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Cell-cycle arrest and inhibition of G_1 cyclin translation by iron in *AFT1-1^{up}* yeast

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Although iron is an essential nutrient, it is also a potent cellular toxin, and the acquisition of iron is a highly regulated process in eukaryotes. In yeast, iron uptake is homeostatically regulated by the transcription factor encoded by *AFT1*. Expression of *AFT1-1^{up}*, a dominant mutant allele, results in inappropriately high rates of iron uptake, and *AFT1-1^{up}* mutants grow slowly in the presence of high concentrations of iron. We present evidence that when *Aft1-1^{up}* mutants are exposed to iron, they arrest the cell division cycle at the G_1 regulatory point Start. This arrest is dependent on high-affinity iron uptake and does not require the activation of the DNA damage checkpoint governed by *RAD9*. The iron-induced arrest is bypassed by overexpression of a mutant G_1 cyclin, *cln3-2*, and expression of the G_1-specific cyclins Cln1 and Cln2 is reduced when yeast are exposed to increasing amounts of iron, which may account for the arrest. This reduction is not due to changes in transcription of *CLN1* or *CLN2*, nor is it due to accelerated degradation of the protein. Instead, this reduction occurs at the level of Cln2 translation, a recently recognized locus of cell-cycle control in yeast.

Keywords: cell-cycle regulation/G_1 cyclins/iron/translational regulation/yeast

Introduction

Iron is an essential nutrient for virtually all life forms because it functions as a critical cofactor in a variety of cellular processes (Cammack *et al*., 1990). The capacity of iron to occupy multiple redox states underlies both its usefulness as a cofactor and its potential for toxicity (Halliwell and Gutteridge, 1989). Excess iron accumulation is toxic to humans and is a feature of a diverse array of human disorders, including the neurodegenerative diseases and hereditary hemochromatosis (HH) (Sofic *et al*., 1991; Gerlach *et al*., 1994; Powell *et al*., 1994; Kienzl *et al*., 1995; Montgomery, 1995; Jenner, 1996; Watt, 1996; Gorell *et al*., 1997). Iron causes cellular damage through unknown mechanisms, but reduced iron can catalyze the formation of hydroxyl radicals, which have the capacity to damage essentially all cellular components (Halliwell and Gutteridge, 1989). Cells are protected from the toxic effects of iron by a precisely regulated system of iron acquisition and utilization (Cammack *et al*., 1990), but the cellular responses to iron toxicity remain unknown. Additional pathways may exist that allow the cell to modulate growth and cell-cycle progression during exposure to toxic levels of iron.

We have used the budding yeast *Saccharomyces cerevisiae* to study the effects of iron toxicity on the mitotic cell cycle. Highly regulated pathways of iron uptake have evolved that allow yeast to survive a wide range of environmental iron conditions. Budding yeast have two distinct systems for the uptake of elemental iron, a low-affinity system (Dix *et al*., 1994, 1997) and a high-affinity system. High-affinity uptake of iron is a two step process. First, iron is converted from the ferric form to the ferrous form by a family of surface reductases that include the Fre1 and Fre2 proteins (Dancis *et al*., 1990, 1992; Georgatsou and Alexandraki, 1994). Secondly, uptake across the plasma membrane is facilitated by a transporter complex consisting of a multi-copper oxidase (Fet3p) (Askwith *et al*., 1994) and a putative iron permease (Ftr1p) (Stearman *et al*., 1996).

In yeast, the genes required for high-affinity uptake of iron at the plasma membrane are homeostatically regulated by the transcription factor Aft1 (Yamaguchi-Iwai *et al*., 1995, 1996). Under conditions of iron depletion, Aft1 induces the transcription of the genes required for high-affinity iron uptake; conversely, when iron is abundant, transcription is not induced. Expression of *AFT1-1^{up}*, a dominant mutant allele of *AFT1*, causes inappropriately high iron uptake due to the dysregulated expression of surface reductases and the iron transporter complex (Yamaguchi-Iwai *et al*., 1996). Yeast are normally very resistant to the toxic effects of exogenous iron, but the bypass of normal regulatory controls caused by Aft1-1^{up} expression has permitted development of a genetic model of iron intoxication. By using *AFT1-1^{up}* strains, we have begun to examine the response of yeast to excess iron stress.

In *S.cerevisiae*, certain environmental stresses such as elevated temperature, nutrient deprivation and excess oxygen radicals are sensed during the G_1 phase of the cell cycle and can delay passage through Start (Cross, 1995; Lee *et al*., 1996). We have used the *AFT1-1^{up}* allele to investigate the effects of iron on the mitotic cell cycle. In this paper, we present evidence that *AFT1-1^{up}* mutants respond to exposure to iron by arresting the cell cycle at

