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Functional Genomics Analysis of the *Saccharomyces cerevisiae* Iron Responsive Transcription Factor Aft1 Reveals Iron-Independent Functions

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ABSTRACT

The *Saccharomyces cerevisiae* transcription factor Aft1 is activated in iron-deficient cells to induce the expression of iron regulon genes, which coordinate the increase of iron uptake and remodel cellular metabolism to survive low-iron conditions. In addition, Aft1 has been implicated in numerous cellular processes including cell-cycle progression and chromosome stability; however, it is unclear if all cellular effects of Aft1 are mediated through iron homeostasis. To further investigate the cellular processes affected by Aft1, we identified >70 deletion mutants that are sensitive to perturbations in *AFT1* levels using genome-wide synthetic lethal and synthetic dosage lethal screens. Our genetic network reveals that Aft1 affects a diverse range of cellular processes, including the *RIM101* pH pathway, cell-wall stability, DNA damage, protein transport, chromosome stability, and mitochondrial function. Surprisingly, only a subset of mutants identified are sensitive to extracellular iron fluctuations or display genetic interactions with mutants of iron regulon genes *AFT2* or *FET3*. We demonstrate that Aft1 works in parallel with the *RIM101* pH pathway and the role of Aft1 in DNA damage repair is mediated by iron. In contrast, through both directed studies and microarray transcriptional profiling, we show that the role of Aft1 in chromosome maintenance and benomyl resistance is independent of its iron regulatory role, potentially through a nontranscriptional mechanism.

LIKE all organisms, the yeast *Saccharomyces cerevisiae* maintains tight regulation of cellular iron uptake and utilization to prevent toxicity caused by iron overload (reviewed in Kaplan *et al.* 2006). *S. cerevisiae* responds to iron depletion through transcriptional remodeling governed primarily by the iron-responsive transcription factor Aft1 (reviewed in Rutherford and Bird 2004). Aft1 is routinely shuttled between the nucleus and the cytoplasm where the export of Aft1 from the nucleus is promoted in the presence of iron-sulfur clusters (ISC) in the cell (Yamaguchi-Iwai *et al.* 2002; Chen *et al.* 2004; Rutherford *et al.* 2005; Ueta *et al.* 2007). Upon iron depletion and decreased levels of ISCs, Aft1 accumulates in the nucleus where it activates the transcription of 25 genes, referred to as the "iron regulon," that are required for increasing cellular iron content (Yamaguchi-Iwai *et al.* 1996; Rutherford *et al.* 2001; Rutherford *et al.* 2003; Shakoury-Elizeh *et al.* 2004; Courel *et al.* 2005).

The iron regulon genes can be grouped into three categories (extensively reviewed in Kaplan *et al.* 2006; Philpott and Protchenko 2008). The majority of the genes encode proteins that increase iron uptake from the environment, including genes that encode siderophore transporters (*ARN1*, *ARN2*, *ARN3*, *ARN4*), cell-wall siderophore binding/uptake proteins (*FIT1*, *FIT2*, *FIT3*), iron-reducing metalloreductase proteins (*FRE1-FRE5*), and the high-affinity iron transport complex composed of a ferroxidase (*FET3*) and a permease (*FTR1*). As copper is required for the activity of Fet3, the iron regulon also includes the copper chaperone *ATX1* and copper transporter *CCC2*. A second class of genes encode proteins that allow the cell to mobilize the significant amounts of iron the cell stores in the vacuole (*SMF3*, *FET5*, *FRE6*, *FTH1*, *COT1*) or in the mitochondria as heme or ISC (*HMX1*, *MRS4*). A third class of genes encode proteins that allow the cell to remodel its metabolic activities to decrease the use of iron-dependent enzymes/pathways in favor of iron-independent processes. This includes the upregulation of the biotin transporter *VTH1*, which allows the cell to obtain essential biotin from the environment instead of utilizing the iron-dependent biotin biosynthesis pathway and *CTH2/*

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TIS11, which encodes a mRNA binding protein that destabilizes mRNAs that encode enzymes that require iron cofactors.

In the absence of Aft1, its paralog Aft2 can compensate and regulate transcription of many iron regulon genes (Blaiseau *et al.* 2001; Rutherford *et al.* 2001; Rutherford *et al.* 2003; Courel *et al.* 2005; Rutherford *et al.* 2005). Although Aft2 and Aft1 have overlapping functions, their roles in the transcriptional regulation of the iron regulon are nonredundant (Blaiseau *et al.* 2001) with Aft1 having the prominent role in the transcriptional activation of the iron regulon (Rutherford *et al.* 2003). Additionally while *aft1*Δ mutant cells exhibit low-ferrous-iron uptake and poor growth under low-iron conditions (Yamaguchi-Iwai *et al.* 1995; Casas *et al.* 1997), *aft2*Δ mutant cells shows no growth defects under these conditions (Rutherford *et al.* 2003; Courel *et al.* 2005). However, consistent with the ability of Aft2 to regulate the iron regulon, an *aft1*Δ*aft2*Δ double mutant is more sensitive to low-iron growth conditions than a single *aft1*Δ null mutant alone (Blaiseau *et al.* 2001; Rutherford *et al.* 2001).

In addition to iron depletion, numerous environmental conditions result in the Aft1-dependent induction of the iron regulon, including zinc (Pagani *et al.* 2007), hydroxyurea (HU) (Dubacq *et al.* 2006), and cisplatin treatments (Kimura *et al.* 2007), during the diauxic shift (Haurie *et al.* 2003) and upon loss of mitochondrial DNA (mtDNA) (Veatch *et al.* 2009). The iron regulon is also induced during both the alkaline response (Lamb *et al.* 2001) and adaptation to lactic and acetic acid (Kawahata *et al.* 2006); however, in these cases Aft1 dependence has not been confirmed. Under many of these conditions, Aft1 is mediating a critical role as *aft1*Δ mutants display hypersensitivity to HU (Dubacq *et al.* 2006), cisplatin (Lee *et al.* 2005), zinc (Pagani *et al.* 2007), and high pH (Serrano *et al.* 2004). Why is Aft1 needed under these diverse conditions? One possibility is that iron is either limited under these conditions and/or additional cellular iron is required to buffer some of these challenges. While it has been shown that iron or iron uptake becomes limiting under alkaline pH conditions (Serrano *et al.* 2004) and cisplatin treatment (Kimura *et al.* 2007) and that loss of mtDNA results in decreased ISC (Veatch *et al.* 2009), in the case of the diauxic shift, the activation of Aft1 is controlled by a Snf1/Snf6-dependent pathway and not by extracellular iron concentrations (Haurie *et al.* 2003). Therefore, activation of Aft1 is not solely limited to conditions that decrease ISC levels. Further, while it has been shown that increasing exogenous iron levels can suppress the HU sensitivity of *aft1*Δ cells (Dubacq *et al.* 2006), it has yet to be established if the Aft1-dependent transcriptional induction of the iron regulon and maintaining cellular iron levels is mediating all of the cellular functions of Aft1 or if Aft1 has additional iron-independent cellular roles.

Indeed, the transcriptional effects of Aft1 may not be limited to the iron regulon genes. Microarray analysis of wild-type *vs.* *aft1*Δ cells grown in iron replete YPD media showed that deletion of *AFT1* resulted in the upregulation of 239 genes and the downregulation of 350 genes (Pagani *et al.* 2007). Furthermore, expression of the constitutively active *aft1-1*^{up}, a mutation that localizes Aft1 to the nucleus, results in transcriptional modulation of more than 200 genes implicated in a variety of processes (Shakoury-Elizeh *et al.* 2004). Although it is not known how many of these genes are directly regulated by Aft1 or result from downstream transcriptional cascades, it suggests that Aft1 function may not be limited to the transcriptional induction of just iron regulon genes.

In addition, several studies have suggested that Aft1 may play a role in cell-cycle regulation. Systematic screens have determined that *aft1*Δ mutant cells are significantly larger than wild-type cells (Jorgensen *et al.* 2002) and are delayed in G1 (White *et al.* 2009). Further, overexpression of *AFT1* or *aft1-1*^{up} results in G1 arrest due to the inhibition of translation of G_1 cyclins by an undetermined mechanism (Casas *et al.* 1997; Philpott *et al.* 1998). Aft1 also has been linked to chromosome stability (Measday *et al.* 2005; Yuen *et al.* 2007). Synthetic genetic array (SGA) studies determined that *aft1*Δ mutants could not tolerate either overexpression or loss-of-function of kinetochore genes (Measday *et al.* 2005). Chromosome transmission fidelity assays, which measure the ability of a cell to maintain an artificial chromosome fragment, determined that *aft1*Δ mutant cells display an increase in chromosome loss compared to wild-type cells (Measday *et al.* 2005; Yuen *et al.* 2007). Furthermore, Aft1 has been shown to colocalize with kinetochore proteins (Measday *et al.* 2005) as well as interact with kinetochore proteins Cbf1 (Measday *et al.* 2005) and Iml3 (Wong *et al.* 2007) in yeast two-hybrid assays. *AFT2*, or other iron regulon genes, have not been identified in the genome-wide kinetochore genetic or genome instability screens (Measday *et al.* 2005; Kanellis *et al.* 2007; Yuen *et al.* 2007; Andersen *et al.* 2008), suggesting that the cellular role of Aft1 in chromosome stability may be independent of its role in iron homeostasis. Presently the molecular mechanism by which Aft1 contributes to genome maintenance is unknown.

To explore the global cellular functions of Aft1 under iron replete conditions we performed genome-wide *AFT1* synthetic lethal (SL) SGA and synthetic dosage lethal (SDL) SGA analyses. Our genetic interaction map reveals that >70 deletion mutants are sensitive to perturbations in *AFT1* levels under normal iron conditions. While some of these genetic interactions are attributable to the role of Aft1 in iron homeostasis, including the *RIM101* pH pathway, and DNA damage repair, we determine that the role of Aft1 in chromosome stability

TABLE 1

Strain list

Strain name	Genetic background	Origin
YKB673	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1, aft1Δ::KanMX*	Measday *et al.* (2005)
YKB676	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, aft1Δ::NAT*	This study
YKB731	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+*	Gift from C. Boone
YPH499	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Sikorski and Hieter (1989)
YKB794	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, pGAL1-AFT1*	This study
YKB795	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, pRS416*	This study
YKB1008	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, rim101Δ::NAT*	This study
YKB1009	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, fet3Δ::NAT*	This study
YKB1010	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, aft2Δ::NAT*	This study
YPH1735	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 aft1ΔkanMX6*	Measday *et al.* (2005)
YKB1110	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 rim101Δ::NAT*	This study
YKB1111	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 rim101Δ::NAT aft1ΔkanMX6*	This study
YKB788	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 aft2Δ::TRP1*	This study
YKB793	*MAT***a**/α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52, CFIII(CEN3.L URA3 SUP11)*	This study
YKB1	*MAT***a**/α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 ctf13-30/ctf13-30, CFIII(CEN3.L URA3 SUP11)*	This study
YKB671	*MAT***a**/α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 aft1Δ::kanMX6/aft1Δ::kanMX6, CFIII(CEN3.L URA3 SUP11)*	This study
YKB1095	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 aft1ΔkanMX6*	This study
YKB479	*MAT***a** *his3Δ1 leu2Δ1 met15Δ0 ura3Δ0 AFT1-TAP::HIS*	Invitrogen

is distinct from its role in regulating the iron regulon and cellular iron levels.

