THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 43, Issue of October 22, pp. 44656–44666, 2004

Printed in U.S.A.

Cdc123 and Checkpoint Forkhead Associated with RING Proteins Control the Cell Cycle by Controlling eIF2γ Abundance*

Received for publication, June 2, 2004, and in revised form, July 13, 2004
Published, JBC Papers in Press, August 19, 2004, DOI 10.1074/jbc.M406151200

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Eukaryotic initiation factor 2 (eIF2) is a central regulator of translational initiation in times of growth and times of stress. Here we discovered three new conserved regulators of eIF2 in *Saccharomyces cerevisiae*. *cdc123*, homolog of mammalian *D123*, is a new cell division cycle mutant with a G_2 delay at permissive temperature and a terminal, mating-proficient G_1 arrest point. Cdc123 protein is regulated by nutrient availability. *CHF1* and *CHF2*, homologs of mammalian checkpoint forkhead associated with RING genes, are required for G_2 delay and G_1 arrest of *cdc123-4* and promote G_1 delay when overexpressed. Cell cycle delaying activity and the natural instability of Chf1 and Chf2 depend on the integrity of both domains and association with Cdc123. Genetic analysis maps the Chf1 forkhead associated domain-binding site to the conserved Thr-274 of Cdc123, suggesting that mammalian D123 is a key target of Chfr. Gcd11, the γ subunit of eIF2, is an additional Cdc123-interacting protein that is an essential target of the Cdc123 cell cycle promoting and Chf cell cycle arresting activity whose abundance is regulated by Cdc123, Chf1, and Chf2. Loss of *cdc123* activity promotes Chf1 and Chf2 accumulation and Gcd11 depletion, accounting for the essentiality of Cdc123. The data establish the Cdc123-Chf-Gcd11 axis as an essential pathway for nutritional control of START that runs parallel to the Tor-Gcn2-Sui2 system of translational control.

Eukaryotic cell division cycles consist of a number of dependent and independent gene-controlled sequences that are coordinated by gene-controlled checkpoints that ensure fidelity (1). After cell separation, early in the G_1 phase of the *Saccharomyces cerevisiae* cell cycle, several genes including those encoding Cdc25 (2, 3), a guanine nucleotide exchange factor for Ras, and Cdc35 (4, 5), encoding the Ras-stimulated adenylyl cyclase, and Cdc33 (6), encoding eukaryotic initiation factor 4E, function to detect nutrients and generate signals that result in increased protein synthesis necessary for commitment to a new cell cycle. Later in G_1, at "START," mating pheromones arrest haploid cell cycles to promote conjugation at the arrest point of *cdc28* (7), which encodes cyclin-dependent kinase 1, the highly regulated and highly conserved protein kinase required to drive entry into S phase and M phase in all eukaryotic systems examined (8–14). Although nutrient limitation arrests yeast cells in early G_1 prior to a great deal of protein synthesis and pheromone treatment arrests haploids at late G_1, many of the connections between nutritional signaling and pheromone signaling and the potential for specific translational requirements in late G_1 remain to be explored.

In this study we discover a new conserved *cdc* gene that is nutritionally regulated at the protein level whose mutants arrest, like *cdc28* mutants, as large unbudded cells at the pheromone-sensitive stage in late G_1. In addition, reminiscent of many *cdc28* alleles and like *cdc2* mutants in *Schizosaccharomyces pombe* and other systems, *cdc123-4* mutants have a G_2 delay phenotype in addition to a G_1 block. We show that Cdc123 protein (GenBank™ accession number BK005577) is depleted in nutritionally arrested cells but present in dividing cells and in pheromone-treated cells and that it functions to reduce the level of Chf1 and Chf2 (GenBank™ accession numbers BK005578 and BK005579). Chf1 and Chf2 are the two yeast homologs of the human checkpoint forkhead associated (FHA)[1] with RING (Chfr) protein, which is required for G_2 arrest in response to microtubule-destabilizing agents (15) and is frequently mutationally inactivated in cancer (16–21). Our study provides a fine genetic mapping of the interaction between the FHA domain of Chf1 and the conserved Thr-274 of Cdc123 and shows that this interaction is required for cell cycle delay by Chf1 and RING-dependent destruction of the checkpoint proteins themselves. This result is interesting not only in identification of a specific functional target of the Chfr-homologous FHA domain but also because the finding runs counter to the expectation that a RING protein bound to a phosphoprotein would tag the phosphoprotein for ubiquitin-mediated proteolysis.

Because deletion of *chf1* and *chf2* relieved the G_2 cell cycle delay and G_1 cell cycle block but not the essential function of *cdc123*, we looked for an essential target of Cdc123 regulation and discovered that Gcd11 (22–24), the γ subunit of eukaryotic initiation factor 2 (eIF2γ), is an additional protein interactor of Cdc123. The data indicate that mating-proficient arrest of *cdc123* is achieved by Chf-dependent alteration of eIF2γ abundance, thus connecting the nutritional and pheromone control of START with regulation of translational initiation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—New plasmids are summarized Table I. All plasmids were made by cloning PCR products using restriction sites

* This research was supported by NCI Research Grant CA77738 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) BK005577–BK005579.

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[1] The abbreviations used are: FHA, forkhead associated; Chfr, checkpoint forkhead associated with RING; E3, ubiquitin-protein isopeptide ligase enzyme 3; HA, hemagglutinin; eIF2, eukaryotic initiation factor 2.

TABLE I
Plasmids generated in this study

Name	Cloned gene	Vector
pB119	*CDC123*	pRS414
pB121	*CDC123*	pRS416
pB174	Human*D123* cDNA	p425*GAL1*
pB179	*3xFLAG-CDC123*	pRS414
pB232	*GCD11*	p425*GAL1*
pB271	*CDC123*	pOBD22
pB295	*CDC123*	p425*GAL1*
pB299	*CHF1*	p425*GAL1*
pB310	*CHF1*	pACT2
pB311	*3xFLAG-CDC123*	pRS415
pB312	*CHF2*-2xHA	p425*GAL1*
pB313	*CHF1*-2xHA	p425*GAL1*
pB319	*3xFLAG-CDC123*	pRS416
pB323	*CHF2*	p425*GAL1*
pB331	*chf1-C345S, H350A*	p425*GAL1*
pB332	*chf2-C451S, H456A, 2xHA*	p425*GAL1*
pB333	*chf1-C345S, H350A, 2xHA*	p425*GAL1*
pB334	*chf2-C451S, H456A*	p425*GAL1*
pB350	*CHF1–2xHA*	pRS425
pB351	*CHF2–2xHA*	pRS425
pB396	*chf1-C345S, H350A*	pACT2
pB403	*chf1-G192E*	p425*GAL1*
pB404	*chf1-S220A, H223L*	p425*GAL1*
pB405	*chf1–G192E, 2xHA*	p425*GAL1*
pB406	*chf1–S220A, H223L, 2xHA*	p425*GAL1*
pB408	*chf1-G192E*	pACT2
pB409	*chf1-S220A, H223L*	pACT2
pB424	*3xFLAG-cdc123-T274A*	pRS414
pB426	*cdc123-T274A*	pOBD22
pB463	*GCD11–2xHA*	pRS416
pB464	*cdc123-4*	pRS414