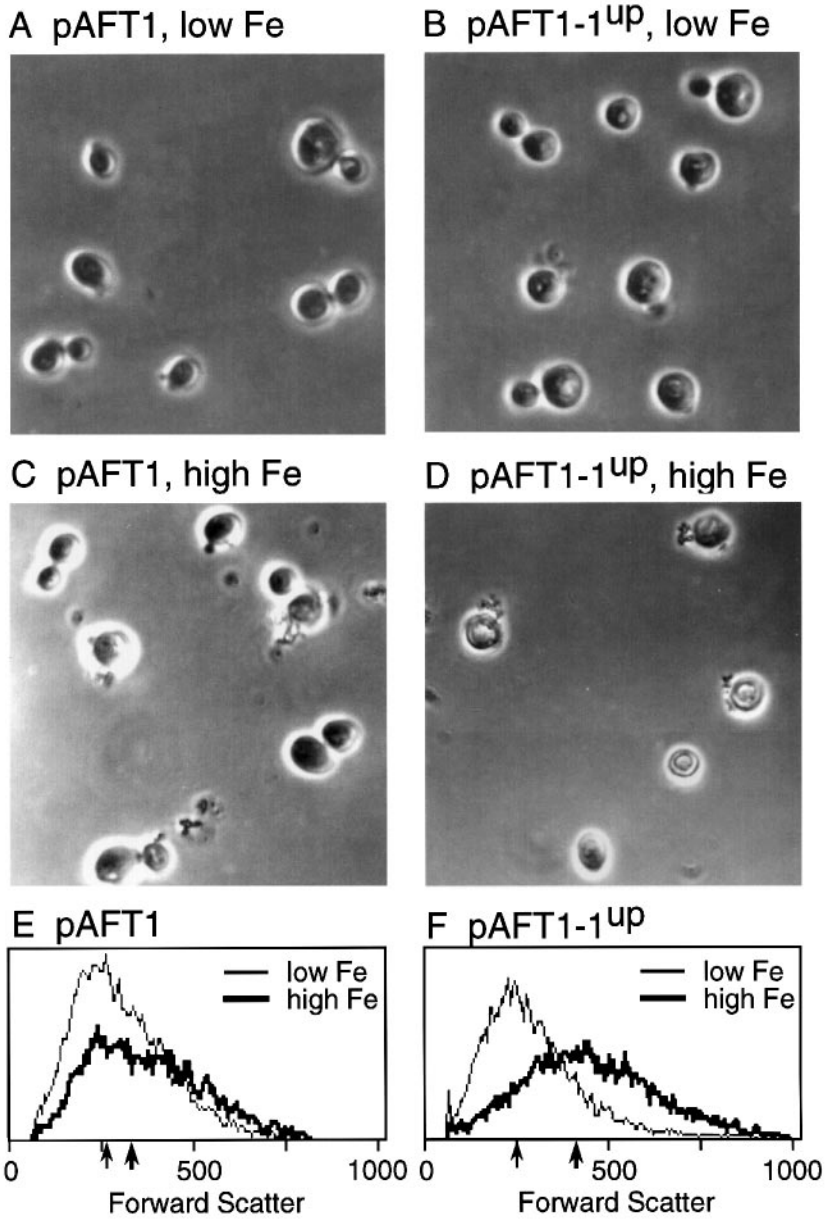

Fig. 1. Accumulation of large, unbudded cells in *AFT1-1*^{up} strain exposed to excess iron. The *aft1*-deleted strain (CPY101) was transformed with a low copy number plasmid carrying either wild-type *AFT1* [pAFT1, (A), (C) and (E)] or the *AFT1-1*^{up} allele [pAFT1-1^{up}, (B), (D) and (F)]. Logarithmically growing strains were inoculated into defined-iron media containing 100 [(A) and (B)] or 500 μM [(C) and (D)] ferrous ammonium sulfate (FAS) and grown for 4 h at 30°C. Cultures were fixed in 70% ethanol and an aliquot removed for morphological analysis. (**A**–**D**) Yeast were photographed at the same magnification using phase-contrast optics on a Zeiss photomicroscope equipped with a plan-apo-X63/1.4 N.A. objective. Flocculent material in (C) and (D) is precipitates of iron. The remainder of the fixed culture was stained with propidium iodide and analyzed by flow cytometry. (**E** and **F**) Increase in mean cell size due to iron. Histograms represent the relative size distribution obtained from the forward scatter parameter. Arrowheads indicate geometric mean: small arrowhead, 100 μM iron; large arrowhead, 500 μM iron.

Start, the key regulatory period in late G_1. This arrest is due to a lowered level of expression of the G_1-specific cyclins Cln1 and Cln2. Although expression of Cln1 and Cln2 is regulated both transcriptionally and post-translationally (Nasmyth and Dirick, 1991; Ogas *et al*., 1991; Dirick *et al*., 1995; King *et al*., 1996), neither of these regulatory mechanisms account for the iron-induced reduction in expression. Recent reports have indicated that Cln3 expression is translationally regulated under conditions of nutrient deprivation (Barbet *et al*., 1996; Gallego *et al*., 1997; Polymenis and Schmidt, 1997). We report that iron causes impaired translation of Cln2 mRNA, which suggests that translation is an important locus of cell-cycle control in budding yeast.

Results

Cell-cycle response to excess iron uptake

To investigate the observation that yeast expressing Aft1-1^{up} exhibit slow growth when exposed to excess iron, *aft1* strains were transformed with plasmids containing either wild-type *AFT1* or *AFT1-1*^{up} (Yamaguchi-Iwai *et al*., 1995). The transformants were transferred to iron-rich or iron-poor media, and examined microscopically for

morphological changes. Cells expressing Aft1 in either iron-poor or iron-rich media (Figure 1A and C) demonstrated morphology typical of logarithmically growing cells, i.e. approximately half of the cells exhibited buds in all stages of development. In contrast, cells expressing Aft1-1^{up} grew normally in iron-poor medium (Figure 1B), but failed to grow in iron-rich medium, accumulating instead as unbudded cells (87 ± 2% unbudded; Figure 1D). Because bud emergence is coordinated with the onset of replication and S phase (Lew *et al*., 1997), an accumulation of unbudded cells suggests a block in the G_1 phase of the cell cycle.

The G_1 phase of the mitotic cell cycle is the period during which budding yeast grow in size, and monitor their environment for nutrients and mating pheromone, which regulate passage through Start (Cross, 1995). Start is a critical regulatory period that marks the end of G_1; cells are small in early G_1 and increase in diameter as they approach Start. Yeast grown in iron-poor and iron-rich media were analyzed by flow cytometry for changes in relative size (Figure 1E and F). Growth in iron-rich media was associated with a larger mean cell size, which was most prominent in the *pAFT1-1^{up}* strain. This result is due in part to a reduction in the number of small, unbudded cells typical of early G_1. Thus, the morphological data were suggestive of a cell-cycle arrest in late G_1.

To confirm that excess iron uptake induces a G_1 cell-cycle arrest and to examine the DNA content of the arrested cells, the cultures of the *AFT1* and *AFT1-1^{up}* strains were stained with propidium iodide and examined by flow cytometry. Cells with 1N DNA content are in the G_1 phase of the cell cycle, cells with 2N DNA are in G_2/M, and cells with >1N but <2N are in S phase (undergoing replication). Again, wild-type cells were relatively unaffected by an increase in iron concentration in the medium (Figure 2A and C), while the *AFT1-1^{up}* strain demonstrated an increase in cells with 1N DNA content from 43 to 72% after exposure to high concentrations of iron (Figure 2B and D). We questioned whether these *AFT1-1^{up}*- and iron-dependent phenotypes were also dependent on the high-affinity iron uptake system; we therefore examined the effects of *AFT1* alleles in a strain bearing a deletion of an essential component of the high-affinity iron transport complex, *FET3*. When the *fet3*Δ strains, which lack high-affinity iron uptake, were grown in iron-rich medium, the presence of the *AFT1-1^{up}* allele no longer produced an increase in the fraction of cells with 1N DNA (Figure 2D and F), confirming the requirement for high-affinity iron uptake in the G_1 arrest.

In *Escherichia coli* and *S.cerevisiae*, deregulation of iron acquisition leads to DNA damage (Touati *et al*., 1995; C.Philpott, unpublished observations) and in *S.cerevisiae*, DNA damaging agents are known to activate the cell-cycle checkpoint governed by the *RAD9* gene. Activation of this checkpoint results predominately in a G_2 arrest (Schiestl *et al*., 1989), although *RAD9* has some arresting activity in G_1 as well (Siede *et al*., 1993). We tested whether the iron-dependent G_1 arrest required activation of the *RAD9* checkpoint. We found that, after deletion of *RAD9*, exposure of the *AFT1-1^{up}* strain to iron-rich media still caused a significant fraction of the cells to accumulate with 1N DNA (62%; Figure 2H), although this fraction is slightly smaller than the fraction of cells with iron-induced G_1 arrest in the *RAD9 AFT1-1^{up}* background

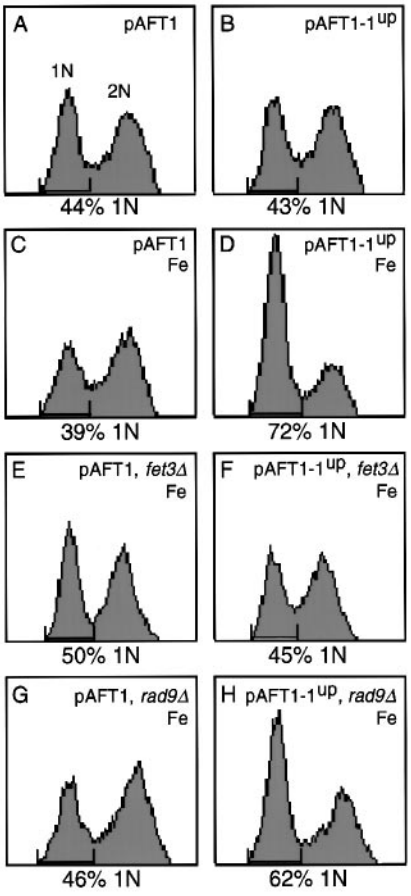

Fig. 2. Iron-dependent accumulation of cells with 1N DNA content. Congenic *aft1* [CPY101, (**A**–**D**)], *aft1 fet3*Δ [CPY103, (**E**) and (**F**)], or *aft1 rad9*Δ [CPY107, (**G**) and (**H**)] strains were transformed with either pAFT1 or pAFT1-1^{up}. Transformants were grown as in Figure 1 with 100 μM [(A) and (B)] or 500 μM [Fe, (C–H)] FAS, fixed in 70% ethanol, stained with propidium iodide and analyzed by flow cytometry for DNA content (see Materials and methods). Histograms reflect the frequency distribution of fluorescence intensity from propidium iodide staining of DNA. The fraction of the cells with 1N DNA (bracketed region) is indicated. Representative histograms from a single experiment are shown; the percentage of cells with 1N DNA content was determined from three or more independent experiments.