MATERIALS AND METHODS

Yeast strains and plasmids: The yeast strains used in this study are listed in Table 1. The *MAT***a** deletion mutant array was purchased from OpenBiosystems (catalog no. YSC1053). The SGA starting strain Y7092 (Tong and Boone 2006) and the media used in the SGA analysis have been described previously (Tong *et al.* 2001; Tong *et al.* 2004). Deletion strains made for this study were designed using a standard PCR-mediated gene insertion technique (Longtine *et al.* 1998). Plasmid *pGAL1–AFT1* (pKB38) (a galactose-inducible promoter followed by the gene *AFT1* carrying a *URA3* resistance marker) was isolated from the yeast overexpression array (Sopko *et al.* 2006) and confirmed by sequencing.

Media and dot assay experiments: Cells were grown in standard YPD or SD medium supplemented with amino acids (Abelson *et al.* 2004), unless otherwise described. For growth in liquid SD–uracil medium at a specific pH, SD–uracil containing 150 mm Hepes was titrated to pH 4 or pH 8 and filter sterilized. To assess growth under various conditions, wild-type and mutant strains were grown to mid-log phase in YPD at 25° and dot assays were performed by spotting 5 μl of fivefold serial dilutions (OD_{600} = 0.1, 0.01, 0.001, 0.0001) onto media containing caffeine (Sigma, C0750), calcofluor white (CFW, Sigma, F3543), cisplatin (Sigma, 479306), methyl methanesulfonate (MMS, Sigma, 129925), or benomyl (Sigma, 381586) as indicated. All dot assay experiments were repeated using two or three different isolates of each strain. For iron-limited YPD plates, 90 μm bathophenanthrolinedisulfonic acid disodium salt hydrate (BPS, Sigma, B1375) was used with the addition of $FeSO_4$ (Sigma, F8048) as previously described (Davis-Kaplan *et al.* 2004).

Synthetic lethal and synthetic dosage lethal screens: Robotic manipulation of the deletion mutant array was conducted using a Singer RoToR HDA robot (Singer Instruments, United Kingdom). Genome-wide SL–SGA screens were conducted three times at 30° for the query strains *aft1Δ::natMX4* (YKB676), *rim101Δ::natMX4 (YKB1008)*, *aft2Δ::natMX4* (YKB1010), and *fet3Δ::natMX4* (YKB1009) as described previously (Tong *et al.* 2001). Double mutants were scored for slow growth or lethality by visual inspection. Putative genetic interactions identified in a minimum of two of three screens were confirmed by tetrad dissection. Genome-wide SDL–SGA screens were conducted in triplicate for the query strain carrying the plasmid *GAL1–AFT1* (YKB 794) as previously described (Measday *et al.* 2005). After replica pinning onto galactose media to induce overexpression of *AFT1*, colonies were grown for 2 days at 16°, 25°, or 37° and scored for slow growth or lethality by visual inspection. Putative genetic interactions identified in a minimum of two of three screens were confirmed by transforming the deletion mutant strain with either the plasmid p*GAL1–AFT1* or the vector control pRS416 and growth defects were assessed by either streak tests or dot assays on plates containing galactose.

β-Galactosidase assays: Reporter constructs *pMELb2-lacZ* and *pMELb2-FET3-lacZ* (Kimura *et al.* 2007) were transformed into wild-type (YPH499), *aft1Δ* (YPH1735), *rim101Δ* (YKB1110), and *aft1Δrim101Δ* (YKB1111) cells. For assessing pH effects, yeast cells carrying the reporter plasmids were cultured in

SD–URA media to an OD_{600} of 0.6 and then inoculated at an OD_{600} of 0.2 in SD–URA media at pH 4 or pH 8 and grown to an OD_{600} of 0.8. For assessing the effects of benomyl, wild-type, and *aft1*Δ yeast cells carrying the reporter plasmids were cultured in SD–URA media to an OD_{600} of 0.6 and then treated with 20 μg/ml benomyl for 1 hr. β-Galactosidase assays were performed in triplicate using crude extracts exactly as described (Burke *et al.* 2000).

Chromosome transmission fidelity assays: Quantitative half-sector analysis was performed essentially as previously described (Koshland and Hieter 1987), except strains were streaked onto YPD or YPD + 90 μM BPS + 100 μM $FeS0_4$ plates prior to the selection of single colonies for the plating assay. The red pigmentation caused by the addition of BPS in the plates, did not allow for consistent scoring of nondisjunction (white:pink) events; hence only chromosome loss (pink:red) half sector events were scored.

RNA microarray experiment: *Sample preparation:* Wild-type (YKB779) and *aft1*Δ (YKB673) cells were grown in YPD at 30° to OD_{600} of 0.4 and benomyl was added to a final concentration of 20 μg/ml. Cells were harvested prior to benomyl addition and at 20 min post-benomyl treatment by centrifugation and flash frozen in liquid nitrogen. For each strain and time point, three independent treatments and microarray hybridizations were performed. Total RNA was isolated using TRI reagent (Sigma) as per the manufacturer's protocol, followed by RNAeasy column purification (QIAGEN). The RNA was quantified using a NanoDrop-1000 spectrophotometer and quality was monitored with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Cyanine-3 (Cy3) labeled cRNA was prepared from 0.5 μg RNA using the one-color microarray-based gene expression analysis protocol version 5.7 (Agilent) according to the manufacturer's instructions, except using half of the reagent amount in step 2, followed by RNAeasy column purification (Qiagen). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 Spectrophotometer.

Microarray hybridization and scanning: Using Agilent Technologies' eArray online tool, a total of 13,189 60-mer oligonucleotides (up to 2 per ORF) were designed using 6649 target ORF sequences (orf_coding_all.20080606.fasta downloaded from http://downloads.yeastgenome.org/) to construct custom *S. cerevisiae* microarray 8X15K slides (Agilent, G2509F). A total of 0.6 μg of Cy3-labeled cRNA was hybridized to each microarray as per manufacturer's instructions and incubated for 17 hr at 65° in a rotating Robbins Model 400 hybridization oven (Robbins Scientific) and an Agilent rotator rack. Following hybridization, microarrays were washed using the wash procedure with stabilization and drying solution (Agilent) as described in the protocol. Slides were scanned immediately after washing using a GenePix 4200A (Molecular Devices) using only the Cy3 channel, scanning each array individually (scan area 2088 × 3112 pixels). The scan resolution was set at 10 μm, lines to average 1, focus position 0 μm. The laser was set at 100% and PMT between 330 and 370 according to strength of the individual array.

Data analysis: The scanned images were analyzed with Genepix 6.0 (Molecular Devices). A normalization factor was calculated for each array using the "mean of F532 median" acquired in the array quality control report in Genepix. The normalization factor was determined so the average intensity of each array was 3400 (3400/mean of F532 median = normalization factor). This value was applied respectfully to each array in Genepix. Using BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) and Microsoft Excel, duplicate spots for each gene were averaged and any nonreproducible values were removed for the rest of the analysis. The Log 2(ratio) for each ORF was calculated and *P*-values were determined using a one-way ANOVA for Multiple Groups. These data sets were used to filter genes that had an observed change of more than twofold and a P-value <0.05 (see supporting information, File S1, for full analyzed data set; raw data can be accessed at GEO, accession no. GSE20531).

Modified chromatin immunoprecipitation assays: Aft1–TAP-tagged and untagged wild-type strains grown in YPD medium at 30° to an OD_{600} of 0.8 were collected by centrifugation and modified chromatin immunoprecipitation (mChIP) was performed as previously described (Mitchell *et al.* 2008). Immunoprecipitated DNA was amplified using multiplex PCR with the following primer pairs: *CTF19* F (5′ CCTGGATGAAACCCA CTCGAA) and *CTF19* R1 (5′ GAGTAACTTGCACAGCTAT TGG); *FET3* IRE F (5′ GGTCCCTACAGTACGCTGAG), and *FET3* IRE R (5′ GGATCGACTGTTTGAGTGCATCC); *TEL-V* F (5′ GGCTGTCAGAATATGGGGCCGTAGTA) and *TEL-V* R (5′ CACCCCGAAGCTGCTTTCACAATAC). PCR products were resolved on a 3% agarose gel and visualized with ethidium bromide.

RESULTS

Synthetic lethal and synthetic dosage lethal analysis reveals novel roles for Aft1: In an effort to identify cellular processes potentially affected by Aft1 under normal or replete iron conditions, we performed two genome-wide genetic screens using SGA methodology (Tong *et al.* 2001): SL screens to identify mutants that cannot tolerate the deletion of *AFT1* and SDL screens to identify deletion mutants that cannot tolerate overexpression of *AFT1*. The *aft1*Δ SL–SGA screen was performed in triplicate and any double mutants that resulted in inviability (SL) or reduced fitness (synthetic sickness, SS) that were identified a minimum of two of three screens were confirmed by tetrad analysis (Table 2). The resulting confirmed data set contains 45 genetic interactions of which 22% (10/45) were SL interactions. Similarly, the *AFT1* SDL–SGA screen was performed in triplicate and at three different temperatures (16°, 25°, and 37°). Any deletion mutants that displayed inviability (SDL) or reduced fitness (synthetic dosage sickness, SDS) upon overexpression of *AFT1* in a minimum of two of three screens were confirmed by streak test and/or dot assay analysis (see material and methods for details). The resulting confirmed data set (Table 3) contains 32 genetic interactions of which only one was SDL, while the remainder were SDS interactions.