incorporated into the primer sequences indicated in Table II. Yeast genomic DNA was used as a template for PCR unless stated otherwise. The yeast *CDC123* (*YLR215C*) gene was amplified with primers 4952 and 4953. The DNA fragment obtained in this reaction was cloned into vector pRS414 to create plasmid pB119. Plasmid pB121 was obtained by cloning of the BamHI-EcoRI fragment from pB119 into vector pRS416. The NdeI site was created in the pB119 plasmid by site-directed mutagenesis using primer 4960. This restriction site was used to insert a linker obtained by annealing and ligating oligonucleotides 5112 and 5113 to generate plasmid pB179 that expresses a 3xFLAG-Cdc123 fusion protein. Human *D123* coding sequences were amplified from cDNA made from HeLa cells with primers 5106 and 5107. Plasmid pB174 was made by cloning of this PCR product into BamHI and XhoI sites of the p425*GAL1* vector. Plasmid pB319 was obtained by cloning the *CDC123* gene from pB179 into vector pRS416. C-terminal HA-tagged alleles of *CHF1* and *CHF2* were made by PCR with primers 7055 and 7236 (*CHF1*) or 7009 and 7235 (*CHF2*). The products of the yeast genomic DNA amplification with these pairs of primers were cloned into vector p425*GAL1,* and the resulting plasmids were named pB313 and pB312, respectively. These plasmids were used to create RING domain mutants by site-directed mutagenesis. Plasmid pB312 and mutagenic primer 7129 were used to create plasmid pB332 that carries the *CHF2* gene with two point mutations, C451S and H456A. Plasmid pB333, containing a *chf1-C345S, H350A* allele, was obtained by mutagenesis of the pB313 plasmid with mutagenic primer 7128. Primers 7055 and 7008 were used to amplify the *CHF1* gene, which was then cloned into p425*GAL1* to generate plasmid pB299. pB299 was used as a template for site-directed mutagenesis using primer 7128 that resulted in plasmid pB331, carrying *chf1-C345S, H350A*. Plasmid pB323 contains the *CHF2* gene cloned as a PCR product obtained with primers 7003 and 7005 in vector p425*GAL1*. This plasmid and primer 7129 were used to generate plasmid pB334 containing the *chf2-C451S, H456A* allele. The G192E substitution in the FHA domain of *CHF1* was generated from plasmids pB299 and pB313 with primer 7110 resulting in plasmids pB403 and pB405. Plasmids pB404 and pB406, containing the *chf1-S220A, H223L* allele, were obtained by mutagenesis of plasmids pB299 and pB313 with primer 7111. *chf1* alleles were amplified from plasmids pB331, pB403, and pB404 by PCR with primers 7008 and 7010, and the products of these reactions were cloned into plasmid pACT2 and named pB396, pB408, and pB409, respectively. Plasmid pB310 was obtained by cloning of the PCR fragment generated with primers 7008 and 7010, containing wild-type *CHF1*, into the pACT2 vector. To make plasmid pB271, *CDC123* was amplified with primers 7073 and 7074 and cloned into vector pOBD22. Plasmid pB424 carrying the *3XFLAG-cdc123-T274A* allele was made using mutagenic primer 7303 and single-stranded DNA obtained from plasmid pB179. The tag-free *cdc123* allele of plasmid pB424 was amplified with primers 7073 and 7074 and cloned into plasmid pOBD22. This plasmid was named pB426 and was used for two-hybrid assays. The HA-tagged allele of *GCD11* was created by two-step PCR. In the first step, the fragment of yeast genomic DNA was amplified with primers 7796 and 7797. The product of this reaction was re-amplified with primers 7796 and 7798 and cloned between SacI and XhoI sites of plasmid pRS416. The resulting plasmid, pB463, contains the *GCD11* promoter controlling expression of *GCD11* with two C-terminal HA epitopes. To create plasmid pB232, the *GCD11* open reading frame was amplified using primers 7830 and 7831. The product of this reaction was cloned in plasmid p425*GAL1* using BamHI and XhoI restriction sites incorporated in the primers. Plasmid pB295 carrying the *CDC123* gene under *GAL1* promoter control was created by cloning the PCR product generated with primers 7832 and 7833 between BamHI and XhoI sites of plasmid p425*GAL1*. Plasmid pB350 was made by replacing the *GAL1* promoter in plasmid pB313 with the *CHF1* promoter amplified with primers 7045 and 7046. Replacement of the *GAL1* promoter in plasmid pB312 with the PCR product generated in a reaction with primers 7047 and 7048 yielded plasmid pB351 that contains the *CHF2* gene controlled by its native promoter.

Construction of Yeast Strains—Gene disruptions were made by direct transformation of the appropriate yeast strains with the PCR products (25). Plasmid pRS400 was used as a template for PCR amplification of the geneticin resistance marker with primers 4949 and 4950. The *cdc123*-disrupting product from this reaction was used to transform diploid *S. cerevisiae* strain SEY6210.5. Integration of the marker into the *YLR215C* locus was confirmed by PCR with primers 4948 and 4951. A resulting *cdc123* heterozygous strain BY116 was transformed with the plasmids pB121, pB174, or pB179. Transformants carrying these plasmids were induced to sporulate, and resulting tetrads were dissected using a micromanipulator (26). Each dissection yielded viable G418-resistant haploids, whereas dissection of nontransformed BY116 did not yield G418-resistant haploids. Dissections were performed on YPD plates, except for the transformant carrying plasmid pB174, which was dissected on YPGal medium. Haploids selected for the further experiments were named BY116a[pB121], BY116a[pB174], and BY116a[pB179]. The strain obtained by screening of the mutagenized *CDC123* library for temperature-sensitive mutants was named BY116a [pB464].

The *CHF1* gene in strain SEY6210 was replaced by the *HIS3* marker amplified from pRS413 with the primers 7011 and 7012. A homologous deletion by recombination was confirmed by PCR with primers 7013 and 7014. This strain was named BY225. Strain BY226 was constructed from strain SEY6211 by *CHF2* gene replacement with the *TRP1* marker amplified from plasmid pRS414 with primers 7006 and 7007. Proper integration was confirmed with primers 7002 and 7004. A diploid strain heterozygous for both *CHF1* and *CHF2* was created by mating strains BY225 and BY226. One of the *CDC123* gene alleles in a resulting diploid was replaced by a geneticin resistance marker, as described above, to create the triply heterozygous strain BY229. This strain was transformed with plasmid pB319, induced to sporulate, and dissected. One of the haploids obtained from this dissection, with the genotype *cdc123*::*kanMX*, *chf1*::*HIS3*, *chf2*::*TRP1*, carrying plasmid pB319 was named BY232.

The *GCD11* open reading frame was disrupted in strain BY116 (heterozygous for *cdc123*::*kanMX*) by integrative transformation with a DNA fragment obtained by amplification of the *HIS3* marker from plasmid pRS413 with primers 7799 and 7800. Correct integration of the marker into the *GCD11* locus was confirmed by PCR with primers 7801 and 7802. The resulting strain was transformed with plasmids pB464 and pB463. Selected double transformants were subjected to sporulation and tetrad analysis. This procedure yielded isolates named BY290-1a and BY290-14a with both *cdc123* and *gcd11* genes disrupted, carrying plasmids pB464 and pB463. Two other strains generated in this procedure, BY290-3a and BY290-4d, are wild type at the *CDC123* locus and carry a *gcd11*::*HIS3* mutation covered by plasmid pB463. *bar1* deletion was created by transformation of the diploid strain with the PCR product generated with primers 7787 and 7788 using plasmid pRS413 as a template for this reaction. Transformants were tested for integration of the *HIS3* marker into the *bar1* locus with primers 7785 and 7786. A *MATα* haploid generated after sporulation of this strain was crossed to strain BY116a carrying plasmid pB464. Sporulation and dissection of the resulting diploid yielded strain BY286 of genotype *MATa cdc123*::*kanMX bar1*::*HIS3* [pB464] for assays of pheromone sensitivity.

Two-hybrid Screening and β-Galactosidase Assays—Two-hybrid

TABLE II
Oligonucleotides used in this study (5′ to 3′)

4948	CCATTTATGCAGTGGGAAGATTAAGAAGCTAATGATACCGTAACTCCACCAGATTGTACTGAGAGTGCAC
4949	GCGGATATTTTCGATGTCC
4950	ATACAACAGGGTAATAAAAAGACGAATATATTCAAATGCGGGTTCTAAATCTGTGCGGTATTTCACACCGC
4951	CTCTTCCTTGAAGATTGACG
4952	GATC**GGATCC**TTACCTGACGATTATCAAGTG
4953	CTAG**GAATTC**TTGACGTTGAACTAGAAGAAG
4958	CAGTTGACA**CATATG**AAGAAGGAGCATGTGCTTCACTGC
4959	CACAGT**GTCGAC**TCAGTCGTCCTCCTGCTGATTTCTC
4960	GACATAAATTACTTA**CATATG**TTCTCACAAGAA
5088	AGATCTC**GGATCC**AGCCAAATGACGCTG
5089	CCTTGTTAAGAATTCTTGCACGCATAC
5106	GGTCATCGC**GGATCC**ATGAAGAAGGAGCATGTGC
5107	CCAGTACCG**CTCGAG**TCAGTCGTCCTCCTGCTG
5112	TATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGC
5113	TAGGCCTTGTCATCGTCATCCTTGTAATCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCCA
7002	GACT**GAATTC**TCCCTTGCCCCAGTGTACAC
7003	CAGT**CTCGAG**TTAAGGATGGCTATCAACATCC
7004	ACGAGTTCAATTCTAAGTTACAGAGATGTGAC
7005	GACT**GAATTC**ATGCCTATATGATTATGTATCG
7006	GCAACGCATTGTATACGTTTGAAGCTTTCAGCAAAGGATATACCGTGGAAAGATTGTACTGAGAGTGCAC
7007	GGAGTTCGGTGGTTTTTCTTTATTTTTCAAACTGTGTATTTTCTTTGACCCTGTGCGGTATTTCACACCG
7008	TCAGT**CTCGAG**TCAATCTACTAGGCGAACACC
7009	GACT**GAATTC**ATGTACACGCCTATCCCTGCTAATACC
7010	TCGC**CCCGGG**TCTACTAATACTGTTCCCTC
7011	ACAGTGTCGATTTCCTGTGCACATACGTTCTGTATAGTGGTTATTCCTGGAGATTGTACTGAGAGTGCAC
7012	GTGGATGAAGTATCTATAAACGGGGTTAGCCTAATGCTGAAAAGCCCGTGCTGTGCGGTATTTCACACCG
7013	CACATACGTTCTGTATAGTGG
7014	AGACCCTGGTTAGCCACAGAC
7045	CCAGG**GAGCTC**TTATGTTCGGTATATACAAGTATGTAGAG
7046	GGTCC**GGATCC**AGGAATAACCACTATACAGAACGTATGTG
7047	GGACC**GAGCTC**GGAGGGGTGTTCATGCCATGG
7048	GCCTT**CTGCAG**CCACGGTATATCCTTTGCTGAAAGCTTC
7055	GACT**GGATCC**ATATGTCTACTAATACTGTTCCCTC
7073	CATGA**CCATGG**TCTCACAAGAATATACAAC
7074	CATGT**CTGCAG**CTAAGTTTCACTCTCAC
7110	TCTTTCGGTGTATCTTTCGATGATAATTTGCGA
7111	AACTTTGAAACACCCAAGGGTCCTGGCTATCACTTTTGATTT
7128	TCTTACACAACGGAAAGCCCAGCTATGTGCGGATGGTGATATGAAAAT
7129	TCTTACACAATGAAAAGCCCAACTATGGGCAGATGGTGATATAAAAT
7235	CAA**CTGCAG**ATGTACCCATACGACGTACCTGATTACGCATATCCGTATGATGTGCCGGACTATGCTTACACGCCTATCCCTGCTAA
7236	ACG**GGATCC**ATGTACCCATACGACGTACCTGATTACGCATATCCGTATGATGTGCCGGACTATGCTTCTACTAATACTGTTCCCTC
7303	GTTCCATGAAAATAGCAAAGAATCTGCCTTCCTGGCAAACGGATTTATATC
7785	ATAAAAAGAGTGTCTAGAAGGGTC
7786	TATGCTATAAAGAAATTGTACTCCAG
7787	ATGTCTGCAATTAATCATCTTTGTTTGAAACTTATTTTGGCGAGTTTCGCAGATTGTACTGAGAGTGCAC
7788	TTAATATGTTGGTTTACAGACATTTATTAAGACTGTAGAATACTTCGTGACTGTGCGGTATTTCACACCG
7796	GCCGGAGCTCTTCGGCATCTTCAGCTAC
7797	CATACGGATATGCGTAATCAGGTACGTCGTATGGGTAAGCGATGGGTTCCAATGTAG
7798	CGC**CTCGAG**TCATTAAGCATAGTCCGGCACATCATACGGATATGCGTAATCAG
7799	AATAAACATCGGTACCATCGGTCATGTCGCCCACGGTAAATCCACAGTAGAGATTGTACTGAGAGTGCAC
7780	TGCTTTTGACCATCTGTTTTGACACCTAATAGACGACGTAGCAAAAAGAGACACGCCATAAAGTGTGGC
7801	CGAAATTATCAACAGGCAAGCTAC
7802	GACCATAAGAACTTCATTTGGCTC
7830	GACTC**GGATCC**ATGAGTGACTTACAAGACC
7831	AGTCG**CTCGAG**TTAAGCGATGGGTTCCAATG
7832	GACAT**GGATCC**ATATGTTCTCACAAGAATATACAAC
7833	GCTGT**CTCGAG**CTAAGTTTCACTCTCACTATCACTG