(72%; Figure 2D). These results indicated that the iron-induced G_1 arrest was largely independent of the checkpoint pathway governed by *RAD9*.

Bypass of the iron-mediated G_1 block by overexpression of Cln3-2

The iron-mediated late G_1 block suggested a defect at Start. The G_1-specific cyclin Cln3 promotes Start by activating the transcription of G_1-specific genes, including the major G_1 cyclins *CLN1* and *CLN2* (Tyers *et al*., 1993; Dirick *et al*., 1995), but Cln3 can also substitute for the activities of Cln1 and Cln2 in a *cln1 cln2 CLN3* strain (Richardson *et al*., 1989; Cross, 1990). Overexpression of a truncated form of *CLN3*, *cln3-2*, causes premature execution of Start and a shortened G_1 phase (Cross, 1988). To determine whether overexpression of *cln3-2* could result in a bypass of the iron- and *AFT1-1^{up}*-mediated G_1 block, we introduced the *cln3-2* allele under the control of the *GAL1,10* promoter to *AFT1* and *AFT1-1^{up}* strains and analyzed the effects of iron by flow cytometry. Overexpression of *cln3-2* resulted in a reduction in the

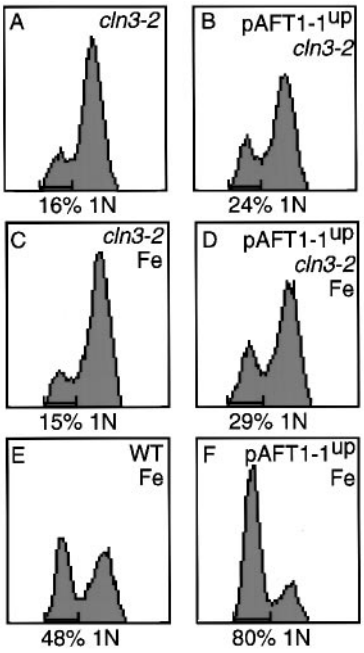

Fig. 3. Bypass of the iron-mediated arrest by the *cln3-2* allele. A *pGAL1,10::CLN3-2* strain and the congenic parent strain, W303, were transformed with pGalAFT1-1^{up}TRP (**B**, **D** and **F**) or pRS414 (**A**, **C** and **E**). Transformants were grown to mid-log phase in raffinose-based SD media then exposed to galactose, ferrozine and 50 μM FAS for 1 h. Cultures were then divided and grown without additional iron (A and B) or with the addition of 500 μM FAS (C–F) for 4 h and analyzed by flow cytometry. The fraction of the cells with 1N DNA is indicated (bracketed region).

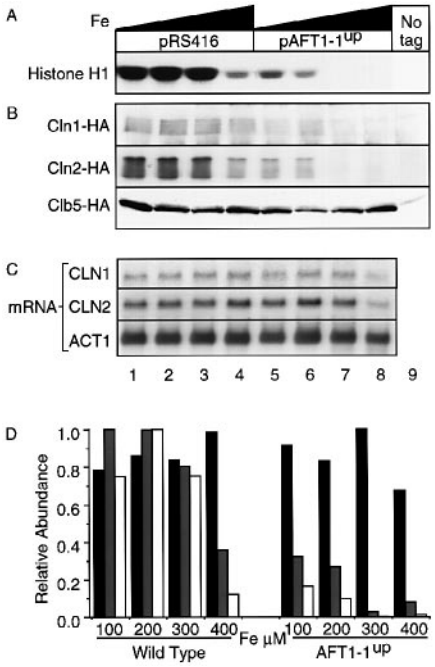

Fig. 4. Iron regulation of Cln1 and Cln2 protein levels. Strains expressing an hemagglutinin (HA)-tagged version of Cln1, Cln2, or Clb5 transformed with either pAFT1-1^{up} or a control plasmid (pRS416, Stratagene), were inoculated at 4×10^6 cells/ml and grown for 4 h in defined iron media containing 100, 200, 300 or 400 μM FAS. Lysates (5 mg) were subjected to immunoprecipitation with monoclonal antibody HA.11 and equal portions of the Cln2 precipitates were assayed for histone H1 phosphorylation activity. (**A**) SDS–PAGE of supernatant from histone H1 phosphorylation assay of Cln2-HA precipitates. The untagged parent strain (No tag) was used as a negative control. (**B**) Immunoblot of precipitated Cln1-HA and Cln2-HA, and Western blot of Clb5. Phosphorylated forms of Cln1 and Cln2 migrate as higher molecular weight proteins. (**C**) Northern blot of RNA derived from the Cln2-HA strain probed serially for Cln1, Cln2 and actin mRNAs. (**D**) Quantification of Cln2 signals from (A), (B) and (C) by PhoshorImage analysis. Solid bars, mRNA; shaded bars, protein; open bars, kinase activity. RNA levels for Cln2 were normalized against Act1 levels. Experiments were replicated twice; representative autoradiograms and quantification from a single experiment are shown.

fraction of cells in G_1 to 16% from 48% (Figure 3A and E) and blocked the accumulation of cells in G_1 when *AFT1-1^{up}* yeast were exposed to iron-rich media (Figure 3D and F). Because overexpression of *cln3-2* resulted in a bypass of the iron-mediated G_1 arrest, we suspected that iron exerted its regulatory effects by interfering with the activities of the G_1 cyclins.

Regulation of Cln1 and Cln2 by iron

To determine how excess iron uptake in *AFT1-1^{up}* mutants leads to G_1 arrest, we directly examined the effects of iron and Aft1-1^{up} expression on the activities of proteins required for the G_1–S phase transition. Passage through the G_1 phase of the cell cycle is dependent on the activities of the G_1 cyclins, Cln1, Cln2 and Cln3, in association with the cyclin-dependent kinase, Cdc28 (Nasmyth, 1993, 1996; Sherr, 1994; Cross, 1995). Cln1 and Cln2 bind to and activate Cdc28, and the resulting active kinase complexes then phosphorylate target proteins that include Sic1, an inhibitor of Cdc28–cyclin B complexes which is degraded at the G_1–S transition (Peter and Herskowitz, 1994). We measured Cdc28–Cln2 kinase activity by immunoprecipitating kinase complexes from extracts prepared from strains that expressed an epitope-tagged version of Cln2 and assaying phosphorylation of the substrate histone H1. Cdc28–Cln2 activity was lost when yeast were exposed to increasing amounts of iron, both in an *AFT1* (Figure 4A, lanes 1–4, and 4D) and an *AFT1-1^{up}* background (Figure 4A, lanes 5–8, and 4D), with kinase activity becoming undetectable in the latter. Proportional decreases in Cln1 (from a strain expressing epitope-tagged Cln1) and Cln2 protein were observed in both *AFT1* and *AFT1-1^{up}* backgrounds under these conditions of increasing iron (Figure 4B and D), with protein again becoming undetectable at higher iron concentrations. Not all cyclins were similarly affected by the iron, however. Expression of an epitope-tagged B-type cyclin, Clb5, which is also expressed in G_1, was relatively unaffected by iron in both *AFT1* and *AFT1-1^{up}* strains (Figure 4B). Thus, the iron-mediated G_1 arrest could be attributed to a deficiency of Cln1 and Cln2 protein, resulting in insufficient Cdc28–Cln activity for progression through Start.