Previous work has determined that SL and SDL screens are complementary in nature, identifying largely nonoverlapping sets of genetic interactions (Measday *et al.* 2005; Baetz *et al.* 2006). Therefore as expected, there was limited overlap between the *AFT1* SL and SDL screens, with only one gene identified in both screens (Figure 1). Despite the lack of overlap, both screens identified genes associated with cellular processes previously associated with Aft1, including iron homeostasis, transcription, and chromosome stability. In addition the genetic network suggests that Aft1 function may affect a wider range of cellular processes than previously thought, including the *RIM101* pH response pathway,

TABLE 2

***AFT1*, *AFT2*, and *FET3* synthetic lethal interactions**

ORF	Standard name	Genetic interaction[a]			2.5 μM $FeSO_4$[b]	Cellular function[c]
		AFT1	*AFT2*	*FET3*		
Iron regulation						
YMR038C	*CCS1*	SS		SS	SG	Copper chaperone, involved in oxidative stress protection
YMR319C	*FET4*	SL		SL[d]		Low-affinity Fe(II) transporter of the plasma membrane
YPL202C	*AFT2*	SL				Iron-regulated transcriptional activator
Chromosome stability[e]						
YBR107C	*IML3*	SS				Outer kinetochore protein.
YDR254W	*CHL4*	SS			J	Outer kinetochore protein
YDR318W	*MCM21*	SS				Component of the COMA kinetochore complex
YJR060W	*CBF1*	SS				Transcription factor and component of the kinetochore
YJR135C	*MCM22*	SS				Component of the kinetochore
YPL018W	*CTF19*	SS				Component of the COMA kinetochore complex
YPR046W	*MCM16*	SS				Component of the kinetochore
Rim101 pathway						
YHL027W	*RIM101*	SS		SL[d]	SG, J	Transcriptional repressor involved in the response to pH
YNL294C	*RIM21*	SL		SS	SG, J	Protein involved in the response to alkaline pH
YOR275C	*RIM20*	SS		SL[d]	SG, J	Protein involved in proteolytic activation of Rim101
DNA damage						
YDR078C	*SHU2*	SS				Involved in homologous recombination repair
YKL113C	*RAD27*	SS			SG	5′ to 3′ exonuclease, required for Okazaki fragment processing
YLR320W	*MMS22*		SS			Acts with Mms1 to repair DNA damage caused by blocked replication forks.
YMR224C	*MRE11*	SS				Subunit of the MRX complex DNA double-strand break repair
Transcription or chromatin						
YAL011W	*SWC3*	SS				Component of SWR1 complex
YDL020C	*RPN4*	SS				Transcription factor, regulates proteasome genes
YOL012C	*HTZ1*	SS			SG, J	Histone H2A variant H2AZ,
YPcR070W	*MED1*	SS	SS		L	Subunit of RNA polymerase II mediator complex
Cell cycle						
YGL003C	*CDH1*	SS			SG	Activator of the anaphase-promoting complex
YGL019W	*CKB1*	SS				A Ser/Thr protein kinase with roles in cell growth
YOR039W	*CKB2*	SS				A Ser/Thr protein kinase with roles in cell growth
YPL256C	*CLN2*	SS				G1 cyclin involved in regulation of the cell cycle
Cytoskeleton and cell wall						
YDR389W	*SAC7*	SS				GTPase activating protein (GAP) for Rho1p
YLR110C	*CCW12*	SS			SG	Component of cell wall
YLR121C	*YPS3*	SS				Component of cell wall
YLR341W	*SPO77*	SS				Meiosis-specific protein required for spore wall formation
YOL052C	*SPE2*	SS	SS	SS		Required for biosynthesis of spermidine and spermine
Protein transport						
YBR283C	*SSH1*	SS				Subunit of the Ssh1 translocon complex
YCR094W	*CDC50*	SL			SG, L	Cdc50-Drs2 endosome complex that regulates cell polarity.
YAL026C	*DRS2*	SS				Cdc50-Drs2 endosome complex that regulates cell polarity.
YCL008C	*STP22*	SL		SL	SG, J	Component of the ESCRT-I complex[f]
YLR119W	*SRN2*	SS				Component of the ESCRT-I complex[f]
Mitochondrial						
YCR071C	*IMG2*	SL			L	Mitochondrial ribosomal protein of the small subunit
YHR091C	*MSR1*			SS		Mitochondrial arginyl-tRNA synthetase
Ribosomal						
YJL136C	*RPS21B*	SS				Protein component of the small (40S) ribosomal subunit
YOL080C	*REX4*	SL			SG	Putative RNA exonuclease possibly involved ribosome assembly
Unknown function						
YEL007W		SS	SS	SS		Protein of unknown function
YGL168W	*HUR1*	SL				Dubious open reading frame, overlaps with *PMR1*
YOR331C		SL			SG, D	Dubious open reading frame, overlaps with *VMA4*
YOR355W	*GDS1*	SL				Protein of unknown function

(*continued*)

TABLE 2

(Continued)

ORF	Standard name	Genetic interaction[a] *AFT1*	*AFT2*	*FET3*	2.5 μM $FeSO_4$[b]	Cellular function[c]
Other						
YGR157W	*CHO2*	SS				Phosphatidylethanolamine methyltransferase (PEMT)
YJR131W	*MNS1*	SS				α-1,2-Mannosidase involved in ER quality control
YOL081W	*IRA2*	SS			SG	GTPase-activating protein that negatively regulates RAS
YOL143C	*RIB4*			SL		Lumazine synthase, catalyzes synthesis of immediate precursor to riboflavin
YOR297C	*TIM18*	SS			J	Component of the mitochondrial Tim54p-Tim22p complex

[a] SS, synthetic sick; SL, synthetic lethal; SG, slow growth.
[b] Iron sensitivity identified in additional screens: J, Jo *et al.* (2009); D, Dudley *et al.* (2005); L, Lesuisse *et al.* (2005); DK, Davis-Kaplan *et al.* (2004).
[c] Adapted from Gene Ontology Annotations/Biological processes listed in *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[d] Genetic interaction also identified in *fet3*Δ or *aft2*Δ SL–SGA screens Costanzo *et al.* (2010)
[e] Chromosome stability genetic interacts were confirmed previously Measday *et al.* (2005).
[f] Role in RIM101 signaling pathway.

cell-cycle regulation, DNA damage response, cell-wall assembly, mitochondrial function, and protein transport.

***AFT1* genetic interaction map is not fully attributable to iron deficiency:** It has recently been shown that *aft1*Δ cells have decreased cellular iron levels when cultured under normal iron conditions (Veatch *et al.* 2009), but it is not known if overexpression of *AFT1* increases cellular iron levels. However, as the constitutively active *aft1-1*^{up} allele results in the induction of the iron regulon and G1 arrest (Philpott *et al.* 1998; Rutherford *et al.* 2003; Shakoury-Elizeh *et al.* 2004), it is likely that overexpression of *AFT1*, which causes G1 arrest (Casas *et al.* 1997), is also inducing the iron regulon and iron influx. Hence, the mutants identified in the *AFT1* SL and SDL screens may be sensitive to cellular iron fluctuations. Alternatively, some of the *AFT1* genetic interactions may reflect novel iron-independent roles for Aft1. In an attempt to differentiate between iron-sensitive and iron-independent roles for Aft1, we undertook secondary chemical and genetic studies.

Numerous studies have systematically screened the yeast deletion mutant arrays for growth sensitivity to iron-limiting conditions (Davis-Kaplan *et al.* 2004; Dudley *et al.* 2005; Lesuisse *et al.* 2005; Jo *et al.* 2009) or iron toxicity (Jo *et al.* 2008). As there is low concordance between these screens, we decided to directly assess the growth of the 77 deletion mutants identified in the *AFT1* SL and SDL genetic network for growth on low (2.5 μM $FeSO_4$) and high (500 μM and 1000 μM $FeSO_4$) iron media (Figure 1 and Table 2 and Table 3). Although we did not identify any mutants that were inhibited by elevated iron levels, 15 deletion mutants were sensitive to decreased iron levels. An additional 10 deletion mutants have been shown to be sensitive to low iron levels in media in other screens (Table 2 and Table 3). As has been previously suggested (Jo *et al.* 2009), the discrepancy between the screens is likely due to media effects. Regardless, between the previously published genome-wide screens and our direct testing, less than one-third of the deletion mutants in the *AFT1* genetic network display sensitivity to iron.

Further, we hypothesized that if an *AFT1* genetic interaction was the result of sensitivity to limited iron availability, the deletion mutant may also have genetic interactions with other mutants of the iron regulon, in particular *AFT2* and *FET3*. To explore this possibility, genome-wide *aft2*Δ and *fet3*Δ SL–SGA screens were performed as described above (Figure 1 and Table 2). As the role of Aft2 in iron response is secondary to Aft1 (Rutherford *et al.* 2003) and as *aft2*Δ cells do not display growth defects under low-iron conditions (Blaiseau *et al.* 2001), we were not surprised to identify only four mutants, *spe2*Δ, *med1*Δ, *mms22*Δ, and *yel007w*Δ, that displayed synthetic sickness with *aft2*Δ. In contrast, as Fet3 is an essential component of the high-affinity iron transport complex and *fet3*Δ mutants are sensitive to iron depletion (Davis-Kaplan *et al.* 2004), we expected to identify numerous mutants implicated in iron homeostasis in the *fet3*Δ SL screen. Ten mutants were identified with synthetic genetic interactions with *fet3*Δ, including deletion of the low-affinity iron transporter *FET4* and the copper transporter *CCS1*, and five of the mutants identified are sensitive to decreases in iron in media (Figure 1 and Table 2). Why did *fet3*Δ mutants interact only with a subset of iron-sensitive mutants that interact with *aft1*Δ mutants? While deletion of *FET3* eliminates the function of the high-affinity iron transport complex, deletion of *AFT1* downregulates not just the high-affinity iron transport from outside the cell, but the mobilization of iron stores in the vacuole or

TABLE 3

***AFT1* synthetic dosage lethal genetic interactions**

ORF	Standard name	Genetic interaction[a]	2.5 μM $FeS0_4$[b]	Cellular function[c]
Iron regulation				
YER145C	*FTR1*	SDS (25)	SG, J, DK	High affinity iron permease
YGL167C	*PMR1*	SDS (16, 25)		High affinity Ca^{2+}/Mn^{2+} P-type ATPase
Rim101 pathway				
YMR063W	*RIM9*	SDS (25, 37)	SG, J	Involved in the proteolytic activation of Rim101
Mitochondrial function				
YAL010C	*MDM10*	SDS (16)		Subunit of the mitochondrial SAM complex
YCL044C	*MGR1*	SDS (25)		Subunit of a mitochondrial protease complex
YDR316W	*OMS1*	SDS (16, 25)		Protein integral to the mitochondrial membrane
YER061C	*CEM1*	SDS (16, 25, 37)		Mitochondrial β-keto-acyl synthase
YHR067W	*HTD2*	SDS (16, 25)		Mitochondrial 3-hydroxyacyl-thioester dehydratase
YHR100C	*GEP4*	SDS (25)		Mitochondrial protein required for respiration
YHR189W	*PTH1*	SDS (16, 25, 37)		Mitochondrial peptidyl t-RNA hydrolases
YKL087C	*CYT2*	SDS (25)		Cytochrome c1 heme lyase
YKL167C	*MRP49*	SDS (25, 37)		Mitochondrial ribosomal protein of the large subunit
YOR221C	*MCT1*	SDS (25)		Mitochondrial fatty acid synthase
YOR334W	*MRS2*	SDS (25)		Mitochondrial inner membrane Mg^{2+} channel
Transcription or chromatin regulation				
YBR289W	*SNF5*	SDS (25)		Component of the SWI/SNF Complex
Protein transport				
YKL041W	*VPS24*	SDS (25)	J, L	Component of the ESCRT-III complex[d]
YLR417W	*VPS36*	SDS (25, 37)	J, L	Component of the ESCRT-II complex[d]
YDR484W	*VPS52*	SDS (25)	J	Component of the GARP complex
YDR495C	*VPS3*	SDS (16, 37)	J	Required for sorting/processing of vacuolar proteins
YOR036W	*PEP12*	SDS (16)	L, J2	Target membrane receptor (t-SNARE)
Unknown function				
YDR493W	*AIM8*	SDS (25)		Protein of known function detected in mitochondria
YGL057C		SDS (25)		Protein of known function detected in mitochondria
YGL226W	*MTC3*	SDS (25)		Protein of known function localized to mitochondria
YHL005C		SDL (25, 37)		Dubious open reading frame, overlaps with *MRP4*
YJR120W		SDS (25)		Protein of known function
YML030W	*AIM31*	SDS (16, 25, 37)		Protein of known function localized to mitochondria
YNL269W	*BSC4*	SDS (25, 37)		Protein of known function
YNR018W	*AIM38*	SDS (25)		Protein of known function detected in mitochondria
Other				
YER052C	*HOM3*	SDS (16, 37)		Aspartate kinase, amino acid synthesis
YFL018C	*LPD1*	SDS (16)	L	Dihydrolipoamide dehydrogenase
Meiosis or sporulation				
YMR139W	*RIM11*	SDS (25)		Protein kinase required for entry into meiosis
YOL052C	*SPE2*	SDS (25)		Biosynthesis of spermidine and spermine

[a] SDS, synthetic dosage sick; SDL, synthetic dosage lethal.

