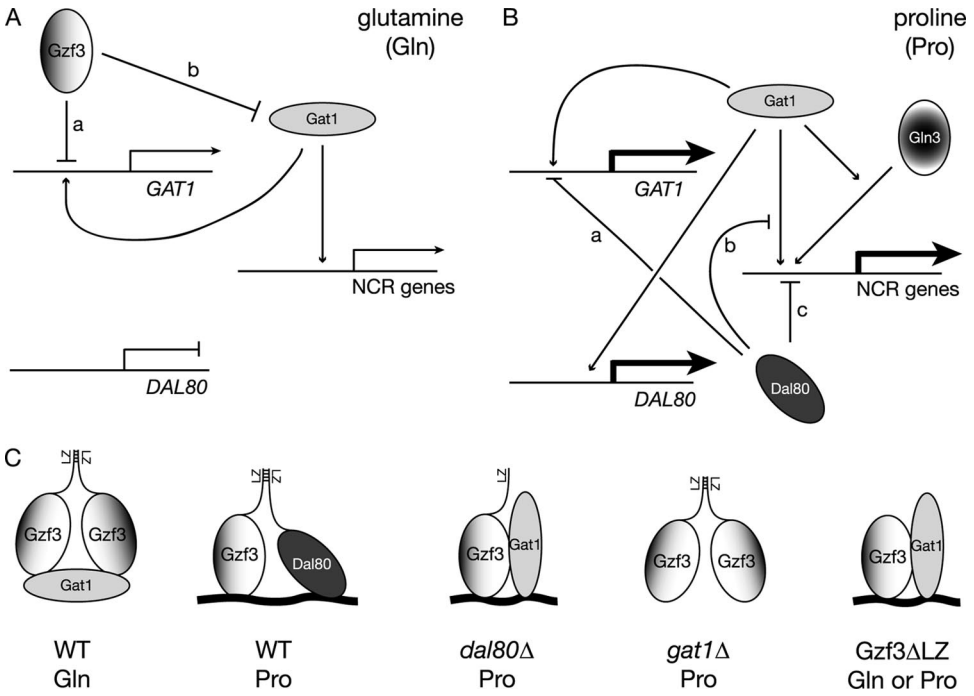

FIG. 10. GATA factor involvement in NCR-sensitive gene regulation. (A) Nuclear GATA factors controlling NCR genes in glutamine-grown cells. *GAT1* is expressed at low levels. Gzf3 represses NCR-sensitive transcription at two levels: (a) it represses *GAT1* expression by binding to its promoter (competition with Gat1); and (b) it sequestrates nuclear Gat1, thereby preventing it from binding GATA sites. *DAL80* is not expressed, and Gln3 is sequestered in the cytoplasm. (B) GATA factors controlling NCR genes in proline-grown cells. The Gat1 and Gln3 activators are nuclear and transactivate NCR genes, including *GAT1* and *DAL80*. Gat1 is required for Gln3 binding to NCR promoters. Dal80-dependent repression occurs at three levels: (a) it represses *GAT1* expression; (b) it competes with Gat1 for binding; and (c) it directly represses NCR gene transcription. (C) Nitrogen-regulated Gzf3 complexes in wild-type (WT) and mutant cells. In glutamine-grown, wild-type cells, Gzf3 is mostly present as a leucine zipper-dependent dimer capable of sequestrating Gat1. A Gat1-Gzf3 heterodimer, which forms independently from the leucine zipper, is found in much smaller amounts and is able to bind NCR promoters (not represented on the model). Growth with proline generates higher *DAL80* and *GAT1* expression. Dal80 efficiently competes with Gzf3 for the formation of a Dal80-Gzf3 heterodimer, responsible for most of the DNA binding of Gzf3. The Gat1-Gzf3 heterodimer also accounts for part of the Gzf3 recruitment to DNA. In the absence of Dal80, the Gat1-Gzf3 heterodimer is solely responsible for the residual recruitment of Gzf3 to NCR promoters. In the absence of Gat1, only the Gzf3 homodimer is found, and it is unable to bind to DNA. Deleting the leucine zipper region of Gzf3 abolishes the formation of the Gat1-Gzf3 heterotrimer and also of the Gzf3-Dal80 heterodimer. In these conditions, only the Gat1-Gzf3 heterodimer is formed and binds to DNA.