We analyzed the abundance of the Cln1 and Cln2 transcripts after treatment with iron and found that the mRNA levels remained relatively unchanged in both *AFT1* and *AFT1-1^{up}* strains (Figure 4C and D). Only at the highest iron concentration in the *AFT1-1^{up}* strain (Figure 4C, lane 8) was a decrease in Cln1 and Cln2 mRNA observed; this was accompanied by a decrease in Act1 mRNA as well and was probably due to degradation in dying cells. We confirmed that the detected transcript was the Cln2 message by demonstrating that the transcript was of appropriate size, was regulated by glucose and galactose

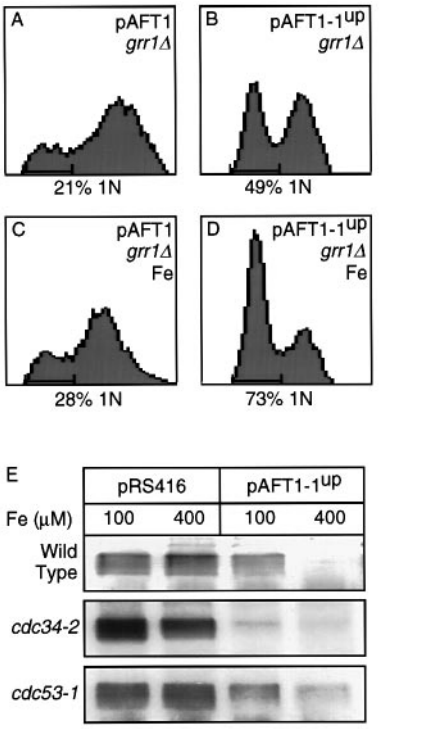

Fig. 5. Iron- and Aft1-1^{up}-dependent G_1 arrest and reduced expression of Cln2 in mutants deficient in the degradation of G_1 cyclins. A *GRR1*-deleted strain (*grr1*Δ) was transformed with pAFT1 (**A** and **C**) or pAFT1-1^{up} (**B** and **D**), cultured as in Figure 1, and analyzed by flow cytometry for DNA content. The fraction of cells with 1N DNA is indicated. (**E**) Congenic wild-type (MTY263), *cdc34-2* (MTY773) and *cdc53-1* (CPY131) strains expressing HA-tagged Cln2 as well as the corresponding congenic *AFT1-1^{up}* strains (CPY135, CPY141, and CPY145) were grown to mid-log phase at 26°C, transferred to defined-iron media containing 100 or 400 μM FAS, and shifted to 37°C for 2 h. Cultures were then harvested and Cln2 was detected as in Figure 4B.

in a *cln2::pGAL1-CLN2^{HA}-LEU2* strain, and became undetectable in stationery phase cells (data not shown). These results indicated that the regulatory effects of iron were post-transcriptional, causing either a diminished rate of mRNA translation or an increased rate of protein degradation.

Effects of excess iron in mutants defective in degradation of G_1 cyclins

To investigate the effects of iron on Cln2 degradation, we examined mutant strains that were defective in the degradation of the G_1 cyclins. *GRR1* is a gene involved in the regulation of glucose transport and catabolite repression (Bailey and Woodword, 1984; Flick and Johnston, 1991); *GRR1* was subsequently identified in a mutant strain that poorly degraded Cln1 and Cln2, but not Cln3, resulting in a marked shortening of G_1 phase (Barral *et al*., 1995). Grr1 interacts with Skp1 and Cdc53, which, along with Cdc34, form a complex that targets Cln1 and 2 for ubiquitin-mediated proteolysis (Li and Johnston, 1997; Skowyra *et al*., 1997). In order to determine whether excess iron uptake would produce G_1 arrest in a *grr1* deletion strain, we analyzed *grr1 AFT1* and *grr1 AFT1-1^{up}* strains by flow cytometry. The *grr1 AFT1* strain had relatively few cells in G_1 in iron-poor or iron-rich media (Figure 5A and C), but the fraction of cells in G_1 increased to 73% when the *grr1 AFT1-1^{up}* strain was transferred to iron-rich medium (Figure 5D), demonstrating that excess iron uptake produced a G_1 arrest in a *grr1* deletion mutant.

CDC34 and *CDC53* encode components of a ubiquitin-protein ligase complex that is required for the proteolysis of Cln2 as well as the S phase inhibitor Sic1 at the end of G_1 (Deshaies *et al*., 1995; King *et al*., 1996; Mathias *et al*., 1996; Willems *et al*., 1996). The temperature-sensitive strains *cdc34-2* and *cdc53-1* exhibit an accumulation of Cln1 and Cln2 protein when placed at the non-permissive temperature due to impaired destruction of the G_1 cyclins. However, exposure of congenic *cdc34-2 AFT1-1^{up}* and *cdc53-1 AFT1-1^{up}* strains to high concentrations of iron at the non-permissive temperature caused reductions in Cln2 that were similar to those seen in the parental strain (Figure 5E). These data indicated that previously identified pathways of G_1 cyclin degradation are not required for the iron-dependent reduction in Cln2 expression.

Reduction of Cln2 biosynthesis by iron

To determine directly the effects of excess iron accumulation on the rate of Cln2 mRNA translation and the stability of Cln2 protein, we evaluated the biosynthetic and decay rates of Cln2 by pulse–chase analysis and immunoprecipitation. Pulse-labeled Cln2 was not detectable when *AFT1-1^{up}* strains were grown in iron-rich defined media; therefore, experiments were performed under a milder iron stress where much smaller changes in Cln2 protein levels were seen. After a 5 min biosynthetic labeling, both hypo- and hyperphosphorylated forms of Cln2 were detected and both diminished rapidly during the chase period (Figure 6A and B). Quantification of the Cln2 signal from the pulse-labeling revealed that a significantly lower amount of Cln2 was synthesized in the *AFT1-1^{up}* strain in iron-supplemented media compared with the *AFT1* strain. (Figure 6A). The overall incorporation of radioactivity was 56% lower in *AFT1-1^{up}* with additional iron than in *AFT1* strains. After correction for this reduced labeling efficiency, there was still a 64% lower level of pulse-labeled Cln2 in *AFT1-1^{up}* with additional iron than in *AFT1*. The difference in the biosynthesis of Cln2 occurred even though essentially identical amounts of Cln2 mRNA were present in the *AFT1* and *AFT1-1^{up}* strains in rich and iron-supplemented media.