screening was performed as described using plasmid pACT2, a yeast cDNA library in λACT2, and yeast strain Y190 (27, 28). The bait plasmid was created by cloning the *CDC123* open reading frame in plasmid pOBD (29). To create prey plasmids for β-galactosidase assays, wild-type and mutant *CHF1* alleles were cloned into plasmid pACT2. Y190 cells transformed with pairs of bait and prey plasmids were grown on selective liquid media to mid-exponential phase. For assays of β-galactosidase activity, equal numbers of exponentially growing cells were harvested. Assays were performed using 2-nitrophenyl β-D-galactopyranoside as a substrate and normalized to units/min/ml/OD according to the methods of Guarente (30). Results are presented as a percentage of the activity in the wild-type *CDC123*-wild-type *CHF1* sample, which, at 14.4 units/min/ml/OD, was the pair of highest interaction.

Generation of cdc123 Temperature-sensitive Alleles—To generate a library of *cdc123* mutants, the gene was used as a template for error-prone PCR using 100 μM dATP, dGTP, and dTTP and 1 mM dCTP, and amplified products were cloned into vector pRS414. The library was selected for complementation at 28 °C, and transformants were screened for temperature sensitivity at 37 °C as described under "Results."

Flow Cytometric Analysis—DNA was stained with SYBR Green I (Molecular Probes), and flow cytometry was performed using a Beckman Coulter FACScan instrument as described (31).

Western and Co-immunoprecipitation Analysis—A FLAG epitope was inserted at the N terminus of Cdc123. HA epitopes were added to the C termini of Chf1, Chf2, and Gcd11. Strains expressing *FLAG-CDC123* and *GAL1*-driven *CHF-HA* alleles were used to assay Cdc123-Chf interactions. A strain expressing *FLAG-CDC123* and *GCD11-HA* from native promoters was used to test Cdc123-Gcd11 association. Whole cell extracts were prepared by glass bead lysis. For Western analysis, anti-HA and anti-FLAG antibodies and secondary rabbit anti-mouse antibodies conjugated to horseradish peroxidase were from Sigma. For co-immunoprecipitation, lysates were incubated with anti-HA antibodies immobilized on agarose (Santa Cruz Biotechnology) at 4 °C for 3 h. Beads were washed five times with phosphate-buffered saline; antigens were eluted with 8 M urea, and Western transfers were analyzed by immunoblotting with anti-FLAG antibody conjugated to horseradish peroxidase (Sigma). Reactions were developed with chemiluminescent peroxidase substrate (Sigma).

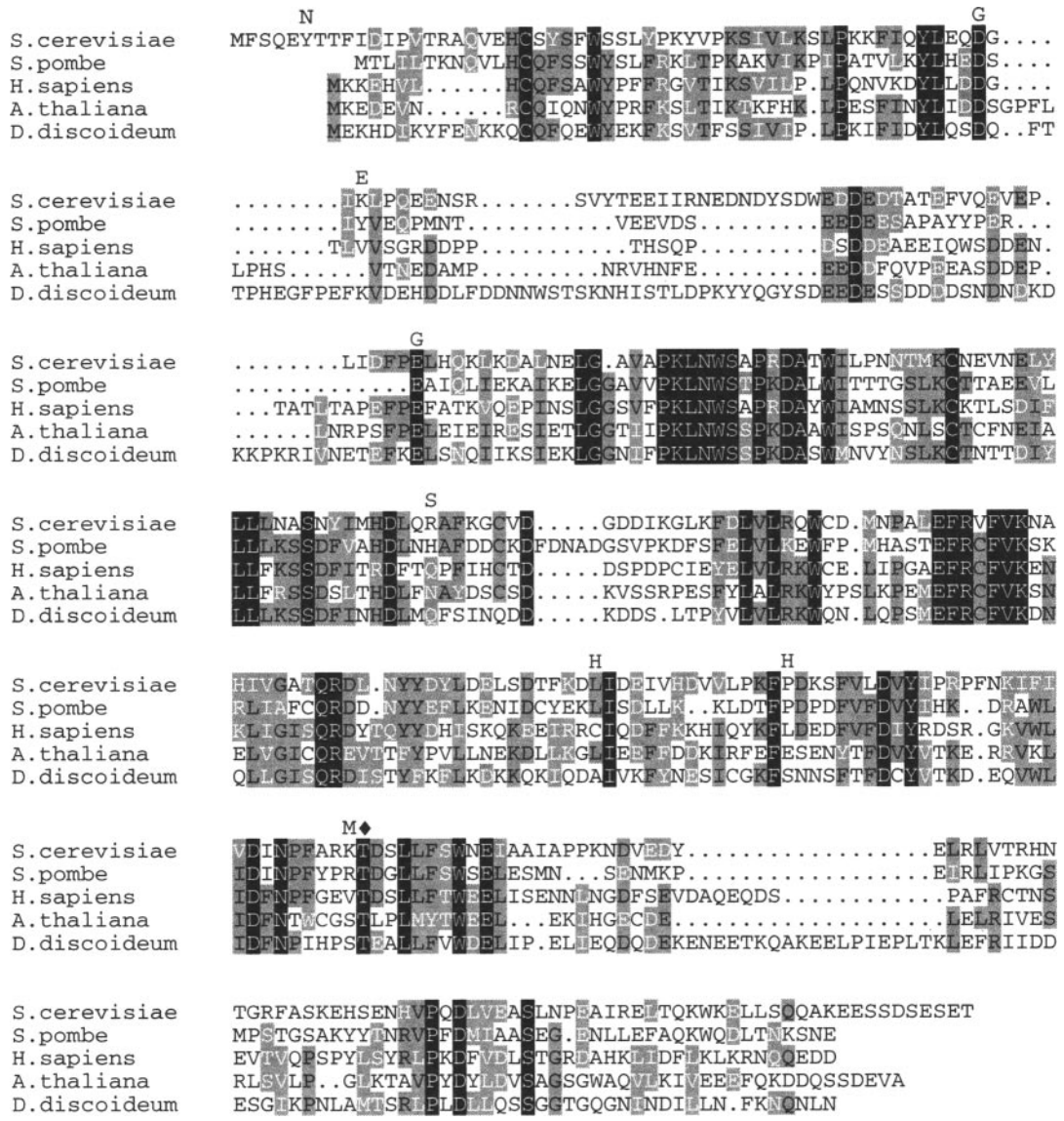

FIG. 1. **Conserved sequences of Cdc123 orthologs.** Protein sequence alignment of Cdc123 and D123 homologs from human, *S. cerevisiae*, *S. pombe*, *A. thaliana,* and *D. discoideum*. *White letters* in *black boxes* indicate residues identical in all sequences. *Black letters* in *gray boxes* indicate residues conserved in three or more sequences. *White letters* in *gray boxes* denote residues that are similar in at least three proteins. Amino acid substitutions in the *cdc123-4* allele are marked *above* the yeast protein sequence. Thr-274 in *S. cerevisiae* Cdc123 is marked with ♦.

RESULTS

CDC123 Is the Functional and Structural Homolog of Mammalian D123—The human *D123*-coding sequence was first isolated from a cDNA expression library as a suppressor of the temperature-sensitive mutation in rat cell line 3Y1tsD123, which arrests in G_1 phase at restrictive temperature (32). The mutant D123 protein has an Ala-109 → Val alteration in sequence (32) and was reported to result in a polypeptide that is degraded at the restrictive temperature (33) in a lactacystin-sensitive manner (34). The *D123* gene is expressed in the cytosol of human and rat tissues, with expression in testis being highest (35).