[b] Iron sensitivity identified in additional screens: J, Jo *et al.* (2009); D, Dudley *et al.* (2005); L, Lesuisse *et al.* (2005); DK, Davis-Kaplan *et al.* (2004).

[c] Adapted from Gene Ontology Annotations/Biological Processes listed in *Saccharomyces* Genome Database (http://www.yeastgenome.org).

[d] Role in the RIM101 pathway.

mitochondria and remodeling of cellular pathways to free iron cofactors from enzymes. Hence, the difference in interactions may be reflective of the difference in the cellular iron levels in the mutants. The fact that many of the mutants identified in the *AFT1* network are not sensitive to extracellular free iron concentrations or

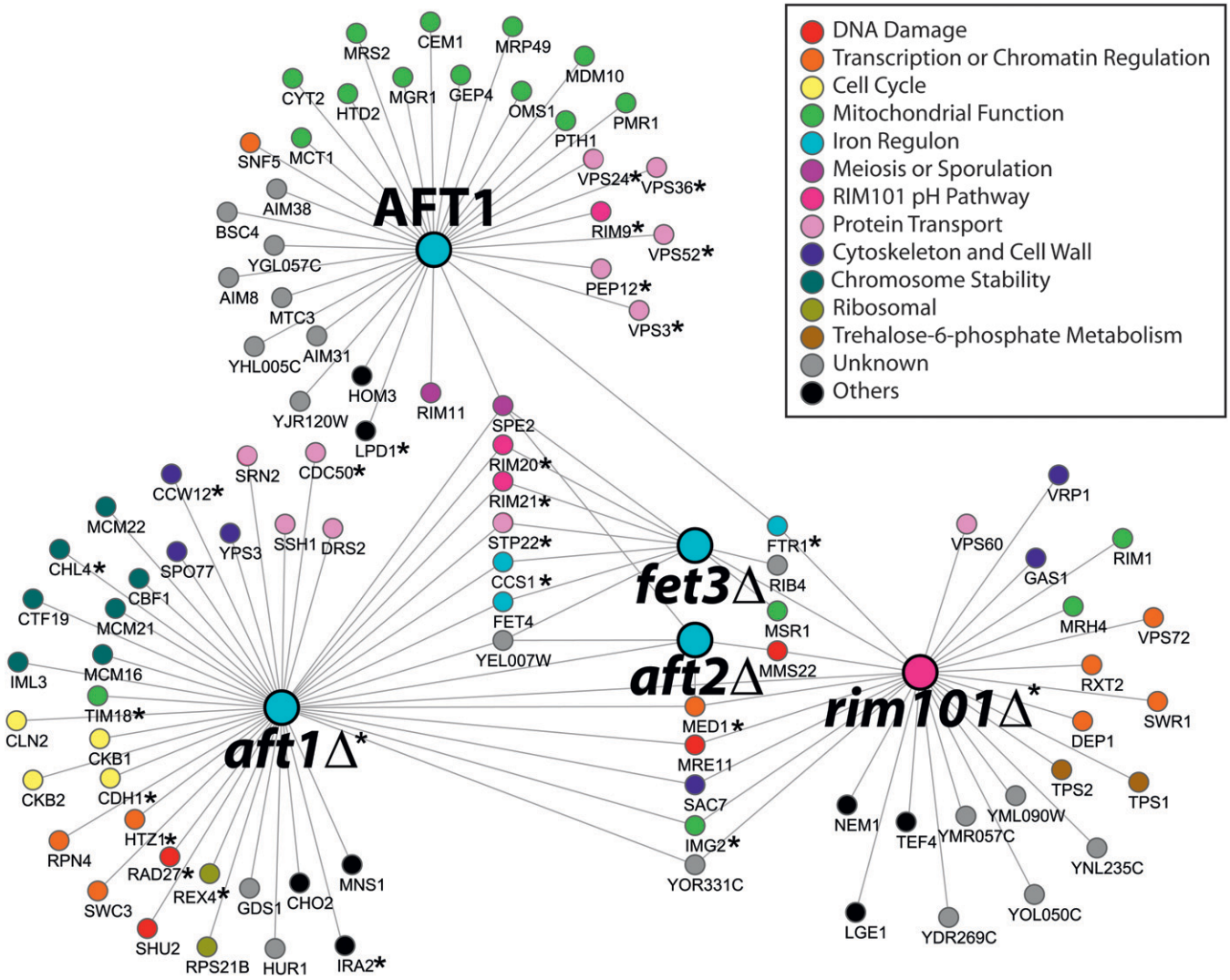

FIGURE 1.—Genetic interaction network of *AFT1*, *AFT2*, *FET3*, and *RIM101*. Genome-wide SL–SGA screens were performed using query strains for *aft1*Δ (YKB676), *aft2*Δ (YKB 1010), *fet3*Δ (YKB 1009), and *rim101*Δ (YKB 1008) and a genome-wide SDL–SGA screen was performed using a query strain containing the galactose inducible pGAL–*AFT1* plasmid (YKB794). Genes are represented by nodes that are color coded according to their SGD cellular roles and/or assigned through review of literature. Interactions are represented by edges. *AFT1* SDL–SGA central node is indicated by *AFT1* whereas SL–SGA central nodes are indicated by Δ. Deletion mutants that are hypersensitive to decreases in iron are indicated by *.

genetically interact with *aft2*Δ or *fet3*Δ suggests that Aft1 may affect numerous processes independently of iron homeostasis.

Aft1 and the *RIM101* pH response pathway function in parallel: A striking feature of the *AFT1* genetic network is the identification of numerous genes with established roles in the *RIM101* pH response pathway (reviewed in PENALVA *et al.* 2008). The *RIM101* pH pathway plays a role in the transcriptional response to alkaline pH (LAMB *et al.* 2001), as well as cell-wall assembly (CASTREJON *et al.* 2006), sporulation (SU and MITCHELL 1993; LI and MITCHELL 1997), and ion homeostasis (LAMB *et al.* 2001). The zinc-finger transcription factor Rim101 is a repressor whose primary targets are two transcriptional repressor genes, *SMP1* and *NRG1* (LAMB and MITCHELL 2003). Hence, Rim101 acts as both a repressor of transcription (through direct binding of promoters) and an activator of transcription (indirectly through the inactivation of repressors). In its full-length form, Rim101 is inactive and requires the proteolytic cleavage of the C-terminal region to become an active repressor. The cleavage of Rim101 is tightly regulated by a variety of processing gene products including the putative transmembrane proteins Rim21 and Rim9, the arrestin-like protein Rim8, the protease Rim13, and the protease scaffold protein Rim20 (reviewed in PENALVA *et al.* 2008). The pH signal transduction and activation of Rim101 also requires ESCRT (endosomal sorting complex required for transport) complexes I, II, and the Snf7–Vps20 subcomplex of ESCRT-III (reviewed in PENALVA *et al.* 2008). In addition to *RIM101*, the *AFT1* genetic network identified *RIM9*,

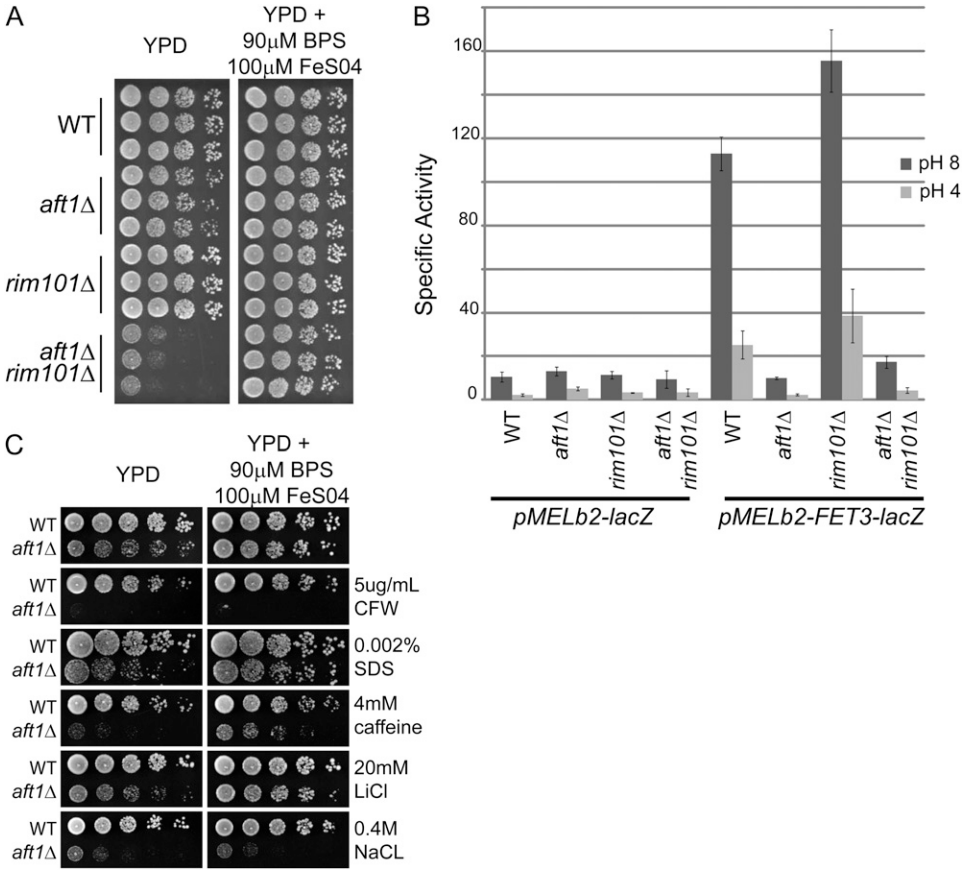

FIGURE 2.—Aft1 and Rim101 function in parallel in alkaline response and Aft1 has a role cell-wall stability and ion homeostasis. (A) Exogenous iron suppresses the synthetic sickness of *aft1*Δ*rim101*Δ. Wild-type (WT) (YPH499), *aft1*Δ (YPH1735), *rim101*Δ (YKB1110), and *aft1*Δ*rim101*Δ (YKB1111) cells were plated in fivefold serial dilution onto YPD or YPD supplemented with exogenous iron (YPD +90 μM BPS, 100 μM $FeS0_4$) as indicated. The plates were incubated for 3 days at 25°. (B) Alkaline induction of *FET3*–lacZ reporter is dependent on Aft1 and independent of Rim101. WT (YPH499), *aft1*Δ (YPH1735), *rim101*Δ (YKB1110), and *aft1*Δ*rim101*Δ (YKB1111) cells were transformed with either the vector control (pMELb2) or *FET3*–lacZ construct (pMELb2–*FET3*–lacZ). The transformed cells were grown in SD–uracil to mid-log phase and then grown for at least two doublings in SD–uracil pH 4 or 8 and the specific activity of β-galactosidase (Miller units) was measured. Data are the mean of three independent transformants and the error bar is 1 standard deviation. (C) Aft1 has a role in cell-wall stability and ion homeostasis. WT (YPH499), *aft1*Δ (YPH1735) cells were plated in fivefold serial dilution onto YPD or YPD supplemented with exogenous iron (YPD + 90 μM BPS, 100 μM $FeS0_4$) that was supplemented with calcofluor white (CFW), SDS, caffeine, LiCl, and NaCl as indicated. The plates were incubated for 2 days at 30°.