The lower amount of pulse-labeled Cln2 was not accounted for by differences in protein half-life, because the half-life of Cln2 was not decreased by iron supplementation in *AFT1-1^{up}* and may have been slightly increased (Figure 6B). Furthermore, the observed half-life for Cln2 of 10 min is in close agreement with previously published data (Barral *et al*., 1995; Blondel and Mann, 1996; Lanker *et al*., 1996). The iron-induced decrease in the biosynthetic rate of Cln2 occurred in the absence of measurable changes in mRNA abundance or protein half-life; this observation was consistent with a model in which excess iron uptake specifically inhibits the translation of Cln2 mRNA.

In cultures of unsynchronized cells, iron causes both a change in the distribution of cells across the different phases of the cell cycle as well as a decrease in the synthesis of Cln2 protein. Although the persistence of Cln2 mRNA supports a model in which the iron-induced

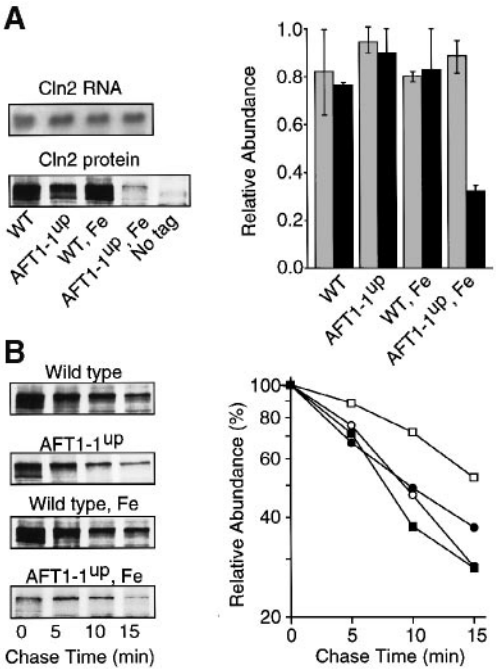

Fig. 6. Iron regulation of Cln2 biosynthesis in the absence of changes in protein half-life. Congenic, wild-type (MTY263) and *AFT1-1^{up}* (CPY137) strains bearing the HA-tagged Cln2 allele and the untagged parent (No tag, K699) in the exponential phase of growth were transferred to YPD without (YPD) or with (YPD, Fe) 1 mM ferrozine and 500 μM FAS for 2.5 h. Aliquots of culture were removed for RNA isolation and Northern analysis with a probe for Cln2 mRNA. Cells were then pulse-labeled with [^{35}S]methionine and cysteine for 5 min, chased with excess unlabeled methionine and cysteine, and aliquots were removed at 5 min intervals. Lysates were prepared and equal quantities of protein were subjected to immunoprecipitation with HA.11 antibody. (**A**) Steady state levels of Cln2 mRNA compared to the biosynthetic rate of Cln2 protein. Quantification of the Northern blot and pulse-labeled Cln2 immunoprecipitate by PhosphorImage analysis is shown at right. Data shown were corrected for reductions in labeling efficiency according to total incorporation of TCA precipitable counts. Graph represents the mean (± SEM) from three separate experiments. Shaded bars, RNA; solid bars, protein. (**B**) Decay of pulse-labeled Cln2 protein. Quantification of all hyper- and hypophosphorylated forms of labeled Cln2 protein at each chase time-point was performed and expressed as a percentage of the initial pulse-labeled (0 min) signal. Graph shows data from three separate experiments. (●) *Cln2HA* in YPD, (○) *Cln2HA AFT1-1^{up}* in YPD, (■) *Cln2HA* in YPD with iron, (□) *Cln2HA AFT1-1^{up}* in YPD with iron.

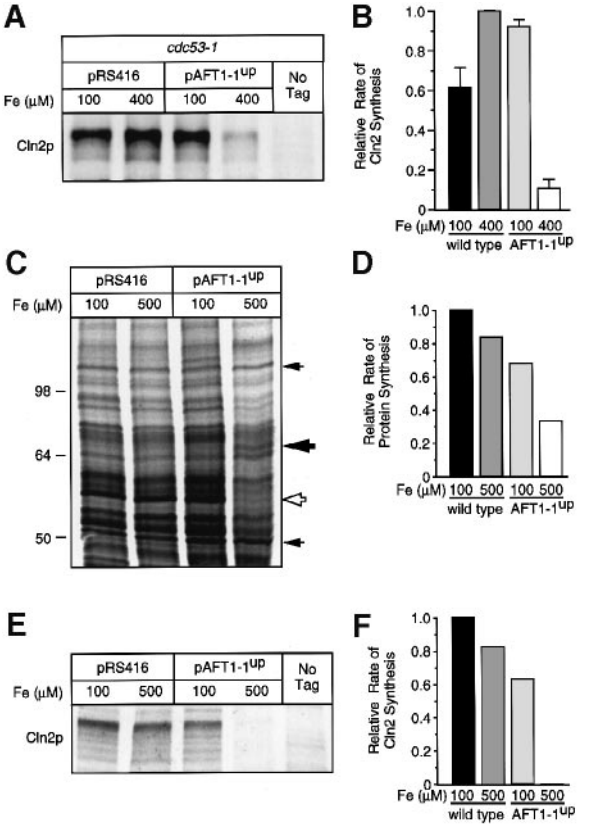

Fig. 7. Iron-dependent block in Cln2 protein synthesis in synchronized cultures and in translation-competent cells. Congenic *cdc53-1* [(A) and (B)] and *CDC+* (C–F) strains expressing an HA-tagged version of Cln2 were transformed with either pAFT1-1^{up} (pAFT1-1^{up}) or a control plasmid (pRS416). *Cdc53-1* transformants were shifted to 38°C for 3 h prior to exposure to defined iron media containing 100 or 400 μM FAS for an additional 3 h. Cells were then pulse-labeled for 10 min and immunoprecipitation performed as in Figure 6. *CDC+* (MTY263) transformants were grown in defined-iron media containing either 100 or 500 μM FAS for 3 h prior to pulse-labeling for 10 min and immunoprecipitation as in Figure 6. (**A**) Immunoprecipitation and SDS–PAGE of pulse-labeled Cln2 protein from *cdc53-1*. (**B**) Relative biosynthetic rate of Cln2 in *cdc53-1*. (**C**) SDS–PAGE and autoradiography of equivalent number of trichloroacetic acid (TCA)-precipitable counts of total protein lysate from labeled cells. Representative proteins in which the biosynthesis is unchanged (small arrows), increased (large arrow) or decreased (open arrow) in the *AFT1-1^{up}* strain by growth in 500 μM FAS are indicated. (**D**) Relative rates of total protein synthesis. Ratio of ^{35}S-labeled, TCA-precipitable material to total protein concentration is expressed relative to the wild-type strain grown in 100 μM FAS. (**E**) Immunoprecipitation and SDS–PAGE of pulse-labeled Cln2 protein from MTY263. Lysates containing equivalent amounts of ^{35}S-labeled, TCA-precipitable material were subjected to immunoprecipitation with HA.11 antibody. (**F**) Biosynthetic rate of Cln2 in MTY263. Quantitation of hypo- and hyperphosphorylated forms of Cln2 from (E) is expressed relative to the wild-type strain grown in 100 μM FAS. The Cln2 signal from the *AFT1-1^{up}* strain grown in 500 μM FAS was not detectable above background (No Tag control).

alterations in the cell cycle are secondary to an iron-induced inhibition of translation, we considered whether the converse was true: that the lower levels of G_1 cyclins were secondary to iron-induced changes in the distribution of cells across the cell cycle. In order to determine the effect of iron on Cln2 synthesis in absence of changes in cell-cycle disribution, we measured the biosynthesis of Cln2 in synchronized cultures. We used the temperature-sensitive *cdc53-1* strain because these cells continue to synthesize G_1 cyclins at the restrictive temperature when they are arrested in late G_1. Congenic *cdc53-1 AFT1* and *cdc53-1 AFT1-1^{up}* strains were placed at the non-permissive temperature and allowed to accumulate in late G_1. Cultures were then exposed to iron-poor or iron-rich media at the restrictive temperature, and newly synthesized Cln2 was detected (Figure 7A and B). Although Cln2 protein was actively synthesized in the *cdc53-1 AFT1* strain in both iron-poor and iron-rich media, and in the *cdc53-1 AFT1-1^{up}* strain in iron-poor media, Cln2 biosynthesis was 15-fold lower in the *cdc53-1 AFT1-1^{up}* strain in iron-rich media. The iron-induced reduction in Cln2 biosynthesis cannot, therefore, be explained solely by an iron-induced change in the distribution of cells across the cell cycle.