Comparison of human D123 protein with sequences deposited in GenBank™ revealed apparent orthologs in animals, fungi, plants, and protists. An alignment of *D123* proteins from human, *S. cerevisiae*, *S. pombe, Arabidopsis thaliana,* and *Dictyostelium discoideum* is presented in Fig. 1. To study function of the 35% identical *S. cerevisiae* protein, the sequence of which was deposited in GenBank™ (accession number BK005577), we made a geneticin-resistant disruption of the YLR215C gene (hereafter *CDC123*), in yeast diploid cells to generate strain BY116, heterozygous at *cdc123*. After meiosis, sporulation and dissection of spores, we found that haploid isolates segregated 2:2 for viability, and none of the viable isolates were geneticin-resistant (Fig. 2*A*). Examination of the nongrowing isolates revealed micro-colonies of ~8–16 cells, suggesting, as in the case of *CAK1* (36, 37), that *CDC123* encodes an abundant and/or long lived essential protein that is depleted in mutant cells after three to four divisions. To exclude the possibility that the gene is required for spore germination and is later dispensable, a human *D123* cDNA from HeLa cells was amplified and cloned into a yeast vector under the control of the *GAL1* promoter. Dissection of the BY116 strain transformed with this plasmid yielded tetrads with four viable progeny (Fig. 2*B*). In these tetrads, geneticin resistance segregated 2:2, and geneticin-resistant colonies exhibited galactose-dependent growth (Fig. 2*C*). Additionally, *URA3*-based plasmid pB121 constructed to express yeast *CDC123* from its own promoter, introduced into heterozygous diploids and recovered in *cdc123*Δ haploids, could not be cured although the same plasmid could be easily lost from *CDC123* wild-type cells (data not shown). These results show that the *CDC123* gene is essential for

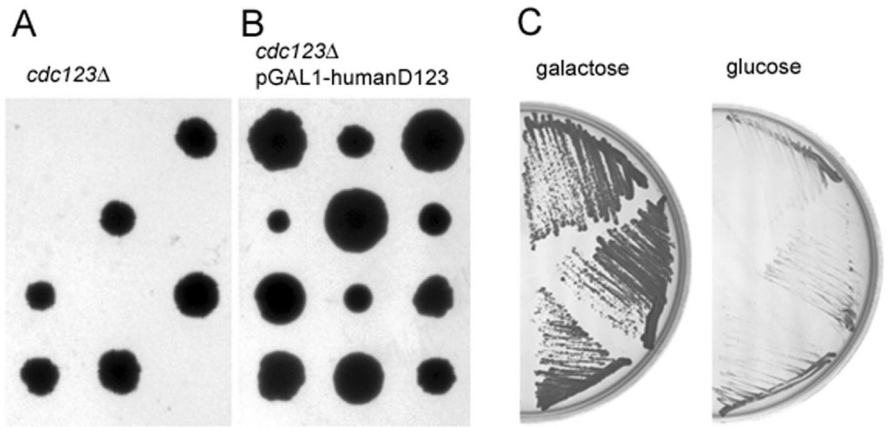

FIG. 2. ***CDC123* is a *D123* homologous essential gene.** *A*, *CDC123* is an essential gene. Dissection of a *CDC123*/*cdc123*::*kanMX* diploid strain yielded two viable geneticin-sensitive spores per tetrad. *B*, human *D123* cDNA complements deletion of yeast *CDC123*. The *cdc123* heterozygous diploid strain transformed with a plasmid expressing *GAL1*-driven human *D123* dissected on galactose plates yielded four viable spores, two of which carry *cdc123* deletion and a plasmid with the human *D123* cDNA. *C*, growth of *cdc123* deletion isolates depends on human *D123* expression. Three independent isolates exhibited galactose-dependent growth.

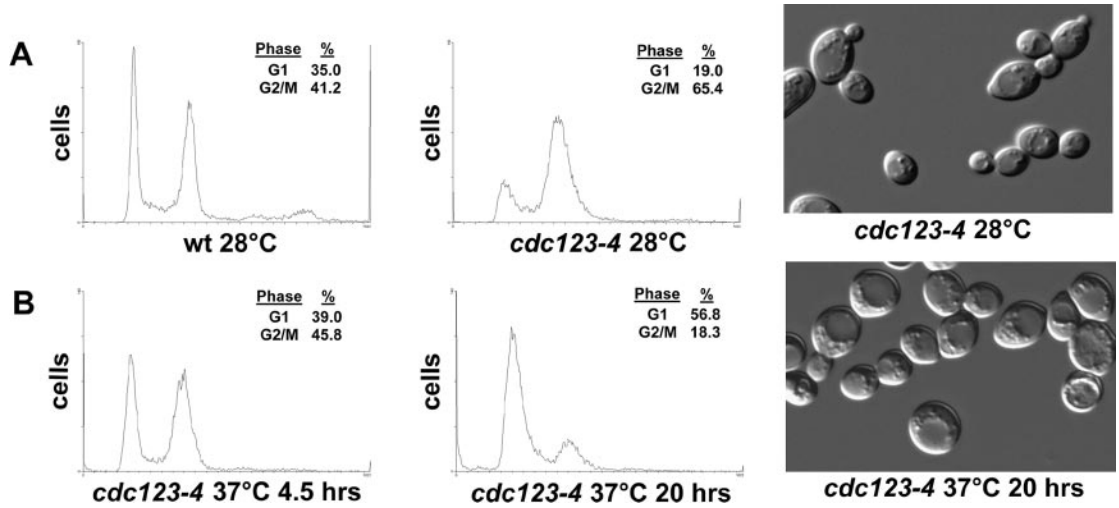

FIG. 3. ***cdc123-4* cells have a G_2 delay at permissive temperature and a large G_2 *cdc* arrest at restrictive temperature.** *A*, flow cytometric analysis of logarithmically growing wild-type and *cdc123-4* cells at 28 °C and microscopic analysis of the mutant illustrate that mutants have a G_2 delay phenotype at permissive temperature. *B*, a culture of *cdc123-4* mutant cells transferred to 37 °C for 4.5 and 20 h illustrates the terminal G_1 *cdc* phenotype.

viability and that the mammalian *D123* gene is a structural and functional homolog of *CDC123*.

Mutation of cdc123 Results in G_2 Delay and G_1 Cell Cycle Arrest—To determine the essential function or functions of *CDC123*, the products of error-prone PCR amplification of the gene were cloned in a yeast vector with a *TRP1*-selectable marker to generate a mutagenized library. This library was transformed into the *cdc123*Δ haploid strain carrying plasmid pB121. Transformants were selected on 5-fluoroorotic acid to select for pB121 plasmid loss (38), indicating complementation of *cdc123*Δ at 28 °C, and then screened for temperature sensitivity at 37 °C. This procedure yielded a number of temperature-sensitive alleles. The sequence of the *cdc123-4* allele was determined, and its phenotypes were studied. As shown in Fig. 1, conceptual translation of *cdc123-4* indicates eight amino acid substitutions, two altering the strictly conserved residues Asp-55 and Glu-106.

As shown in Fig. 3*A*, when analyzed by flow cytometry at the permissive temperature of 28 °C, *cdc123-4* cells have an excess of G_2 cells compared with isogenic *CDC123* wild-type cells, and the overall generation time is slowed (data not shown). Upon shifting to the nonpermissive temperature of 37 °C, G_1 cells accumulate, although the terminal phenotype of large G_1-arrested cells takes ~20 h to achieve (Fig. 3*B*). Consistent with the observation that *cdc123*Δ spores from a *CDC123*/*cdc123*Δ tetrad dissection divide three to four times before dying, these data suggest that *CDC123* encodes an essential and abundant and/or relatively stable protein. Moreover, despite the partial G_2 block that is seen at the *cdc123-4* permissive temperature, the ability for *cdc123-4* temperature-shifted cells to accumulate in G_1 indicates that the most stringent requirement for the Cdc123 polypeptide is at G_1 cell cycle entry.