grown cells, Gzf3-ΔLZ-Myc[13] interacted with Gat1-TAP, although to a lesser extent than the wild-type Gzf3-Myc[13] (Fig. 9D). Surprisingly, deleting the leucine zipper of Gzf3 severely reduced the interaction between Gat1-TAP and Gzf3-ΔLZ-Myc[13] in glutamine-grown cells. This result indicates that the leucine zipper region of Gzf3 is mostly required to enable the Gat1-Gzf3 interaction in repressing conditions, whereas it is dispensable on proline, suggesting that another domain is responsible for the interaction. Since a previous two-hybrid analysis has shown a weak but significant interaction between the zinc fingers of Gln3 and Gzf3 (37), it is possible that the Gzf3 zinc finger region alone can allow an interaction with Gat1 and/or basal level DNA binding. Finally, deleting the leucine zipper of Gzf3 impaired its ability to repress *GAP1* expression on glutamine (Fig. 9E) without affecting its basal binding to P_{GAP1}, demonstrating that low-level binding of Gzf3 to P_{GAP1} is not responsible for its repressing function on glutamine.

DISCUSSION

This study generates a thorough understanding and uncovered novel aspects of the network of four GATA zinc finger proteins that are responsible for the fine tuning of yeast's response to the quality of nitrogen supply (Fig. 10). Cross-regulation of these four GATA factors has been extensively documented, but some of the results obtained differed from one lab to another, mainly due to differences in the techniques used (7, 8, 13, 30, 34). Our qRT-PCR results corroborate previous observations that *GLN3* was constitutively expressed and that *GAT1* and *DAL80* expression was NCR sensitive. However, whereas some reports in the literature describe *GZF3* as being regulated by the nitrogen source (8, 34), we could not demonstrate any significant difference in *GZF3* expression under various nitrogen conditions and in cells lacking Gat1, with qRT-PCR and Western blotting (data not shown).

On proline, Dal80 function involves *GAT1* expression control, competition for DNA binding, and direct repression. Dal80-negative action can take place in vivo at three different levels (Fig. 10B). First, our results confirmed earlier data showing that Gat1 and Dal80 regulators are linked by reciprocal transcriptional control (7, 12, 34). Indeed, *GAT1* expression increased in *dal80*Δ proline-grown cells compared to that in the wild type and *DAL80* expression was severely affected in proline-grown *gat1*Δ cells. Thus, in proline-grown cells, Dal80 downregulates NCR-sensitive genes by lowering the amount of

Gat1 produced. Second, at the *UGA4* promoter, which contains a canonical URS_{GATA} (1, 2, 15), Dal80 competes with Gat1 for binding to similar or overlapping sites: (i) Dal80 binds in vitro to P_{UGA4} (14); (ii) the in vivo binding of Dal80 and Gat1 to P_{UGA4} was elevated in nitrogen derepression conditions; and (iii) the binding of constitutively expressed Gat1-Myc^{13} to P_{UGA4} was raised upon *DAL80* deletion. Third, impressive changes in *UGA4* expression have been observed following *DAL80* deletion (about 20×), although constitutively expressed Gat1-Myc^{13} binding was only weakly affected (about 2×). These data suggest that Dal80 could have a third, yet-unreported effect that does not rely on *GAT1* expression nor on its binding and rather corresponds to Dal80-mediated repression interfering with transcriptional activation.