Specificity of iron-induced repression of Cln2 mRNA translation

The rate of translation of Cln2 was markedly diminished in the *AFT1-1^{up}* strain grown in iron-supplemented media. To determine whether this effect could be explained by a general iron-induced repression of translation, we examined general protein synthesis patterns as well as the synthesis of Cln2 in *AFT1* and *AFT1-1^{up}* strains exposed to iron-rich and iron-poor media. The effects of iron on the pattern of general protein synthesis is shown in Figure 7C. Changes in the overall pattern of protein synthesis when the *AFT1-1^{up}* strain was exposed to high concentrations of iron were evident, with the synthetic rates of different individual proteins often either increasing or decreasing. These changes in the pattern of protein synthesis may be due to changes in mRNA abundance (alterations in transcription or mRNA degradation) as well as translation. Although the overall rate of protein synthesis in the *AFT1-1^{up}* strain in iron-rich medium was diminished ~3-fold compared with the *AFT1* strain in iron-poor medium (Figure 7D), cells were clearly competent for new protein synthesis. Newly synthesized hypo- and hyperphosphorylated forms of Cln2 were readily detectable in the *AFT1* strain in both iron-rich and iron-poor media and in the *AFT1-1^{up}* strain in iron-poor medium (Figure 7E). In contrast, Cln2 was undetectable in the *AFT1-1^{up}* strain in iron-rich medium, and the ~3-fold reduction in general protein synthesis did not account for the absence of Cln2 synthesis in iron-rich media. Quantification of the immunoprecipitated Cln2 indicated that both exposure of the *AFT1* strain to iron-rich medium and expression of *AFT1-1^{up}* in iron-poor medium resulted in decreased synthesis of Cln2 (Figure 7F), suggesting that less extreme levels of iron uptake can also produce inhibition of Cln2 translation.

Discussion

Iron as an environmental stressor

The regulation of cell division is dependent on specific trophic factors as well as the availability of essential nutrients; cell division is also regulated by environmental stresses. Here we report that an essential micronutrient, iron, can also act as an environmental stressor and delay the cell division cycle at the G_1 regulatory point Start. While the iron-induced arrest that we have observed is most prominent in a mutant background that exhibits dysregulated iron uptake, we also note that exposure of wild-type strains (with intact iron-regulatory systems) to high concentrations of iron leads to both decreased steady-state levels of Cln2 (Figure 4B, lane 4) as well as reduced biosynthesis of Cln2 (Figure 7D, wild-type, 500 μM Fe). These observations indicate that the response to an abrupt increase in environmental iron that we describe in this paper is not restricted to the mutant strain, but can occur in wild-type yeast; this may indicate the presence of a pathway in which translation is repressed in response to the presence of potentially toxic environmental agents. A model that summarizes the regulation of Cln1 and Cln2 during G_1 is presented in Figure 8. While uptake of iron is necessary for growth, excess free iron has the potential to disrupt cellular processes through several mechanisms.

Iron can participate in oxidation-reduction chemistry in the cell, leading to the generation of hydroxyl radical and other reactive oxygen species (Halliwell and Gutteridge, 1989). DNA-bound iron could directly catalyze oxidative DNA damage and both dysregulated iron uptake and defects in oxygen radical metabolism result in increased rates of DNA damage in *E.coli* and *S.cerevisiae* (Farr *et al*., 1986; Imlay *et al*., 1988; Gralla and Valentine, 1991; Brennan *et al*., 1994; Keyer *et al*., 1995; Touati *et al*., 1995; Woodford *et al*., 1995; Keyer and Imlay, 1996; C.C.Philpott, unpublished data). Despite the potential for iron to increase rates of DNA damage, evidence does not support the concept that the iron-mediated arrest occurs subsequent to the sensing of DNA damage for three reasons. First, the cell-cycle block caused by DNA-damaging agents such as gamma- and UV-irradiation occurs mainly in G_2 (Friedberg *et al*., 1995), whereas the iron-induced block occurs in G_1. Secondly, cell-cycle arrest caused by DNA damage depends upon the activation of a checkpoint pathway governed by *RAD9* (Lydall and Weinert, 1996); however, we find the iron-induced G_1 arrest to be largely independent of *RAD9*. Similarly, *RAD9*-independence has been reported for the G_1 arrest associated with heat shock and paraquat-generated superoxide radicals (Nunes and Siede, 1996). Thirdly, although excess iron uptake in yeast deficient in DNA repair causes a decrease in survival, it does not cause cells to accumulate in G_1; instead, DNA repair mutants that are exposed to excess iron arrest the cell cycle in both G_1 and G_2 (C.C.Philpott, unpublished data).

Cellular components other than DNA may be the targets of iron-generated reactive oxygen species. Oxygen radicals cause damage to proteins by oxidizing amino acid side chains and by cleaving the protein backbone (Berlett and Stadtman, 1997). Oxidatively damaged proteins may also be selectively targeted for proteasomal degradation (Iwai *et al*., 1998). Lipid peroxidation may lead to membrane fragility and a loss of integrity of membrane-bound compartments (Favier, 1994). Alternatively, iron may exert

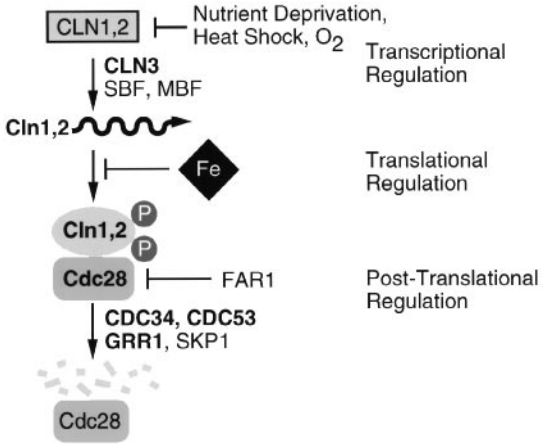

Fig. 8. Model of regulation of Cln1 and Cln2 during G_1 phase of cell cycle. Transcription of Cln2 is dependent on the Cln3 activation of the transcription factors SBF and MBF (Nasmyth and Dirick, 1991; Dirick *et al*., 1995). *FAR1* encodes an inhibitor of Cln–Cdc28 kinase activity that is expressed in response to mating pheromone (Peter and Herskowitz, 1994). *SKP1* encodes a component of the ubiquitin-protein ligase complex needed for rapid degradation of Cln1 and Cln2 (Bai *et al*., 1996). Arrows indicate positive regulatory events; bars indicate inhibitory events. Loci in bold type were examined in this paper.

deleterious effects by oxygen-independent pathways, for example by displacing other metals that act as essential cofactors for proteins involved in gene expression, DNA repair, or other cellular processes. The possibility exists that the effects of iron on the cell cycle occur not as a result of iron-induced damage, but as a result of the cell sensing a potentially damaging agent and initiating a response to prevent entrance into S phase, when the genome is especially sensitive to the mutagenic effects of DNA damage.