Cell division cycle mutants with G_1 phenotypes have been placed into two classes on the basis of whether they arrest as small, unbudded cells prior to sensitivity to mating pheromone or, as is the case of the classic START mutant *cdc28*, concomitant with the pheromone stage (7, 39). The large size of arrested *cdc123-4* mutants suggested a late G_1 block but, as shown in Fig. 4*A*, temperature-arrested cells released into media with α-mating pheromone at 28 °C formed shmoos rather than proceeding to bud emergence and shmooing after the next cell division. This experiment indicates that the *cdc123-4* arrest point is prior to or at the mating pheromone-sensitive point in the cell division cycle. To distinguish between these possibilities, cells arrested at the nonpermissive temperature were treated with pheromone. As shown in Fig. 4*B*, these

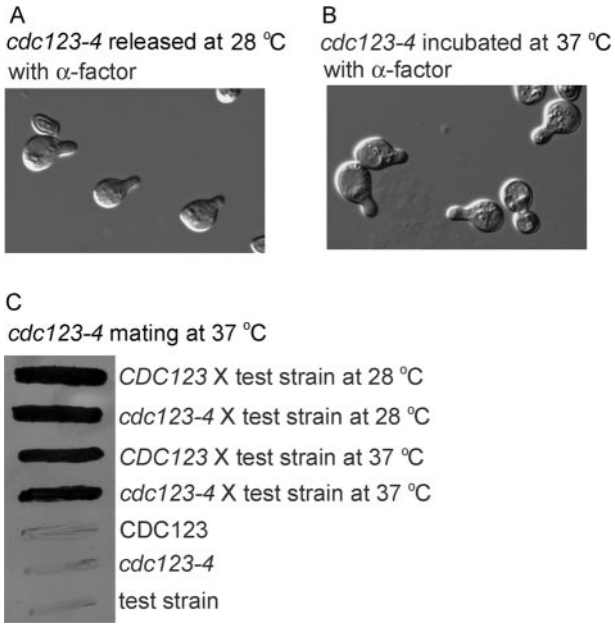

FIG. 4. ***cdc123-4* mutants arrest in a pheromone-sensitive and mating-competent state.** *A*, *MAT**a*** *bar1 cdc123-4* cells arrested for 16 h at 37 °C were released to the permissive temperature in the presence of α-factor. Cells released from temperature arrest formed shmoos without prior cell division. *B*, photomicroscopy of α-factor added to the same temperature-arrested culture at 37 °C indicated that the terminal arrest phenotype is pheromone-sensitive. *C*, wild-type and *cdc123-4* strains were tested for the ability to mate at the restrictive temperature. Cell division cycle arrest of the mutant does not preclude mating.

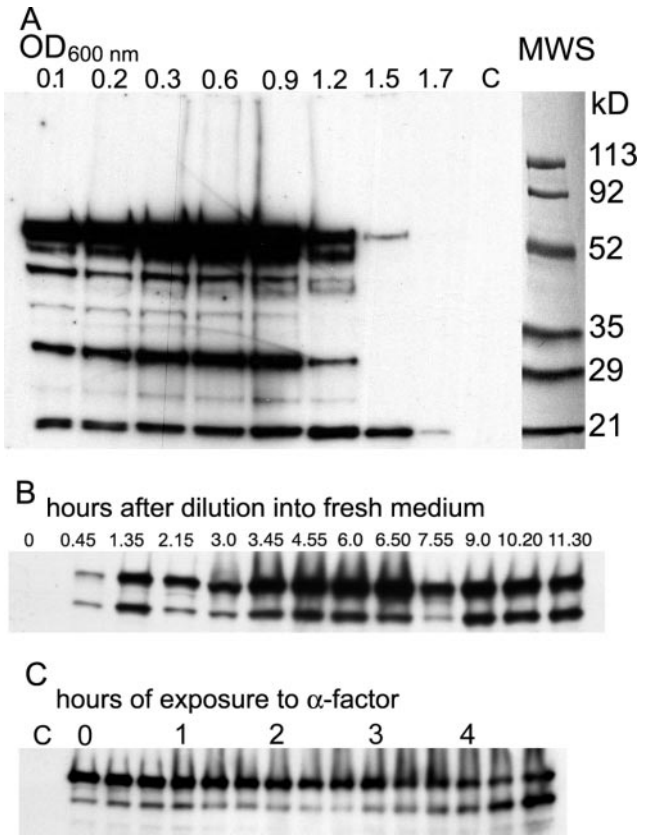

FIG. 5. **Nutrient-sensitive and pheromone-insensitive expression of Cdc123 protein.** Western analysis of Cdc123 expression. Protein extracts from equal numbers of yeast cells expressing FLAG-Cdc123 as a sole source of this protein were loaded in each lane. *A*, samples prepared from cultures of increasing cell density indicate that expression of Cdc123 decreases in cultures reaching saturation. The *C lane* is a control strain not expressing FLAG-tagged Cdc123 to establish that all signals are due to FLAG-Cdc123. *B*, Cdc123 is undetectable in stationary cultures (time 0) but reappears during the first cell cycle after conditions for growth are restored. The culture was continually diluted to maintain logarithmic phase. *C*, Cdc123 protein levels are unaffected by addition of mating pheromone.

arrested cells shmoo upon addition of pheromone. Additionally, as shown in Fig. 4*C*, arrested cells mate at the nonpermissive temperature. Thus, the data indicate that Cdc123 is a previously unrecognized component of START.

Nutritional Control of Cdc123 Protein Expression—To follow expression of the Cdc123 protein, we constructed a plasmid to express Cdc123 with an N-terminal FLAG tag, and we introduced this plasmid into a *CDC123/cdc123*Δ heterozygous strain. Recovery of viable haploid cells bearing the geneticin resistance marker after sporulation and tetrad dissection confirmed that FLAG-tagged Cdc123 retained biological activity. Western analysis of wild-type cells or cells bearing FLAG-tagged Cdc123 in place of wild type established the specificity of the anti-FLAG antibody in reporting the covalent sizes of Cdc123 in yeast cells. Bands of ~60, 50, 40, 30, and 20 kDa were found in cells expressing N-terminally FLAG-tagged Cdc123 and were absent in cells not expressing FLAG-tagged Cdc123 (the predicted size of the FLAG-tagged protein is ~45 kDa). Moreover, when equal cell numbers were extracted from a culture proceeding from exponential growth into stationary phase, the level of Cdc123 protein per cell was shown to decline as cells enter stationary phase (Fig. 5*A*). In cells recovering from stationary phase into fresh media, protein increases dramatically within the first cell division cycle and continues at a high level as long as cells are in exponential phase (Fig. 5*B*). Given that Cdc123 protein disappears and reappears in concert with cell cycle exit and entry and that the *CDC123* arrest point is in late G_1 at the time of pheromone sensitivity, we tested whether pheromone leads to depression of Cdc123 protein levels, thereby causing G_1 arrest. However, the data shown in Fig. 5*C* indicate that α-factor treatment does not alter Cdc123 protein accumulation. Thus, Cdc123 protein accumulation is dependent on nutrient availability, is insensitive to pheromone-mediated arrest, and functions as a positive regulator of cell cycle entry. Additionally, the long lived forms of Cdc123 are consistent with genetic results that show that it takes multiple cell generations to deplete *CDC123* function; proteolysis of Cdc123 takes place over multiple cell cycles.

Interaction of Cdc123 with Checkpoint Forkhead Associated with RING Proteins Mediates FHA Domain and RING Domain-dependent Destabilization of the Chf Proteins—To identify additional components of the cell cycle machinery controlled by Cdc123, we performed a two-hybrid screen and identified both yeast homologs of human checkpoint forkhead associated with RING protein, Chfr. Chfr has been described as a mitotic checkpoint protein (15) whose RING domain is an E3 ubiquitin ligase that reportedly targets Polo-like kinase 1 for destruction (40) and is required for inhibiting chromosome condensation in response to Taxol treatment (41). However, the mechanism of Chfr function in mitotic checkpoint function has recently been questioned with the observation that Chfr participates with the Ubc13-Mms2 heterodimer to lead to auto-polyubiquitination via Lys-63 linkages, a type of modification that does not target proteins for proteolysis but rather functions in stress signaling (42).

The two yeast homologs of Chfr, YHR115C and YNL116W, here named Chf1 and Chf2, are 46% identical to each other at the amino acid sequence level and share the domain structure of Chfr. As shown in Fig. 6*A*, the two characteristic features are an N-terminal forkhead associated (FHA) domain, typically a module for binding Thr-phosphorylated proteins found in cell cycle arrest and/or DNA repair proteins (43, 44), and a C-terminal RING domain, which is thought to be the signature sequence for

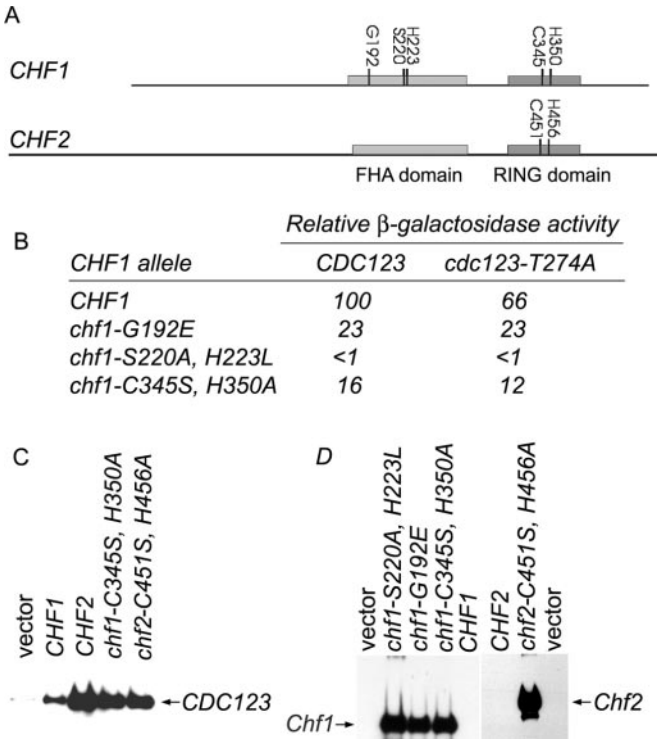

FIG. 6. **Checkpoint forkhead associated with RING proteins interact with Cdc123 and are greatly stabilized by mutations that reduce Cdc123 interaction.** *A*, schematic structures of Chf1 and Chf2 proteins with the indicated FHA and RING domains and mutations used in this study. *B*, relative β-galactosidase activities from assays of wild type of mutant *CHF1* with wild type or mutant *CDC123*. Note that mutations in Chf1 FHA and RING domains decrease Cdc123-Chf1 interactions, whereas mutation of Cdc123 Thr-274 reduces interaction with wild type and a RING domain mutant of Chf1 but shows no additive loss of interaction with alleles of Chf1 that have a mutated FHA domain. *C*, anti-FLAG Western blot of an anti-HA immunoprecipitation from co-expression of different alleles of HA-Chf1 with FLAG-Cdc123. Note that when the anti-HA reagent is not limiting, mutations in the FHA and RING domains of Chf1 do not decrease the amount of Cdc123 that can be brought down with Chf1. *D*, with constant protein loading of whole cell lysates, wild-type Chf1 and Chf2 are undetectable, but alleles with mutations in FHA or RING domain of Chfs cause massive accumulation of these proteins.