RIM20, *RIM21*, along with ESCRT components *SRN2*, *STP22*, *VPS24*, and *VPS36*. Most of the *RIM101* pH response pathway mutants identified in the *AFT1* screen are sensitive to decreased extracellular iron levels and *fet3*Δ also displayed genetic interactions with deletion mutants of *RIM101*, *RIM20*, *RIM21*, and *STP22*. This suggests that the interaction between these two transcriptional pathways is due to decreased cellular iron levels of *aft1*Δ cells. Indeed, this is the case as we observed that exogenous iron can suppress the slow-growth defects of *aft1*Δ*rim101*Δ cells (Figure 2A).

The strong genetic interactions between *aft1*Δ and the *RIM101* pathway mutants suggest these two transcriptional pathways are functioning in parallel to regulate similar biological processes. Like *RIM101* pathway mutants, *aft1*Δ cells are also sensitive to alkaline pH (SERRANO *et al.* 2004) and have sporulation defects (GIL *et al.* 1991). Furthermore, microarray studies have shown that upon alkaline pH treatment, the expression levels of iron regulon genes are induced (LAMB *et al.* 2001) presumably to compensate for decreases in iron availability in alkaline conditions (SERRANO *et al.* 2004). However, while the alkaline induction of some iron regulon genes, like *TIS11*, appears independent of Rim101, others like *ARN4* are dependent on Rim101 (LAMB *et al.* 2001; BARWELL *et al.* 2005). Hence it is unclear if Rim101 and Aft1 are functioning in parallel or within a single pathway during alkaline response. Nor is it known if the *RIM101* pH pathway also plays a role in any other cellular processes that are also affected by Aft1.

To explore these questions, a genome-wide *rim101*Δ SL–SGA screen was performed (Figure 1 and Table 4). The resulting confirmed data set contains 26 genetic interactions of which 22% (6/26) were synthetic lethal. *aft1*Δ and *rim101*Δ only share five common synthetic genetic interactions with *IMG2*, *MED1*, *SAC7*, *YOR331C*, and *MRE11* (Figure 1). However, both screens identified genes implicated in iron regulation, cell-wall assembly, and sporulation, further providing credence that both Aft1 and Rim101 participate in these cellular functions. The other cellular roles of Aft1, such as chromosome stability and cell-cycle regulation, were not identified in the *rim101*Δ SL–SGA screen suggesting that the participation of Aft1 in these processes is not shared between the pathways. Likewise, the *rim101*Δ network identified pathways not identified in the *aft1*Δ network, such as

TABLE 4

***RIM101* synthetic lethal interactions**

ORF	Standard name	Genetic interaction[a]	Cellular function[b]
Transcription or chromatin			
YAL013W	*DEP1*	SS	Component of Rpd3 histone deacetylase complex
YBR095C	*RXT2*	SS	Component of Rpd3 histone deacetylase complex
YDR334W	*SWR1*	SS	Component of SWR1 complex
YDR485C	*VPS72*	SS	Component of SWR1 complex
YPR070W	*MED1*	SS	Subunit of RNA polymerase II mediator complex
Mitochondrial function			
YCR028C-A	*RIM1*	SS	Protein required for mitochondrial genome maintence
YCR071C	*IMG2*	SL	Mitochondrial ribosomal protein of the small subunit
YGL064C	*MRH4*	SL	Mitochondrial RNA helicase
Cytoskeleton or cell-wall function			
YDR389W	*SAC7*	SS	GTPase activating protein for Rho1p
YLR337C	*VRP1*	SS	Actin-associated protein involved in cytoskeletal organization
YMR307W	*GAS1*	SS	β-1,3-Glucanosyltransferase, required for cell-wall assembly
Iron metabolism			
YER145C	*FTR1*	SS	High affinity iron permease
YGL071W	*AFT1*	SS	Iron responsive transcription factor
YMR058W	*FET3*	SS	Ferro-O_2-oxidoreductase required for high-affinity iron uptake
YDR269C		SS	Dubious open reading frame, overlaps with *CCC2*
DNA damage			
YLR320W	*MMS22*	SS	Acts with Mms1 to repair DNA damage caused by blocked replication forks.
YMR224C	*MRE11*	SS	Subunit of the MRX complex DNA double-strand break repair
			Trehalose-6-phosphate metabolism
YBR126C	*TPS1*	SL	Synthase subunit of trehalose-6-phosphate synthatase/phosphatase complex
YDR074W	*TPS2*	SS	Phosphatase subunit of the trehalose-6-phoshate synthase/phosphatase complex
Others			
YPL055C	*LGE1*	SS	Null mutant forms abnormally large cells
YDR486C	*VPS60*	SS	Membrane protein involved in late endosome to vacuole transport
YHR004C	*NEM1*	SS	Phosphatase implicated in phospholipid biosynthesis and nuclear growth.
YKL081W	*TEF4*	SS	Translation elongation factor EF-1 gamma
Unknown			
YML090W		SS	Dubious open reading frame
YNL235C		SL	Dubious open reading frame, overlaps with *SIN4* mediator
YOL050C		SL	Dubious open reading frame, overlaps with *GAL11* mediator
YOR331C		SL	DUBIOUS open reading frame, overlaps with *VMA4*

[a] SS, synthetic sick; SL, synthetic lethal.

[b] Adapted from Gene Ontology Annotations/Biological processes listed in *Saccharomyces* Genome Database (http://www.yeastgenome.org).

trehalose-6-phosphate synthase (*TPS1* and *TPS2*). The lack of overlap between the screens suggests that although these transcriptional pathways affect similar cellular functions they are doing so in parallel.

To further confirm that Aft1 and the *RIM101* pH pathway are functioning independently during the alkaline response, β-galactosidase assays were performed using a *FET3* promoter–lacZ fusion (Kimura *et al.* 2007) in wild-type, *aft1*Δ, *rim101*Δ, and *aft1*Δ*rim101*Δ cells after growth at pH 4 and pH 8 (Figure 2B). While the lacZ fusion vector with no insert was not active at either pH, as expected the *FET3*–*lacZ* fusion expression was induced upon alkaline treatment and this induction was dependent on Aft1. Deletion of *RIM101* did not suppress induction of the *FET3*–*lacZ*; rather the *FET3*–*lacZ* alkaline induction was increased by 50% in the absence of *RIM101*. Similar phenomena have been reported for *ARN1* (Lamb and Mitchell 2003; Barwell *et al.* 2005) and *FRE1* (Lamb *et al.* 2001), which suggest that Rim101 may be a negative regulator of some iron regulon genes.

The *RIM101* pH pathway also contributes to cell-wall assembly (Castrejon *et al.* 2006) and ion homeostasis (Lamb *et al.* 2001); hence we were curious to determine if Aft1 plays a role in these processes too. We asked if *aft1*Δ cells are hypersensitive to cell-wall-damaging agents calcoflour white (CFW), which interferes with cell-wall assembly by binding to chitin, sodium-dodecyl sulfate (SDS), which interferes with membrane synthesis, and caffeine, which activates a map kinase cascade altering cell-wall assembly (Figure 2C). Although *aft1*Δ cells are only mildly sensitive to 0.002% SDS, they are hypersensitive to 4 mm caffeine and 5 μg/ml CFW

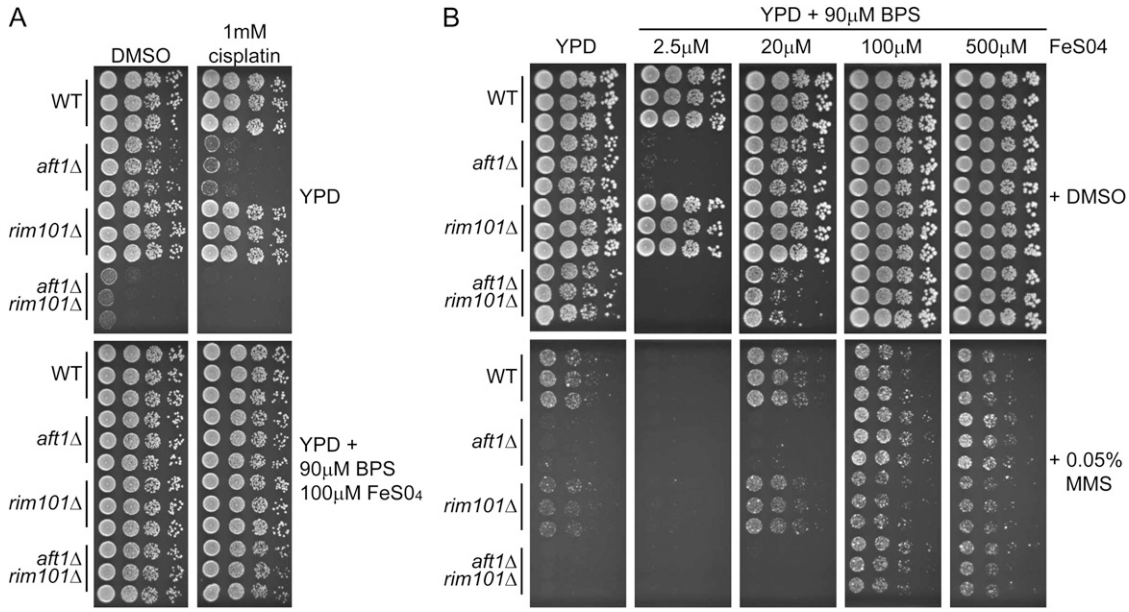

FIGURE 3.—Exogenous iron buffers the effects of MMS and cisplatin. (A) Exogenous iron suppresses the hypersensitivity of *aft1*Δ mutants to cisplatin treatment. WT (YPH499), *aft1*Δ (YPH1735), *rim101*Δ (YKB1110), and *aft1*Δ*rim101*Δ (YKB1111) cells were 10-fold serially diluted onto YPD or YPD supplemented with exogenous iron (YPD +90 μM BPS, 100 μM $FeS0_4$) that contained DMSO (carrier control) or cisplatin as indicated. The plates were incubated for 2 days at 25°. (B) Exogenous iron levels modulate the cellular effects of MMS. The strains indicated above were 5-fold serially diluted onto YPD plates containing DMSO or YPD plates containing 0.05% MMS with varying levels of iron as indicated. The plates were incubated for 4 days at 25°.

treatment indicating that Aft1 contributes to cell-wall maintenance. We also asked if, like *rim101*Δ cells, *aft1*Δ cells display growth defects in the presence of cations NaCl and LiCl. While *aft1*Δ cells are only mildly sensitive to LiCl, they are hypersensitive to NaCl, which suggests that Aft1 also plays a role in ion homeostasis. Exogenous iron suppressed the sensitivity of *aft1*Δ cells to caffeine, SDS, and LiCl treatment, suggesting that the role of Aft1 in response to these challenges is mediated through iron availability in the cell. In contrast, exogenous iron cannot suppress *aft1*Δ cells sensitive to CFW or NaCl, which suggests that the role of Aft1 in response to these environmental challenges is mediated through an iron-independent mechanism.