On glutamine, Gzf3 represses at two levels, by two different modes of action. Initial observations of Gzf3 function, using *GAP1-lacZ* fusions, have demonstrated an antagonistic role for Gzf3 on activation by Gat1 in cells grown under nitrogen repression conditions (30, 34). Our qRT-PCR results confirm that derepression of *GAP1* expression in glutamine-grown *gzf3*Δ cells requires Gat1 and not Gln3. The absence of involvement of Gln3 is explained by its cytoplasmic sequestration by Ure2 in these conditions. Our study provides more details on how Gzf3 negatively influences NCR-regulated gene expression (Fig. 10A). First, several lines of evidence validate previous assumptions that Gzf3 competes with Gat1 at its own promoter (30), thereby reducing the expression of the activator in conditions of nitrogen abundance: (i) *GAT1* expression was higher in glutamine-grown *gzf3*Δ cells than in wild-type cells, confirming earlier *lacZ* fusion data (30); (ii) rendering *GAT1* expression constitutive diminished the difference between wild-type and *gzf3*Δ glutamine-grown strains; and (iii) in vivo binding of Gzf3-Myc^{13} to P_{GAT1} was elevated on glutamine, whereas binding of Gat1-Myc^{13} to its own promoter was weak. Second, the fact that *GAP1* expression still responded to Gzf3 in cells constitutively expressing *GAT1* indicates that Gzf3 also acts downstream of *GAT1* expression.

A revised model for Gzf3-mediated repression on glutamine. Our data disagree with earlier hypotheses accounting for the negative effect of Gzf3 by its competition with Gat1 for binding to similar GATA sites in NCR-sensitive promoters (34). Indeed, on glutamine, the in vivo binding of Gzf3-Myc^{13} to P_{GAP1} was, like that of Gat1-Myc^{13}, very low. In glutamine-grown cells, a physical interaction between Gzf3-Myc^{13} and Gat1-TAP was detected, which suggests that Gzf3, which we showed to be expressed constitutively and located exclusively in the nucleus, negatively acts on nuclear Gat1 by sequestrating it, thereby preventing it from binding to its target GATA sites in conditions of nitrogen repression.

Since Gzf3 and Gat1 are apparently associated in both repressive and derepressive growth conditions, despite the fact that the negative effect of Gzf3 toward Gat1 function is only obvious in conditions of repression, one has to hypothesize that binding of Gzf3 to Gat1 can occur in two manners: one being productive (repressive, on glutamine), and the other being nonproductive (not repressive, on proline). The deletion of Gzf3's leucine zipper, which is known to mediate its homodimerization, affected its repressing capability and impaired its interaction with Gat1, which suggests that the form of Gzf3 able to sequestrate Gat1 is a Gzf3 homodimer. In contrast, deleting Gzf3's leucine zipper did not severely impair its interaction with Gat1 in proline-grown cells, which suggests that a Gzf3 monomer can also interact with Gat1. Our model is summarized in Fig. 10C. In wild-type, glutamine-grown cells, Gzf3 is more abundant than Gat1, and the most common form of Gzf3 is the repressive one. The Gzf3 homodimer can bind to P_{GAT1} (Fig. 10A) and also interacts with Gat1, sequestrating it in the nucleus. Some Gzf3-Gat1 heterodimers can be found at NCR-sensitive promoters. Upon a shift to proline, NCR expression increases, leading to induced expression of *DAL80* and *GAT1*. In these conditions, the Gzf3-Dal80 heterodimer dominates over the Gzf3 homodimer and the Gzf3-Gat1 heterodimer due to a stronger affinity (37). The two heterodimers are responsible for the high-level DNA binding of Gzf3. Lowered DNA binding of Gzf3 in *dal80*Δ cells is explained by the lack of *DAL80*, partially compensated for by a higher *GAT1* expression. Lack of Gzf3 DNA binding in *gat1*Δ cells is due to the joined absence of the two proteins responsible for its recruitment to P_{GAP1}. Finally, deleting Gzf3's leucine zipper resulted in the lack of formation of its repressive form, and of the Dal80-Gzf3 heterodimer, without impairing low-level, Gat1-mediated Gzf3 recruitment to P_{GAP1}.