The yeast response to an increase in iron availability includes the transcriptional downregulation of genes required for high-affinity uptake of iron (Yamaguchi-Iwai *et al*., 1995). Other predicted responses would include detoxification/sequestration of the metal and repair of iron-induced damage. For example, exposure of yeast to high concentrations of copper results in the expression of copper-binding metallothioneins encoded by *CUP1* and *CRS5*, which are involved in the protection against copper toxicity (Zhu and Thiele, 1996). It is not surprising that, given the potential toxic and mutagenic effects of iron, the yeast response to excess iron uptake includes a delay in cell-cycle progression.

Translational regulation of the cell cycle

Heat shock, changes in nutrient status and dioxygen stress are environmental stresses that cause a G_1 delay in yeast and are accompanied by decreased abundance of Cln1 and Cln2 transcripts (Rowley *et al*., 1993; Baroni *et al*., 1994; Tokiwa *et al*., 1994; Lee *et al*., 1996). Our evidence indicates that excess iron uptake in *AFT1-1^{up}* mutants results in the inhibition of Start by a distinct mechanism: the repression of translation of Cln2 (and presumably Cln1) mRNA. Although we have focused on the effects of iron on Cln2 translation, our evidence, that both Cln1 and Cln2 proteins levels diminish in the absence of changes in RNA levels, and work by others suggests that Cln1 and Cln2 are regulated in an identical fashion (Ogas *et al*., 1991; Rowley *et al*., 1993; Tyers *et al*., 1993; Baroni *et al*., 1994; Sidorova and Breeden, 1997; Walker *et al*., 1997).

Start is the period of the cell cycle that appears to be most sensitive to defects in translation initiation and protein synthesis. Two cell-division-cycle mutants which arrest in G_1, *cdc33* and *cdc63*, harbor mutations in the genes encoding eukaryotic translation initiation factor 4E (eIF4E, the mRNA cap-binding protein) and a component of eIF3 (known as Prt1) (Hanic-Joyce *et al*., 1987; Brenner *et al*., 1988; Naranda *et al*., 1994). In mammalian cells, eIF4E has a regulatory role in translation initiation and the control of cell growth (Sonenberg, 1994). Overexpression of eIF4E results in enhanced translation of the G_1 cyclin, cyclin D1, in part by promoting the export of cyclin D1 mRNA from the nucleus (Rosenwald *et al*., 1995). Our preliminary results indicate that excess iron uptake in yeast has no effect upon the partitioning of Cln2 mRNA between the cytosol and the nucleus (C.C.Philpott, unpublished data).

Cln3, which activates the transcription of late G_1-specific genes including *CLN1* and *CLN2*, is also subject to translational control (Barbet *et al*., 1996; Gallego *et al*., 1997; Polymenis and Schmidt, 1997). The translation of Cln3 mRNA is rapidly repressed during nitrogen deprivation conditions that produce G_1 arrest (Gallego *et al*., 1997). Inactivation of the yeast rapamycin-sensitive phosphotidylinositol kinases *TOR1* and *TOR2* simulates a starvation response, resulting in inactivation of translation initiation and a G_1 arrest. This arrest (but not the general translation defect) can be suppressed by substituting the 5′ upstream region of *UBI4* for the 5′ untranslated region (UTR) of Cln3 (Barbet *et al*., 1996). Mutation of the AUG codon in the upstream open reading frame (uORF) present in the 5′ UTR of the Cln3 transcript enhances the translation of Cln3 message and accelerates Start-related events in the presence of rapamycin and in nutritionally deficient media (Polymenis and Schmidt, 1997). However, the majority of Cln2 transcripts do not contain an uORF (Ogas *et al*., 1991; Cross *et al*., 1994; Stuart and Wittenberg, 1994; Shen and Green, 1997); therefore, it is unlikely that the iron-mediated translational regulation of Cln2 relies on this mechanism.

How does iron reduce the efficiency of G_1 cyclin translation? Cln1 and Cln2 may be among a group of transcripts that are poorly translated under conditions of excess iron accumulation; iron may reduce the translation of these transcripts by directly or indirectly altering a component of the translation machinery. Currently, no evidence supports the existence of a specific translational repressor analogous to the iron regulatory proteins that repress translation of iron-regulatory element-containing transcripts in mammalian cells (Rouault and Klausner, 1996). Regardless of the mechanism, some feature(s) must be present in the Cln1 and Cln2 transcripts that mediates their translational repression in the presence of iron; efforts to map the translational control regions of the transcripts are underway. Non-coding regions of mRNAs may contain information that affects the fate of the transcript. Both Cln1 and Cln2 mRNAs have relatively long 5′ UTRs (240 and 174 nucleotides, respectively) compared with other yeast transcripts (Ogas *et al*., 1991; Cross *et al*., 1994; Stuart and Wittenberg, 1994), but analysis of the 5′ UTRs shows that they are remarkable for the absence of regions of conserved sequence and predicted stable secondary structures. However, preliminary data indicate that deletion of the majority of the Cln2 5′ UTR abrogates the iron-induced reduction in Cln2 protein (C.C.Philpott and J.Rashford, unpublished observations). The Cln1 and Cln2 transcripts may share features with other, as yet unidentified transcripts that are poorly translated in the presence of excess iron. Our findings suggest that yeast can delay progression through Start in the presence of potentially damaging environmental agents through the translational repression of G_1 cyclins.

Materials and methods

Yeast strains, plasmids and growth conditions

The plasmid pT20 (Yamaguchi-Iwai *et al*., 1995) was used for disruption of *AFT1* in YPH499 (Yeast Genetic Stock Center, Berkeley, CA), and the presence of the disruption in the genome was confirmed by Southern blot and by the lack of growth on nonfermentable carbon sources, a phenotype of *aft1* mutants. For disruption of *FET3*, the plasmid pΔfet3-HIS3 was constructed by replacing the URA3 gene of pT21 (Yamaguchi-Iwai *et al*., 1995) with a PCR-generated HIS3 gene. The resulting plasmid was linearized with *Apa*I and used to transform YPH499 to histidine prototrophy. Disruption of the *FET3* gene was confirmed by PCR and by lack of growth on iron-chelated media, a previously described phenotype of *fet3* mutants (Askwith *et al*., 1994). The *RAD9*