Iron, Aft1 and DNA Repair: The genetic map links both Aft1 and the *RIM101* pH pathway to DNA damage repair and many lines of evidence have already linked Aft1 to this cellular process. A chemical genomics study found that *aft1*Δ mutant cells are hypersensitive to interstrand cross-linking DNA damaging agents such as carboplatin and cisplatin (LEE *et al.* 2005), and cisplatin treatment has been shown to induce the iron regulon through activation of Aft1 (KIMURA *et al.* 2007). However, other members of the iron regulon have not been identified as being hypersensitive to DNA-damaging agents (BENNETT *et al.* 2001; CHANG *et al.* 2002; LEE *et al.* 2005). Nor have mutants of the *RIM101* pH pathway been identified as being sensitive to DNA damage in systematic chemical genomic screens. Therefore we were interested in further exploring the connection between Aft1, Rim101, and DNA damage. While *aft1*Δ cells hypersensitivity to cisplatin could be suppressed by exogenous iron, *rim101*Δ cells are not sensitive to cisplatin treatment (Figure 3A). Despite the fact that *aft1*Δ, *rim101*Δ, and other mutants in these pathways have not been identified as being hypersensitive to MMS in genome-wide screens (BENNETT *et al.* 2001; CHANG *et al.* 2002; LEE *et al.* 2005), we decided to test their sensitivities directly. We determined that neither *aft1*Δ nor *rim101*Δ cells had significant hypersensitivity to 0.005, 0.02, or 0.035% MMS treatment (data not shown). However, although dramatic growth inhibition occurs for all strains tested at 0.05% MMS treatment, *aft1*Δ and *aft1*Δ*rim101*Δ cells displayed increased sensitivity compared to wild-type cells (Figure 3B). Although subtle, *rim101*Δ cells were slightly more sensitive than wild-type cells to 0.05% MMS treatment. We were surprised to find that MMS effects even on wild-type cells were exacerbated upon depletion of iron (2.5 μM $FeS0_4$) and rescued upon increasing iron levels, with *aft1*Δ cells requiring higher levels of iron for rescue. This strongly suggests that iron has protective effects against DNA damage, likely through its role as a cofactor in a variety of DNA repair proteins (reviewed in LILL and MUHLENHOFF 2008).

The role of Aft1 in chromosome stability is iron independent: Aft1 is required for faithful chromosome transmission under normal iron media conditions (MEASDAY *et al.* 2005; YUEN *et al.* 2007). As many mutants with defects in chromosome stability also have increased sensitivity to the microtubule-destabilizing drug beno-

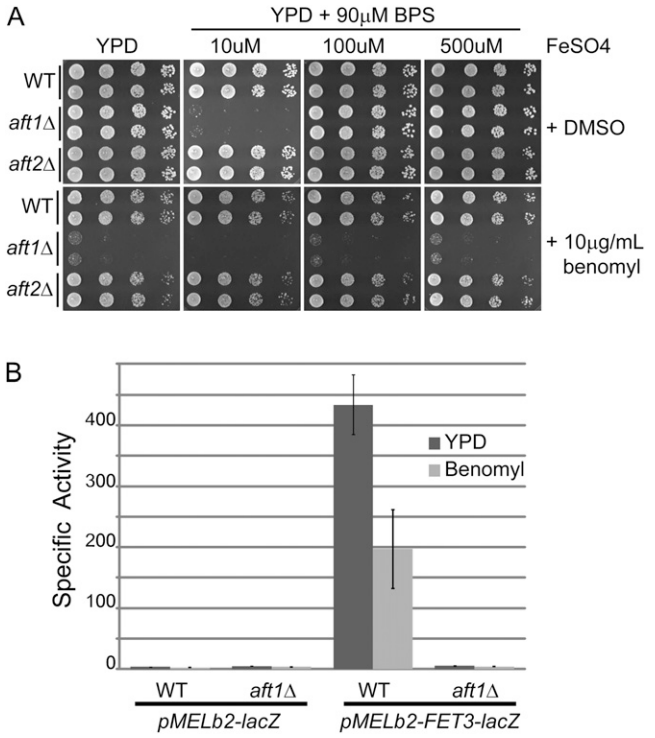

FIGURE 4.—The benomyl hypersensitivity of *aft1*Δ cells is not due to defects in iron homeostasis. (A) *aft1*Δ cells' hypersensitivity to benomyl is not suppressed by exogenous iron. Wild-type (WT, YPH499), *aft1*Δ (YPH1735), and *aft2*Δ (YKB788) cells were fivefold serially diluted onto YPD plates containing either DMSO or 10 μg/ml benomyl and supplemented with varying levels of iron ($FeS0_4$) as indicated. The plates were incubated for 2 days at 30°. (B) Benomyl treatment does not induce a *FET3*–lacZ reporter. Wild-type (WT, YPH499), and *aft1*Δ (YPH1735) cells were transformed with either the vector control (*pMELb2*) or *FET3*–lacZ construct (*pMELb2–FET3–lacZ*). The transformed cells were grown to SD–uracil to mid-log phase and collected (untreated) or treated with 20 μg/ml benomyl for 1 hr and the specific activity of β-galactosidase (Miller units) was measured. Data are the mean of three independent transformants and the error bar is 1 standard deviation.

myl (SORA *et al.* 1982), we asked whether *aft1*Δ mutants are also hypersensitive to this compound. As expected, *aft1*Δ mutant cells are hypersensitive to benomyl treatment compared to wild-type cells (Figure 4A). A deletion mutant of *AFT2* does not display chromosome segregation defects (data not shown and MEASDAY *et al.* 2005; YUEN *et al.* 2007) and as expected *aft2*Δ cells are not hypersensitive to benomyl. Unlike the hypersensitivity of *aft1*Δ cells to HU (DUBACQ *et al.* 2006), SDS, caffeine, LiCl (Figure 2C), cisplatin, and MMS (Figure 3), which are suppressed by exogenous iron, the hypersensitivity of *aft1*Δ cells to benomyl cannot be rescued by increasing levels of exogenous iron in the growth medium (Figure 4A). Further, using a *FET3*–lacZ reporter assay we determined that unlike iron-restricted conditions or alkaline pH, benomyl treatment does not induce *FET3*–lacZ; rather we see reduction of reporter activity upon benomyl treatment (Figure 4B). This is in agreement with microarray studies that have not detected changes in expression of iron regulon genes upon benomyl treatment (LUCAU-DANILA *et al.* 2005). Our work indicates that *aft1*Δ hypersensitivity to benomyl is not the result of defects in the induction of iron regulon genes and cellular iron levels.

If the role of Aft1 in faithful chromosome segregation is mediated by cellular iron levels, one would predict that exogenous iron could suppress chromosome loss defects in *aft1*Δ cells. To test this hypothesis, we performed a series of chromosome transmission fidelity (CTF) assays (KOSHLAND and HIETER 1987) in which wild-type, *aft1*Δ, and *ctf13-30* cells, an inner kinetochore mutant with extremely high rates of chromosome segregation defects (DOHENY *et al.* 1993), were plated onto YPD media or YPD + 90 μM BPS + 100 μM $FeS0_4$ (Table 5). As the CTF assay is measuring chromosome loss in the first cell division, cells plated from YPD onto YPD + 90 μM BPS + 100 μM $FeS0_4$ may not have enough time to readjust intracellular iron levels prior to the first cell division to impact CTF. Therefore, cells were first cultured on media containing exogenous iron (see MATERIAL AND METHODS). *aft1*Δ cells have a chromosome missegregation rate ninefold greater than that of wild-type cells, but less than that of the essential kinetochore mutant *ctf13-30*. Further, the addition of exogenous iron did not affect chromosome loss rates of the wild-type cells and could not suppress CTF defects of *aft1*Δ or *ctf13-30* cells. Together these assays suggest that the role of Aft1 in chromosome stability is distinct from its role in transcriptional regulation of the iron regulon and iron homeostasis.

Microarray experiments suggest the role of Aft1 in chromosome stability and benomyl response is transcription independent: Numerous microarray studies in iron replete media have indicated that the impact of Aft1 on transcription is not limited to iron regulon genes (SHAKOURY-ELIZEH *et al.* 2004; PAGANI *et al.* 2007). Hence, we hypothesized that Aft1 could be regulating the transcription of a non-iron regulon gene required for resistance to benomyl and chromosome stability. We first asked if mutants corresponding to any genes that have been reported to be downregulated in *aft1*Δ cells (PAGANI *et al.* 2007) or upregulated in *aft1-1^{up}* mutants cells (SHAKOURY-ELIZEH *et al.* 2004) have been identified as being both hypersensitive to benomyl (as listed on SGD) and displaying CTF defects in genome-wide screens (YUEN *et al.* 2007). Neither screen identified genes whose mutants are both benomyl sensitive and display CTF defects (Table 6).

To further explore the possibility that Aft1 is regulating the transcription of key genes required for benomyl resistance and chromosome stability, we performed a series of microarray experiments to compare the transcriptional response of *aft1*Δ cells *vs.* wild-type cells grown in YPD media. As it is possible that the hypersensitivity of *aft1*Δ cells to benomyl is due to a role of Aft1 in

TABLE 5

Rates of chromosome loss events

Strain	Genotype	Plated on:	Rate of half-sector formation	Total colonies
YKB793	+/+	YPD	1.67×10^{-4} (1)	23,940
YKB671	*aft1*Δ/*aft1*Δ	YPD	1.55×10^{-3} (9.3)	19,325
YKB1	*ctf13-30/ctf13-30*	YPD	1.64×10^{-2} (98.1)	5,244
YKB793[a]	+/+	YPD + 90 μM BPS + 100 uM $FeSO_4$	1.83×10^{-4} (1)	32,868
YKB671[a]	*aft1*Δ/*aft1*Δ	YPD + 90 μM BPS + 100 uM $FeSO_4$	1.48×10^{-3} (8.1)	32,516
YKB1[a]	*ctf13-30/ctf13-30*	YPD + 90 μM BPS + 100 μM $FeSO_4$	1.61×10^{-2} (88.3)	8,490

Numbers in parentheses are fold increase in rates of chromosome missegregation events above wild-type rates for each treatment.