An unanticipated role for Gzf3 on proline. Our results indicate that proline-induced Gzf3 binding to NCR-sensitive promoters occurs via Dal80- and Gat1-mediated recruitment or is stabilized by them. Attempts to identify a function for Gzf3 during growth on poor nitrogen sources gave contradictory results: in proline-grown cells, deletion of *GZF3* reduced *DAL5* and *GAP1* expression (Fig. 6; also data not shown), suggesting that Gzf3 may carry out an activating function or be required to stabilize activating complexes when bound on some NCR-sensitive promoters. On the other hand, we have also demonstrated that Gzf3 could perform an inhibitory task at the promoter of *DAL5* in glutamine-grown cells treated with rapamycin (19). Hints of a repressive function for Gzf3 have previously been reported in derepressive growth conditions for *DAL80* (34) and *CIS2* (35) regulation.

Binding of all four GATA factors is induced upon growth on poor nitrogen sources. Although we have observed a concomitant increase of binding of the four GATA factors in proline-grown cells, it does not necessarily imply that all are bound to the same promoter at the same time. However, hints of protein-protein interaction have been provided throughout our work: (i) the fact that Gln3-Myc^{13} binding to DNA required Gat1 suggests that the latter may help in recruiting the former or in stabilizing its binding; (ii) Dal80 interference with transcriptional activation, besides its competitive effect with Gat1-Myc^{13} binding to DNA, suggests that it might bind simultaneously and alter the activator potency; and (iii) Dal80- and Gat1-mediated recruitment of Gzf3-Myc^{13} to NCR-sensitive promoters has been demonstrated, with no clearly established function for the latter during transcriptional activation. It is, however, conceivable that Gzf3 may relay transduction signals to Gat1, due to its demonstrated physical interactions with Ure2 (33) and Tor1 (5). However, these interaction data, obtained using the two-hybrid technique, must be taken with care since Ure2 is currently thought to be cytoplasmic, whereas Gzf3 is nuclear.

Controlling Gat1 function is a good strategy to fine-tune NCR. This study reveals a central role for Gat1 in NCR con-

trol. Multiple-level control of Gat1 activity enables yeasts to perform subtle adaptation of their metabolism in response to the wide variety of nitrogen sources they can utilize. First, *GAT1* transcription is controlled by the nitrogen supply by the four GATA factors. The existence of an autoactivation loop on *GAT1* expression makes it an efficient target to control NCR. Attempts to reproduce wild-type variations in *GAT1* expression demonstrated that Gat1 DNA binding and *GAP1* expression levels paralleled *GAT1* expression, suggesting that Gat1 concentration is the limiting factor for NCR activation. Thus, small changes in *GAT1* expression have a high impact on NCR-sensitive gene expression.

Our study demonstrates that cells constitutively expressing Gat1 are still sensitive to NCR, although to a lesser extent, suggesting an important role for sequestration, competition, and presumed posttranslational modifications affecting Gat1 function. Unlike Gln3, Gat1 localization on glutamine is not exclusively cytoplasmic, is only weakly Ure2 dependent (20), and is not exclusively nuclear on proline. Preventing Gat1 from accessing its binding sites occurs not only via cytoplasmic retention but also by nuclear sequestration involving Gzf3. Competition of Gat1 with the two negative GATA factors for DNA binding has also been demonstrated. Conflicting results about Gat1 phosphorylation have been presented in the literature, some suggesting nitrogen-regulated Gat1 phosphorylation (3; data not shown) and others showing indistinguishable SDS-polyacrylamide gel electrophoresis patterns (24; also our unpublished data).

Once having integrated these multiple signals, Gat1 activity in stimulating NCR-sensitive gene transcription occurs not only by direct transcriptional activation but also by controlling the binding of the other transcriptional activator, Gln3.

In sum, Gat1 function does integrate the information about the activity of all four GATA factors, not only at the level of its expression (all four GATA factors are involved in *GAT1* expression control) but also through presumed protein-protein interactions with Gln3, Dal80, and Gzf3.

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