E3 ubiquitin ligases (45–50). Indeed the crystal structure of the FHA domain of human Chfr bound to tungstate is consistent with function as a phospho-receptor (51), although the target of this FHA domain had not been identified. Similarly, the RING domain of Chfr has been investigated from the earliest observations of loss of Chfr in human cancer (15, 40–42), although the means by which the RING domain mediates a checkpoint function remains incompletely understood.

As shown in Fig. 6, we made two site-directed mutants in the *CHF1* gene to reduce function of the FHA domain, and we constructed site-directed alleles of both *CHF1*and *CHF2* to inactivate the RING domains. Assaying the two-hybrid interaction as measured by a β-galactosidase assay, the *chf1-G192E* substitution, targeted to an absolutely conserved Gly in box A of FHA domains (51) shown to be functionally important in the kinase-associated protein phosphatase (52), reduced interaction by more than 4-fold, whereas the double mutation of *chf1-S220A,H223L* targeted to the FHA domain (52) reduced the β-galactosidase signal by more than 100-fold. When two putative zinc-ligating residues of the RING domain were targeted by the *chf1-C345S,H350A* allele, a 6-fold reduction in two-hybrid signal with *CDC123* was observed. Obviously, two-hybrid signals could have arisen from a variety of artifactual phenomena, and reductions in two-hybrid signals could indicate reductions in protein stability or other unanticipated effects of the mutations. To test whether wild-type or mutant Chf proteins form physical complexes with Cdc123, the stability of which might be affected by Chf protein sequences, we expressed HA-tagged *CHF* genes with the *GAL1* promoter in a yeast strain in which FLAG-tagged Cdc123 replaced untagged Cdc123. As shown in Fig. 6*C*, by probing HA immunoprecipitates with an anti-FLAG antibody, we demonstrated that both wild-type Chf1 and Chf2 proteins are physically associated with Cdc123 *in vivo*. Curiously, however, this assay did not detect a reduction in association of the RING domain alleles, Chf1-C345S,H350A and Chf2-C451S,H456A, in association with Cdc123, although the former allele was shown to reduce the two-hybrid interaction. To explore whether the disparity between two-hybrid and immunoprecipitation might be because of differences in protein stability, we simply probed total protein in HA-Chf1 and HA-Chf2 transformants for the abundance of wild-type and mutant Chf proteins. Remarkably, as shown in Fig. 6*D*, under conditions in which HA-tagged wild-type Chf1 and HA-tagged wild-type Chf2 are undetectable by chemiluminescent Western blot, the two mutant Chf1 constructs with mutations in the FHA domain and the Chf1 and Chf2 mutants with alterations in the RING domain are tremendously abundant. These data indicate that Cdc123 protein interacts with Chf1 and Chf2, that the FHA domain and the RING domain of Chf1 contribute to the interactions, and that the dysfunction of either domain results in dramatic *stabilization* of the proteins. The data also indicate that the constant amount of Cdc123 protein co-immunoprecipitated with mutant Chf1 and Chf2 in Fig. 6*C* is driven by the huge increase in Chf1 and Chf2 proteins due to mutation of the RING domains.

Chf Proteins Promote G_1 Cell Cycle Delay via Interaction between Chf Protein FHA Domains and Conserved Thr-274 of Cdc123—The physiological effects of Chf protein overexpression were explored. As shown in Fig. 7*A*, we noticed that on galactose media, *GAL1*-driven *CHF1* or *CHF2* expression leads to growth retardation and a large increase in G_1 cells, reminiscent of the terminal phenotype of *cdc123-4*. With the knowledge that mutations in the FHA domain and the RING domain of Chf proteins stabilize Chf1 and Chf2 while reducing interactions with Cdc123, we examined whether any of these mutations affected the toxicity of Chf overexpression. As shown in Fig. 7*B*, expression of either *GAL1*-driven wild-type *CHF1* or *CHF2* protein greatly retards growth on galactose plates with *GAL1-CHF1* being almost fully growth-inhibitory. Mutation of the FHA domain of Chf1 with *chf1-G192E* or *chf1-S220A,H223L* substitutions, both of which reduced interaction with Cdc123, resulted in alleles with only mild toxicity when expressed from the *GAL1* promoter. In addition, the expression of the RING domain allele, *chf1-C345S, H450A*, exhibited reduced toxicity with respect to wild type. Because designed FHA domain alleles and RING domain alleles are both greatly stabilized *in vivo*, as shown in Fig. 6, these data indicate that the *in vivo* specific activities of the FHA domain and the RING domain mutants of Chf proteins are greatly reduced in growth retardation.

Based upon the two-hybrid and co-immunoprecipitation data, the similar cell cycle kinetics between Chf protein overexpression and *cdc123-4* temperature shift, and the fact that mutant Chf proteins with reduced Cdc123 interaction are stabilized *in vivo*, we hypothesized that the FHA-dependent cell cycle delay caused by Chf protein overexpression is mediated by direct interaction with a phosphorylated residue of Cdc123. In the case of the two FHA domains of Rad53, phosphopeptide selection experiments indicated strong binding specificity for an Asp and a hydrophobic amino acid in the +3 position with respect to phosphothreonine for FHA domains 1

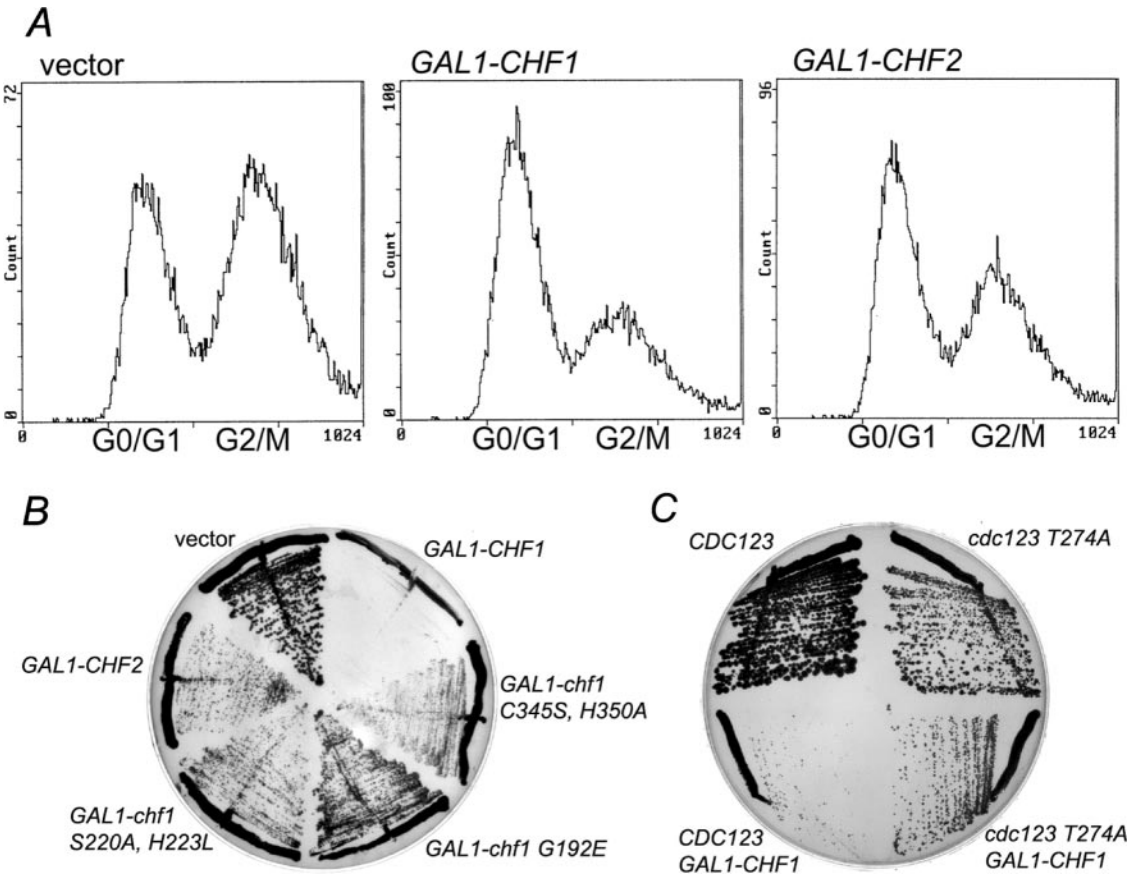

FIG. 7. **Chf protein overexpression imposes G_1-delaying toxicity in a manner that depends on the Chf FHA and RING domains and conserved Thr-274 of Cdc123.** *A*, wild-type yeast cells transformed with a control plasmid or plasmids expressing Chf1 or Chf2 from the *GAL1* promoter were grown on galactose and analyzed by flow cytometry. Overexpression of Chfs causes accumulation of cells in G_1. *B*, cells carrying plasmids with *GAL1*-controlled alleles of *CHF* genes were grown on galactose medium. Overexpression of wild-type *CHF1* and, to a lesser degree, *CHF2* causes a slow growth phenotype. Although the FHA domain and RING domain mutants of Chf1 accumulate greatly, they exhibit less toxicity. *C*, cells expressing wild-type *CDC123* or the hypomorphic *cdc123-T274A* allele were transformed with empty vector or a plasmid overexpressing *CHF1*. Chf1 toxicity largely depends on Thr-274 of Cdc123 and lack of Thr-274 of Cdc123 provides a growth advantage in cells overexpressing Chf1.