[a] Strains were pretreated on YPD + 90 μM BPS + 100 μM $FeSO_4$

the transcriptional response to benomyl (Lucau-Danila *et al.* 2005), genome-wide expression profiles were also compared after treatment with 20 μg/ml benomyl for 20 min. The vast majority of benomyl-dependent transcriptional remodeling is detected using these conditions (Lucau-Danila *et al.* 2005). Similar to other groups, we found that deletion of *aft1*Δ results in a global transcriptional remodeling under YPD culture conditions (File S1). Of the genes we identified whose transcription is decreased twofold or more (*P*-value < 0.05) only deletion mutants of *CTF19* have been demonstrated both to be hypersensitive to benomyl and to display chromosome transmission defects (Hyland *et al.* 1999). *CTF19* encodes a component of the central kinetochore complex COMA (reviewed in Westermann *et al.* 2007). Intriguingly, genome-wide ChIP studies had reported an enrichment of Aft1 to the intergenic region of 5′ of *CTF19* (Harbison *et al.* 2004). We were interested in determining if *aft1*Δ cells hypersensitivity to benomyl could be explained by decreases in *CTF19* gene levels. However, neither an extra genomic clone of *CTF19* (pKH5) or an HA-tagged *CTF19* fusion clone (pKH32) (Hyland *et al.* 1999) could suppress the benomyl sensitivity of *aft1*Δ cells (Figure 5A). Further, although we could detect the localization of Aft1–TAP to the promoter of the iron regulon gene *FET3* by ChIP, we could not detect it on the promoter of *CTF19* (Figure 5B). Together this suggests that Aft1 is likely not regulating the transcription of *CTF19* directly, nor is the decreased levels of *CTF19* in *aft1*Δ cells likely the reason for *aft1*Δ benomyl sensitivity. Although this candidate approach is limited by both the microarrays and genome-wide data sets available for benomyl sensitivity and CTF, especially for essential genes, it suggests that under YPD conditions Aft1 may not be affecting chromosome stability through transcription.

Similar to previous studies, benomyl treatment dramatically affects the transcription profile of wild-type cells, resulting in the twofold induction of 351 genes and the twofold repression of 495 genes (wild type [WT] + BEN/WT; *P*-value < 0.05; see File S1). Similarly, benomyl treatment of *aft1*Δ cells resulted in the induction of 421 genes and repression of 1094 genes (*aft1*Δ + BEN/*aft1*Δ, *P*-value < 0.05). In agreement with our reporter assay (Figure 4B) and previous expression profiles (Lucau-Danila *et al.* 2005), our analysis confirms that benomyl treatment does not induce the transcription of the iron regulon. Despite the dramatic transcriptional remodeling upon benomyl treatment that occurs in these two strains, the transcriptional differences between *aft1*Δ and wild-type cells treated with benomyl (*aft1*Δ + BEN/WT + BEN, *P*-value < 0.05) are mild. Deletion of *AFT1* only negatively impacted the transcription of 35 genes and positively impacted the transcription of 90 genes compared to wild type upon benomyl treatment (minimum twofold change, *P*-value < 0.05). If Aft1 were playing a transcriptional role in the benomyl response, one would predict that a subgroup of the 351

TABLE 6

Aft1-regulated genes that may have a role in benomyl response or CTF

Microarray expression study[a]	Benomyl sensitive[b]	CTF[c]
AFT1-1^{UP} *vs.* wild type (SE) (210 genes > 2× increase)	*KAP123* (D)	*SSZ1*, *YLR235c*, *CDC73*
*aft1*Δ *vs.* wild type (P) (220 genes > 2× decrease)	*KAP123* (D), *TMA19* (R)	*SSZ1*
*aft1*Δ *vs.* wild type, this study(76 genes > 2× decrease)	*PAC10* (L), *CTF19* (H), *RVS167*(D)	*CTF19*

[a] Genome-wide expression profiles reported in SE (Shakoury-Elizeh *et al.* 2004) and P (Pagani *et al.* 2007).

[b] Benomyl sensitivity identified in additional screens: D, Dudley *et al.* (2005); R, Rinnerthaler *et al.* (2006); L, Lacefield and Solomon (2003); H, Hyland *et al.* (1999).

[c] CTF defects reported in Yuen *et al.* (2007)

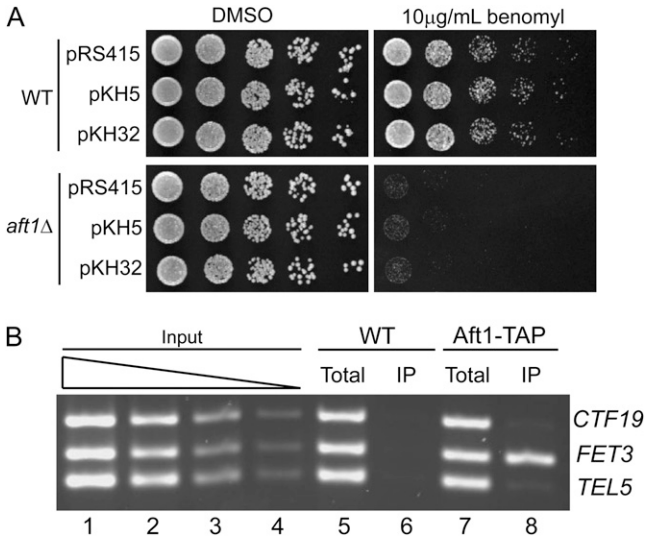

FIGURE 5.—*CTF19* does not rescue the benomyl sensitivity of *aft1*Δ cells. (A) Wild-type (WT, YPH499) and *aft1*Δ (YKB1095) cells transformed with pRS315 (vector control), pKH5 (genomic fragment containing *CTF19*), or pKH32 (HA-tagged *CTF19* fusion clone) were fivefold serially diluted onto YPD plates containing either DMSO or 10 μg/ml benomyl. The plates were incubated for 2 days at 30°. (B) Aft1 does not localize to the promoter of *CTF19*. Modified ChIP was performed using untagged (WT; YPH499) and Aft1–TAP (YKB479) strains. Total or immunoprecipitated (IP) DNA was subjected to multiplex PCR amplification using primers specific to the promoter region of *CTF19*, *FET3*, and a subtelomeric region of chromosome V (TEL-V). The result of this ChIP was representative of three experiments.

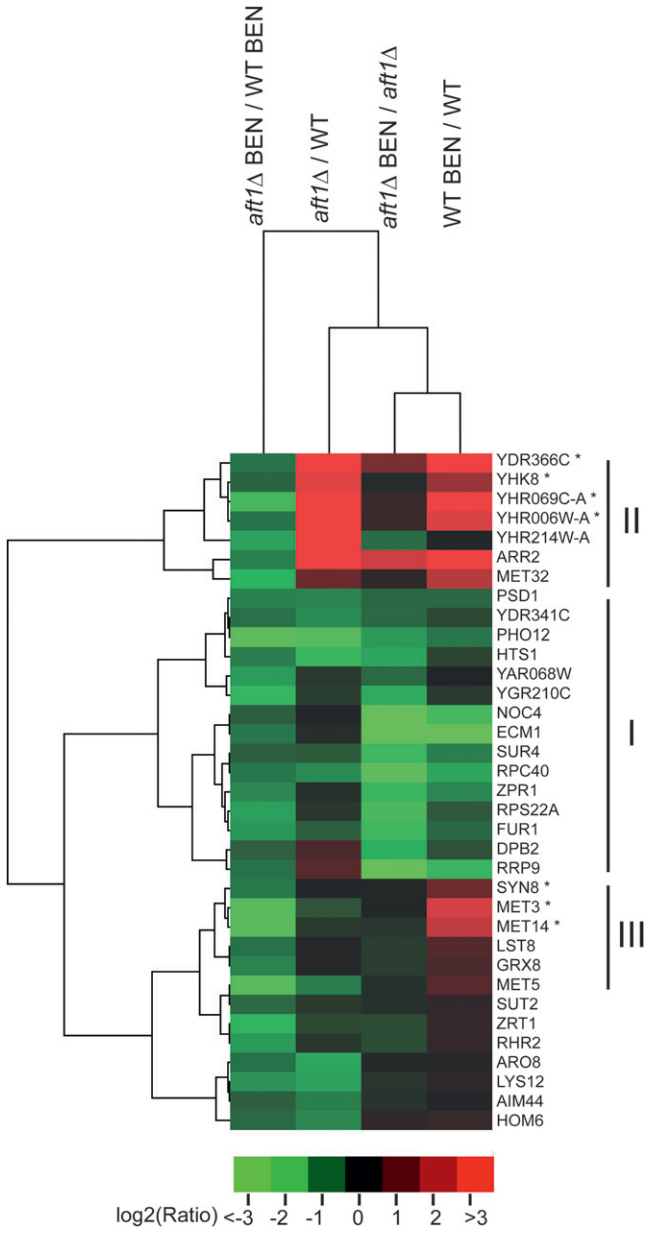

FIGURE 6.—The microarray profiles of the 35 genes whose expression in benomyl is reduced twofold or more (*P*-value < 0.05) in *aft1*Δ cells compared to wild-type cells (*aft1*Δ BEN/WT BEN). The 2D hierarchical cluster analysis of the expression profiles of the 35 genes was performed. Expression data are represented on a log2 scale, with inductions marked with red and repression marked with green. For *aft1*Δ/WT, *aft1*Δ BEN/*aft1*Δ, and WT BEN/WT expression analysis includes expression data with fold-changes less than twofold and/or *P*-values > 0.05. Genes whose transcript was significantly induced twofold or greater in wild-type cells upon benomyl treatment (WT BEN/WT) are marked with an asterisk (*). Gene groups I, II, and III are discussed in the text.

genes whose expression is induced upon benomyl treatment in wild-type cells would no longer be induced in *aft1*Δ cells. However, of the 35 genes whose expression upon benomyl treatment is significantly reduced in *aft1*Δ cells compared to wild-type cells, the majority were already downregulated in *aft1*Δ cells in YPD or the transcription of these genes is decreased in both wild-type and *aft1*Δ cells upon benomyl treatment, with the effect greater in the mutant (group I, Figure 6). Of the nine genes with an observed induction of twofold or greater in wild-type cells upon benomyl treatment (*P*-value > 0.05), the expression of six of these genes is also upregulated in *aft1*Δ cells, but to a lesser extent than in wild-type cells (group II, Figure 6). Only the benomyl induction of three genes, *SYN8*, *MET3*, and *MET14*, appears to be dependent on Aft1. If we extend this analysis to genes that are induced 1.8-fold or more, an additional three genes, *LST8*, *GRX8*, and *MET5*, can be added to the group of genes whose benomyl induction is dependent on Aft1 (group III, Figure 6). Interestingly, *MET3*, *MET5*, *MET14*, and *GRX8* are genes of the newly defined 45 gene Met4 regulon (LEE *et al.* 2010). Met4 is a transcriptional activator that in conjunction with its DNA-binding cofactors Met31/32 or Cbf1 tightly regulates the transcription of the sulfur metabolic network or Met4 regulon in yeast. This suggests that the Met4 regulon is induced upon benomyl treatment in an Aft1-dependent manner. Is it possible that the chromosome stability defects and benomyl sensitivity of *aft1*Δ cells could be due to defects in the sulfur metabolic network? To our knowledge no mutants of Met4 regulon

genes, except for Cbf1 (see DISCUSSION), display chromosome transmission fidelity defects or are hypersensitive to benomyl. Nor have compounds synthesized by the sulfur biosynthetic network, such as methionine and cysteine, *S*-adenosylmethionine, or glutathione been implicated directly in chromosome stability in yeast. Our study suggests that although Aft1 may have a role in the benomyl-induction of the Met4 regulon, the overall contribution of Aft1 to the benomyl transcriptional response is minimal. Taken together, our study argues for a novel nontranscriptional role of Aft1 in chromosome stability.