gene was deleted in the YPH499 background using the plasmid pRR330 (Schiestl *et al*., 1989) and the correct genomic location of the deletion was confirmed by PCR. *aft1* strains were transformed with low copy-number plasmids containing either *AFT1* (pT11, pAFT1) or *AFT1-1^{up}* (pT14, pAFT1-1^{up}) alleles (Yamaguchi-Iwai *et al*., 1995). The strain W303pGALCln3-2 (*leu2::LEU2::URA3::pGal1,10::CLN3-2 HIS3*) and its congenic parent, W303 (gift from I.Herskowitz), were transformed with pGalAFT1-1^{up}TRP, a pRS414 derivative (Sikorski and Hieter, 1989) that contains the AFT1-1^{up}-coding sequence under the control of the GAL1,10 promoter (Liu *et al*., 1992). The strain MTY263 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-1,15 ura3 cln2::CLN2HA-LEU2*) (gift from M.Peter) contains an in-frame fusion of three copies of the hemagglutinin (HA) epitope at the C-terminus of Cln2 (Tyers *et al*., 1993). MTY297 is a congenic strain containing a similarly tagged version of Cln1. Congenic *CLN2-HA cdc34-2* (MTY773) and *CLN2-HA cdc53-1* (CPY131) strains (Willems *et al*., 1996) contain tagged versions of Cln2. Replacement of the chromosomal *AFT1* gene with the *AFT1-1^{up}* allele was accomplished by the 'pop-in/pop-out' method (Rothstein, 1991): first, the *Xho*I–*Not*I fragment of pGalAFT1-1^{up} containing the *AFT1-1^{up}* coding region was inserted into the corresponding sites of the integrating plasmid pRS406 (Stratagene). Then, the resulting plasmid was linearized at the *Bsa*BI site of Aft1-1^{up} and used to transform strains MTY263, MTY773, and CPY131 to uracil prototrophy. Transformants were screened by Southern blot and by iron-uptake phenotype to confirm integration of the *AFT1-1^{up}* allele at a chromosomal site adjacent to the *AFT1* gene. Strains CPY135, CPY141, and CPY145 contain adjacent *AFT1* and *AFT1-1^{up}* alleles. These were used for studies because of genetic instability of the *AFT1-1^{up}* single integrant (C.C.Philpott, unpublished observations). Finally, the *CLN2-HA AFT1-1^{up}* strain (CPY137) was isolated by exposure of CPY135 to 5-fluoroorotic acid (Sikorski and Boeke, 1991) and the presence of a single *AFT1-1^{up}* allele was confirmed by iron uptake phenotype and Southern blotting. Deletion of the entire coding region of *GRR1* in CPY133 was achieved by transformation of MTY263 with a PCR product containing the *HIS3* gene flanked by 40 bp of the *GRR1* 5′ and 3′ UTRs. Histidine prototrophs were examined for the multibudded morphology that is characteristic of *grr1* (Barral *et al*., 1995) and the deletion was confirmed by PCR.

Rich media (YPD) and defined media (SD) were prepared as previously described (Sherman, 1991). Media with defined iron content were prepared using modified SD media in which iron and copper were omitted, and the following were supplemented: 25 mM MES pH 6.1, 1.0 mM ferrozine (a ferrous iron chelator, Fluka), 1 μM copper sulfate and ferrous ammonium sulfate at varying concentrations. Ferrozine (1.0 mM) becomes saturated at ferrous iron concentrations above 330 μM, and a range of added ferrous iron from 50 to 330 μM represents a range of free ferrous iron from 10^{-12} to 10^{-8} M (Chaney, 1988). For flow cytometry and immunoprecipitations, yeast were grown to mid-log phase in SD media, then inoculated at an OD_{600} of 0.2 into defined-iron media and grown for 4 h. For expression of genes under the control of the Gal1,10 promoter, strains were grown in SD media containing 2% raffinose instead of glucose before being exposed to defined-iron media containing 2% raffinose and 0.2% galactose.

Microscopy and flow cytometry

Samples of cultures for microscopy were fixed in 70% ethanol, sonicated briefly, and photographed at the same magnification using phase contrast optics on a Zeiss photomicroscope equipped with a plan-apo-X63/1.4 N.A. objective. The remainder of the fixed culture was stained with propidium iodide and analyzed by flow cytometry (Espinoza *et al*., 1994). Flow cytometry was performed on a Becton Dickenson Fluorescence Activated Cell Scanner and results were analyzed using CellQuest software. Histograms representing the relative size distribution were obtained from the forward scatter parameter. The remaining histograms reflect the frequency distribution of fluorescence intensity from propidium iodide staining of DNA.

Immunoprecipitations, kinase assay and Northern analysis

Immunoprecipitations were carried out as previously described (Peter, 1994). Lysis buffer contained 50 mM Tris pH 7.5, 300 mM NaCl, 5mM EDTA, 1.0 mM desferal, 0.1% NP-40, 10 mM NaF, 80 mM β-glycerophosphate, 1.0 mM DTT, 1% aprotinin, 1.0 mM AEBSF, 2.0 mM benzamidine, 50 μM leupeptin and 10 μM pepstatin A. Protein concentration was determined by the bicinchoninic acid method (Pierce). Lysates (generally 5 mg) were incubated with 0.5 μl of HA.11 ascites fluid (BAbCo) for 1 h and immune complexes precipitated with protein G–Sepharose beads for 1 h. Immunoprecipitates were washed four times with lysis buffer and aliquots were reserved for assay of histone H1 phosphorylation activity. Beads were boiled in SDS sample buffer, subjected to SDS–PAGE, and transferred to nitrocellulose for immunoblot analysis. HA.11 at a 1:5000 dilution served as primary antibody, polyclonal anti-mouse antisera from rabbit (Jackson ImmunoResearch) at 1:1000 as secondary antibody, and ^{125}I-labeled anti-rabbit antisera from donkey (Amersham) at 1:200 as tertiary antibody. Histone H1 phosphorylation assays were performed as described (Tyers *et al*., 1992) for 10 min at 30°C and the incorporation of ^{32}P into histone H1 was linear with respect to time. RNA was prepared using Trizol reagent (Life Technologies) and glass beads according to the manufacturer's instructions. Northern blot analysis was performed as previously described (Yamaguchi-Iwai *et al*., 1995). Probes used were: for Cln1, a 3.9 kb *Hin*dIII fragment; for Cln2, a 2 kb *Nde*I–*Nco*I fragment (Hadwiger *et al*., 1989); and for Actin, a cloned PCR fragment corresponding to the coding region of ACT1 (Dancis *et al*., 1994). Quantification of immunoblots, Northern blots, and immunoprecipitations was performed by PhosphorImage analysis (Molecular Dynamics) using ImageQuant software.

Biosynthetic labeling

For mild iron stress, strains MTY263 and CPY135 were grown in YPD to an OD_{600} of 0.4 then supplemented with 100 μM methionine, or 100 μM methionine, 1 mM ferrozine and 500 μM FAS for an additional 2.5 h. Yeast were isolated by filtration and washed three times with methionine-free SD media at 30°C. Yeast were recovered in 1.5 ml of methionine-free SD media and incubated at 30°C for 5 min. Trans ^{35}S-label (0.5 mCi) was added and labeling proceeded for 5 min. Chase media (0.5 ml of 200 mM methionine, 200 mM cysteine, and 10 mM cycloheximide in SD) were added and 0.5 ml aliquots of culture were transferred to an equal volume of 10% trichloroacetic acid on ice at 5 min intervals. Cells were washed twice with ice-cold H_2O and lysates prepared. Immunoprecipitations were performed as described above, except that washes contained 0.5% deoxycholate and precipitates were spun through a 1 M sucrose cushion prior to the final wash. Precipitates were subjected to SDS–PAGE followed by autoradiography. For biosynthetic labeling in defined-iron media, strains carrying either pRS416 or pT14 were grown in the indicated concentrations of FAS for 4 h prior to labeling and immunoprecipitation as described above. Quantification of labeled proteins by acid precipitation was performed as described (Bonifacino, 1994). The specific activities of the methionine pools were not determined and were assumed to be equal in determining protein biosynthetic rates.

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