and 2, respectively (43). We therefore scanned Cdc123 homologs (Fig. 1) for conserved T*XX*D or T*XX*ϕ (where ϕ indicates a hydrophobic amino acid) motifs, and nominated Thr-274 as a potential site of a putative direct association with the FHA domains of Chf proteins. As shown in Fig. 7*C*, we constructed a strain carrying *cdc123-T274A* as the sole source of *CDC123*, and discovered this to be a hypomorphic allele with a slow growth phenotype. As shown in Fig. 5*B*, by quantitative β-galactosidase analysis of constructed two-hybrid partners, the Cdc123-T274A allele has a reduced interaction with wild type and the RING domain mutant of Chf1. Consistent with the idea that Cdc123 interacts in a phosphothreonine-dependent manner with the Chf FHA domain and in another manner with the RING domain of Chf proteins, the Cdc123-T274A construct reduced the interaction with forms of Chf1 that have an intact FHA domain but did not reduce the interaction with the two Chf1 constructs that possess mutated FHA domains. To test whether the growth retardation of Chf1 overexpression, a phenomenon shown above to depend on the Chf1 FHA domain, depends on interaction with the putative phospho-Thr of Cdc123, we expressed *GAL1*-driven *CHF1* in the *cdc123-T274A* background. As shown in Fig. 7*C*, despite the slow growth phenotype of *cdc123-T274A* with the specific loss of the putative FHA domain-recognized phospho-Thr in Cdc123, overexpression of Chf1 imposes little growth retardation. In fact, in the background of *GAL1-CHF1*, loss of Thr-274 from Cdc123 confers a strong growth *advantage*. These genetic data establish Cdc123 as a direct target of the FHA domain of Chf1 and indicate that recognition of Cdc123 by the FHA domain of Chf1 protein mediates the Chf-dependent cell cycle delaying function.

Cdc123 Expression Mediates Destabilization of Chf Proteins and Not the Other Way Around—Given that Chf protein overexpression causes a G_1 cell cycle delay similar to *cdc123-4* temperature shift and that Chf proteins contain a RING domain reported to be an E3 ubiquitin ligase for cell cycle-promoting factors such as Polo-like kinase 1 (40), we initially hypothesized that recognition of Cdc123 by Chf proteins would target Cdc123 for ubiquitin-mediated proteolysis, leading to cell cycle arrest. However, defying expectations based on the association of Chf RING proteins with the presumed phospho-Cdc123 through the Chf FHA domain, as we demonstrate in Fig. 8*A*, *GAL1*-mediated expression of the G_1-retarding Chf1 or Chf2 proteins does not lead to Cdc123 destabilization. Moreover, expression of the RING domain alleles of Chf1 and Chf2 showed no evidence of affecting Cdc123 accumulation or mobility. Although unprecedented, it was equally plausible that Cdc123 drives cell cycle progressions via interaction with Chf proteins with this interaction leading to destruction of the Chf proteins. To test this hypothesis, tagged forms of each *CHF* gene were placed under the wild-type promoter of each gene, and the constructs were transferred to a *cdc123-4* strain. As shown in Fig. 8*B*, Chf1 and Chf2 proteins are difficult to detect in logarithmically growing *cdc123-4* cells at the permissive temperature of 28 °C. However, when cells were shifted to the

restrictive temperature of 37 °C, cells arrested with abundant Chf1 and Chf2 protein. As Chf overexpression significantly retards the cell cycle, these data suggest that one of the important functions of Cdc123 is to promote, through a mechanism that depends on Cdc123 Thr-274 binding to the FHA domains of Chf proteins, the RING-dependent destruction of Chf proteins.

Deletion of chf1 and chf2 Suppresses the cdc Phenotype but Not the Essentiality of cdc123—To explore further *CHF1*- and *CHF2*-dependent processes, we prepared a *chf1 chf2* deletion strain and a strain in which *cdc123-4* was accompanied by *chf1 chf2* deletion. The *chf1 chf2* deletion was not inviable, and the loss of *chf* genes neither aggravated nor abrogated the temperature sensitivity of *cdc123-4* (data not shown). As shown in Fig. 9, flow cytometric analysis of *cdc123-4 versus cdc123-4 chf1*Δ *chf2*Δ strains indicates that although the latter strain is as temperature-sensitive as the former, deletion of *chf1* and *chf2* reduces the G_2 peak of *cdc123-4* cells grown at the permissive temperature and eliminates the G_1 arrest of *cdc123-4* cells shifted to the nonpermissive temperature. Thus, Chf proteins, which are physically stabilized by loss of *cdc123* function, enforce the G_2 and G_1 checkpoints associated with loss of *cdc123* function.

The eIF2 Complex Is an Essential Target of Chf-Cdc123 Regulation—Although *chf1 chf2* deletion makes *cdc123* arrest randomly rather than at G_1, the checkpoint deletion does not relieve the essential function of *CDC123*, suggesting that there are other targets of Cdc123 regulation. Additionally, although Chf proteins apparently make use of an FHA domain-dependent interaction with Cdc123 and a RING and FHA domain-dependent destabilization of the Chf proteins themselves, the residual toxicity of Chf protein mutants suggests that there may be an additional target of the Chf protein RING domains. One additional gene, *GCD11*, which encodes the eukaryotic initiation factor 2 γ subunit, was obtained by two-hybrid screening with Cdc123 as the bait. eIF2 consists in yeast of the Sui2-Sui3-Gcd11 α-β-γ heterotrimeric complex. This complex delivers aminoacylated $tRNA^{Met}$ to the small ribosomal subunit with the γ subunit bound to GTP (53), the β subunit mediating some of the interactions with mRNA (54), and the α subunit as the key target of eIF2 kinases (55).

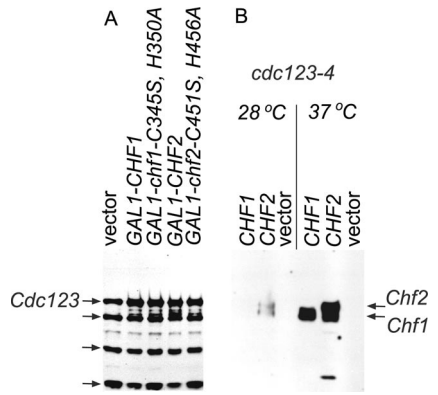

FIG. 8. **Chf expression does not destabilize Cdc123 but Cdc123 dysfunction stabilizes Chf1 and Chf2.** *A,* protein extracts from cells that express FLAG-Cdc123 and carry plasmids containing *CHF* genes under *GAL1* promoter control were probed with anti-FLAG antibody. Overexpression of *CHF1* or *CHF2* does not alter *CDC123* accumulation or mobility. *B,* cells expressing HA-tagged Chf proteins from native promoters in the *cdc123-4* background were grown at 28 °C and shifted to 37 °C for 16 h. Protein extracts from cells before and after temperature shift were assayed by immunoblotting with anti-HA antibodies. Inactivation of Cdc123 leads to accumulation of Chf proteins.

As shown in Fig. 10*A*, HA-tagged Gcd11 can be expressed in place of the essential *GCD11* gene, and when it is immunoprecipitated, FLAG-tagged Cdc123 is specifically detected in the Gcd11-associated fraction. To see if the level of Gcd11 protein is affected by Cdc123 function, we expressed HA-tagged Gcd11 as the sole source of Gcd11 in a *cdc123-4* strain and in an isogenic *CDC123* wild-type strain. As shown in Fig. 10*B*, the Gcd11 protein level is not altered by temperature shift in a wild-type strain, but shifting *cdc123-4* to the nonpermissive temperature leads to loss of Gcd11 protein. As the cycle kinetics of Chf overexpression were shown to be similar to *cdc123* loss of function and, indeed, *cdc123* temperature shift leads to Chf protein stabilization, we wished to test whether Chf1 and Chf2 overexpression might destabilize Gcd11. As shown in Fig. 10*C*, *GAL1*-driven overexpression of Chf1 renders Gcd11 virtually undetectable under the conditions in which a specific, HA-tagged Gcd11 doublet is observed in wild-type cells. Moreover, *GAL1*-driven overexpression of Cdc123 also reduces the amount of steady-state Gcd11 protein. Surprisingly, *GAL1*-driven overexpression of Chf2, which is almost as toxic as *GAL1*-driven expression of Chf1, has the opposite effect on eIF2γ abundance, increasing the levels of Gcd11 with respect to wild type. Because the *GCD11* gene is essential (22–24), reducing the level of the Gcd11 polypeptide is an obvious mechanism for G_1 arrest, precedented by arrest of *cdc33*, encoding mRNA cap-binding eIF4E (6). The fact that too much or too little Cdc123 function depresses Gcd11 levels, whereas Chf2 overexpression boosts Gcd11 levels prompted us to test whether Cdc123 or Gcd11 overexpression are toxic. As shown in Fig. 10*D*, both of these conditions are strongly deleterious, indicating that having too much or too little eIF2γ is growth-inhibitory.