DISCUSSION

Genome-wide genetic screens identify diverse cellular roles for Aft1: In an effort to further define the cellular functions of Aft1, SGA methodology was used to perform complementary genome-wide SL and SDL screens (Tables 2 and Tables 3). As expected, the genetic interaction map identified genes encoding proteins implicated in processes previously linked to Aft1 including iron regulation (reviewed in RUTHERFORD and BIRD 2004), chromosome stability (MEASDAY *et al.* 2005), cell-cycle progression (PHILPOTT *et al.* 1998; JORGENSEN *et al.* 2002; WHITE *et al.* 2009), and DNA damage repair (LEE *et al.* 2005; KIMURA *et al.* 2007). Further, the *AFT1* genetic interaction map also predicts possible functional roles for Aft1 in cell-wall assembly, protein transport, and the mitochondria.

The diverse range of cellular functions suggests two possibilities. One is that Aft1 is not directly affecting these pathways per say; rather the deletion mutants identified cannot tolerate fluctuations in cellular iron content and likely encode proteins that work in parallel with pathways that functionally require iron cofactors. As a third of the mutants identified in the *AFT1* genetic network are sensitive to decreased levels of iron (Figure 1 and Tables 2 and Tables 3), this is likely an explanation for a subset of the interactions. Furthermore, it suggests that *aft1*Δ cells have decreased intracellular iron levels even when cultured under iron-replete conditions, which is in agreement with a recent study that showed that *aft1*Δ mutants have twofold decreases in cellular iron content (VEATCH *et al.* 2009). Additionally, many of the chemical sensitivities displayed by *aft1*Δ mutants, such as to HU (DUBACQ *et al.* 2006), SDS, caffeine (Figure 2), cisplatin, and MMS (Figure 3), can be suppressed by exogenous iron. This suggests that enzymes that are central to DNA replication, DNA damage response, and some cell-wall challenges require iron cofactors to function and the decreased cellular iron levels of *aft1*Δ cells compromise the function of these pathways. This is certainly the case for the DNA damage response where numerous proteins, like Rad3, a DNA helicase involved in nucleotide excision repair (RUDOLF *et al.* 2006), and Pri2, a subunit of DNA primase involved in both DNA replication and double-strand-break repair (KLINGE *et al.* 2007), require ISCs to function. Although the scope of enzymes that require iron cofactors has not been systematically assessed, it is clear from our and other genome-wide screens that iron is an essential cofactor for a myriad of diverse cellular processes (DAVIS-KAPLAN *et al.* 2004; DUDLEY *et al.* 2005; LESUISSE *et al.* 2005; JO *et al.* 2008; JO *et al.* 2009). As mutant sensitivity to iron fluctuations may result in subtle or distinct phenotypes that we or others have not detected, it is likely that we are underestimating the number of iron-sensitive mutants in the *AFT1* network. Nonetheless, the majority of the deletion mutants in the *AFT1* network do not have detectable sensitivities to either decreases or increases in iron in the media, or display genetic interactions with either *fet3*Δ or *aft2*Δ. Hence, our genetic network suggests that Aft1 has cellular roles that are independent from its role in inducing the iron regulon and regulating cellular iron levels.

Aft1 and the *RIM101* pH pathway—connected by iron: One of the most striking features of our genetic screens is the links between *AFT1* and the *RIM101* pH pathway. Most (7/8) of the mutants implicated in the *RIM101* pH pathway that were identified in the *AFT1* genetic network cannot tolerate low levels of iron and many also genetically interacted with the *fet3*Δ mutant (Figure 1 and Table 2 and Table 3). In addition, we show that the synthetic sick interaction displayed by *aft1*Δ*rim101*Δ cells can be suppressed by exogenous iron (Figure 2A and 3), which suggests that the *RIM101* pH response pathway is regulating cellular processes that may work in parallel with iron-dependent pathways. Indeed both *rim101*Δ and *aft1*Δ mutants display similar defects and sensitivities. The *RIM101* pH pathway is known to be involved in numerous cellular processes: alkaline pH response (HAYASHI *et al.* 2005), sporulation (SU and MITCHELL 1993; LI and MITCHELL 1997), ion homeostasis (LAMB *et al.* 2001), and cell-wall assembly (CASTREJON *et al.* 2006). Similarly, Aft1 has been implicated in alkaline pH response (SERRANO *et al.* 2004), sporulation (GIL *et al.* 1991), cell wall (Figure 2C), and ion homeostasis (Figure 2C). Interestingly, while the *aft1*Δ cells' sensitivity to SDS, caffeine, and LiCl can be suppressed by exogenous iron, suggesting that iron is a cofactor for key proteins required for resistance to these treatments, exogenous iron cannot suppress the sensitivity of *aft1*Δ cells to CFW or NaCl. This suggests that Aft1 potentially buffers the effects of these compounds by a novel iron-independent mechanism. These results demonstrate that Aft1 plays a functional role in three cellular processes also regulated by Rim101 and suggests that these two transcriptional pathways work in parallel to govern similar cellular functions.

However, the interplay between these transcriptional cascades is likely more complicated. Reporter assays, Northern blot analysis, and microarray gene expression

studies have shown that both the expression in standard YPD and the alkaline induction of the iron regulon gene *ARN4* is dependent on Rim101 (Lamb *et al.* 2001; Lamb and Mitchell 2003; Barwell *et al.* 2005). In addition, the expression levels of *FRE2*, *FRE3*, *FRE4*, *ARN2*, and *FIT1* are reduced in *rim101*Δ cells under alkaline conditions (Barwell *et al.* 2005). In contrast, the expression of *ARN1* (Lamb and Mitchell 2003; Barwell *et al.* 2005), *FRE1* (Lamb *et al.* 2001), and a *FET3*–lacZ reporter (Figure 2) is increased in the absence of Rim101 in alkaline treatment. Rim101 functions as a repressor through binding of promoters (reviewed in Penalva *et al.* 2008) and indirectly as an activator through the repression of negative-acting genes *SMP1* and *NRG1* (Lamb and Mitchell 2003). Could Rim101 be acting as both a repressor and activator of a subset of iron regulon genes? Directed studies did not detect Rim101 on the promoter of *ARN4* by chromatin immunoprecipitation (Lamb and Mitchell 2003) and global ChIP studies only detected weak enrichment of Rim101 to the promoters of iron regulon genes *FET5* and *ARN1*, while Nrg1 or Smp1 localization was not detected on the promoters of any iron regulon genes (Harbison *et al.* 2004). The mechanism through which Rim101 affects iron regulon gene expression will require further investigation.

An iron-independent role for Aft1 in chromosome stability: We found that increased exogenous iron cannot suppress the chromosome fragment loss (Table 5) or the benomyl sensitivity (Figure 4A) of *aft1*Δ cells. In addition iron regulon genes are not induced upon benomyl treatment (Figure 4B, File S1, and Lucau-Danila *et al.* 2005). It is also important to note that, except for *AFT1*, no other iron regulon genes have been identified in genome-wide screens measuring genome instability by various assays (Kanellis *et al.* 2007; Yuen *et al.* 2007; Andersen *et al.* 2008). Our results indicate that the role of Aft1 in chromosome stability is distinct from its role as a transcriptional inducer of the iron regulon and iron homeostasis.

How could Aft1 be regulating chromosome stability? One possibility is that Aft1 is regulating chromosome stability through transcription of key genes that encode proteins required for genome maintenance and benomyl resistance. Our candidate approach identified only one potential gene (Table 6); however, we show that the transcriptional regulation of *CTF19* is likely not the means through which Aft1 contributes to genome stability (Figure 5). Similarly, we determined that Aft1 plays only a minor role in the benomyl transcriptional response (Figure 6). Intriguingly, of the few genes whose induction upon benomyl treatment is dependent on Aft1, most are members of the Met4 regulon (Lee *et al.* 2010). Met4 cannot bind DNA on its own, but rather localizes to its target promoters through interaction with either the partially redundant zinc finger proteins Met31 or Met32 or through the helix–loop–helix protein Cbf1 (reviewed in Lee *et al.* 2010). As the expression of *MET32* in benomyl is decreased in *aft1*Δ cells, this may explain the overall decrease in the Met4 regulon genes. Could Aft1 play a more direct role in the Met4 transcriptional pathway? Two-hybrid interaction has been detected between Aft1 and Cbf1 (Measday *et al.* 2005), which has an alternative function as an inner kinetochore protein directly binding centromeric DNA (Cai and Davis 1990). As Aft1 also interacts with kinetochore protein Iml3 in two-hybrid studies (Wong *et al.* 2007) and Aft1 can co-immunoprecipitate numerous other kinetochore proteins (performed by A.H. and K.B.), one interpretation is that interaction between Aft1 and Cbf1 is solely reflective of a role of Aft1 at the kinetochore. Alternatively, the two-hybrid interaction between Aft1 and Cbf1 may reflect a direct role of Aft1 in regulation of the sulfur metabolic network. The connection between Aft1 and Met4 pathway will need to be further explored. However, except for Cbf1 (Cai and Davis 1990), no other Met4 cofactors or genes of the Met4 regulon have been implicated in chromosome stability or benomyl resistance. This suggests the sensitivity of *aft1*Δ cells to benomyl treatment is not the result of defects in the transcription of the Met4 regulon.

Although we cannot rule out a transcriptional role for Aft1 in chromosome stability or benomyl resistance, it is more likely that Aft1 is functioning directly at the kinetochore to regulate chromosome loss. As Aft1 has never been identified in the numerous kinetochore affinity chromatography purifications (Westermann *et al.* 2007), it suggests that the Aft1–kinetochore interaction is transient and potentially plays a regulatory role. Intriguingly, Aft1 has recently been shown to interact with and regulate the ubiquitination state of Arn3 (Jeong *et al.* 2009). As ubiquitination of the inner kinetochore protein Ctf13 (Kaplan *et al.* 1997) and the centromeric histone H3 variant Cse4 (Collins *et al.* 2004) contributes to the regulation of kinetochore complex formation at centromeres, it is tempting to speculate that Aft1 may play a similar role at the kinetochore. A detailed dissection of the Aft1-kinetochore interaction will be required to fully understand the role Aft1 is playing in regulating chromosome stability.

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