DISCUSSION

Studies with a mammalian *d123* mutant cell line suggested the likely essentiality of the D123 function, but no analysis was available to identify the pathways by which D123 acts. In this study, we identify *CDC123* as the yeast homolog of mammalian *D123,* and we characterize its functions in cell cycle progression

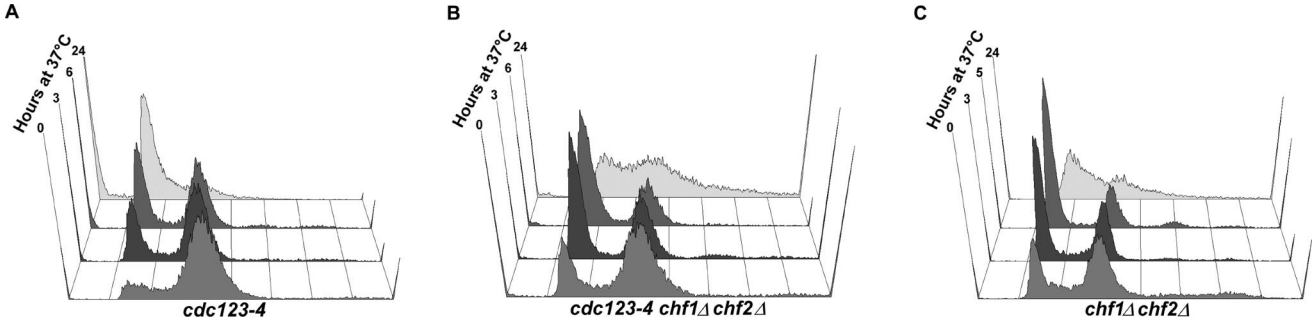

FIG. 9. ***CHF* genes are required for *cdc123-4* G_2 delay and G_1 arrest.** Cells with the indicated genotypes were grown to early logarithmic phase at 28 °C and shifted to 37 °C for flow cytometric analysis at 0, 3, 6, and 24 h. The data indicate that *CHF* genes are required for *cdc123* cell cycle phenotypes.

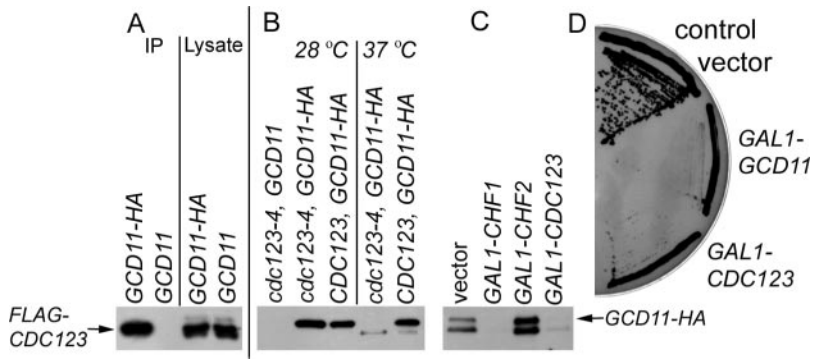

FIG. 10. **Cdc123-interacting Gcd11 protein is destabilized by Cdc123 dysfunction and regulated by Chf1, Chf2, and Cdc123 overexpression.** *A*, HA-tagged Gcd11 complements *gcd11*deletion and does not affect accumulation of Cdc123. Cdc123 protein is specifically co-immunoprecipitated with Gcd11 protein. *B*, Gcd11 protein is specifically destabilized by *cdc123-4* temperature shift. *C*, *GAL1*-driven *CHF1* and *CDC123* overexpression lead to Gcd11 depletion, whereas *GAL1*-driven *CHF2* overexpression leads to Gcd11 accumulation. *D*, addressing the *GAL1-CHF2*-dependent Gcd11 accumulation and *GAL1-CDC123*-dependent Gcd11 depletion, we show that *GAL1*-driven overexpression of Gcd11 and Cdc123 are both toxic.

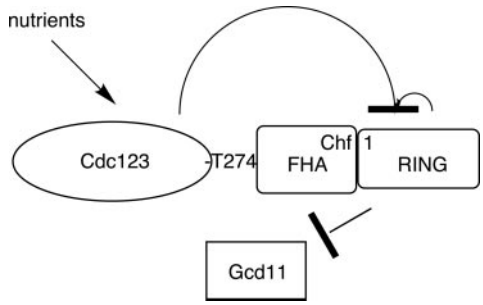

FIG. 11. **Model of the Cdc123-Chf1-Gcd11 axis of nutritional control of START.** Cdc123 levels are maintained by nutrient availability, and nutritional control of the cell cycle is regulated by protein interactions between Cdc123, Chf proteins, and Gcd11. Chf1 levels are negatively regulated by Cdc123 in a manner that requires the Chf1 FHA domain and RING domain. Chf1 toxicity depends on Thr-274 of Cdc123, suggesting that the Cdc123-Chf1 complex, which may be mediated by docking of Thr-274 and the Chf1 FHA domain, may also target destruction of another protein. Gcd11, which is also complexed with Cdc123 *in vivo*, is degraded with temperature-dependent Cdc123 inactivation and Chf1 accumulation.

as a molecule positively regulated by nutrients, as a negative regulator of yeast homologs of Chfr, and as a regulator of eIF2γ abundance. Although Chfr is frequently inactivated in human cancer (15–21), the mechanisms of Chfr function, including the target of its FHA domain and the function of its RING domain were not clear.

As summarized in the model presented in Fig. 11, Cdc123 levels are maintained by the availability of nutrients, and this polypeptide in turn controls the abundance of the two checkpoint forkhead associated with RING proteins, Chf1 and Chf2, and the γ subunit of eIF2. Our analysis indicates that Chf proteins are normally inabundant polypeptides whose turnover depends on FHA domain-dependent association with Cdc123 and the integrity of their own RING domains. Interestingly, although Cdc123 levels are maintained by nutrients and Cdc123 interacts with Chfr homologs via an interaction of Cdc123-Thr-274 and the Chf FHA domains, this interaction does not destabilize Cdc123 but rather leads to RING-dependent destabilization of the Chf proteins themselves. Moreover, toxicity of Chf expression is dependent on the integrity of the FHA domain and the RING domain and, for Chf1, this toxicity was shown to depend on the conserved Thr-274 of Cdc123, the apparent target of the Chf protein FHA domains. As shown by flow cytometry, in the absence of Chf1 and Chf2, the *cdc123*-dependent cell cycle delay at G_2 and the G_1 arrest is not maintained.

Our data indicate that a critical function of Cdc123 is to direct the destabilization of Chf proteins, whose accumulation creates a G_1 block that is similar to *cdc123* loss of function. Because *chf1 chf2* deletion suppressed the cell cycle phenotypes but not the essentiality of *cdc123*, we searched for an essential cellular function regulated by Cdc123, and we discovered that Gcd11 is another Cdc123-interacting protein whose abundance is regulated by Cdc123 and Chf proteins. Thus we show that nutritional deprivation leads to Cdc123 depletion, which results in accumulation of Chf proteins and depletion of Gcd11, an essential component of translational initiation factor 2, which is a central regulator of the transition between bulk translation and stress-associated translation (55). We also show that *cdc123* arrest, which results in Gcd11 depletion, is fully compatible with mating though mating pheromone does not act by depletion of the Cdc123 protein. These experiments establish a Cdc123-Chf-Gcd11 axis of growth and division control functioning in parallel to the well characterized Tor-Gcn2-Sui2 axis of growth and division control (56). Ongoing studies are designed to elucidate the types of stress that lead to Chf-dependent G_2 delay and G_1 arrest and how these signals bear on eIF2-dependent choices of messages to translate.

REFERENCES

1. Hartwell, L. H. (2002) *Biosci. Rep.* **22,** 373–394
2. Haney, S. A., and Broach, J. R. (1994) *J. Biol. Chem.* **269,** 16541–16548
3. Gross, A., Winograd, S., Marbach, I., and Levitzki, A. (1999) *Biochemistry* **38,** 13252–13262
4. Matsumoto, K., Uno, I., and Ishikawa, T. (1984) *J. Bacteriol.* **157,** 277–282
5. Uno, I., Mitsuzawa, H., Matsumoto, K., Tanaka, K., Oshima, T., and Ishikawa, T. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7855–7859
6. Brenner, C., Nakayama, N., Goebl, M., Tanaka, K., Tohe, A., and Matsumoto, K. (1988) *Mol. Cell. Biol.* **8,** 3556–3559
7. Reid, B. J., and Hartwell, L. H. (1977) *J. Cell Biol.* **75,** 355–365
8. Miake-Lye, R., and Kirschner, M. W. (1985) *Cell* **41,** 165–175
9. Lee, M. G., and Nurse, P. (1987) *Nature* **327,** 31–35
10. Dunphy, W. G., Brizuela, L., Beach, D., and Newport, J. (1988) *Cell* **54,** 423–431
11. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988) *Cell* **54,** 433–439
12. Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) *Cell* **55,** 371–378
13. Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B., and Nasmyth, K. (1991) *Cell* **65,** 145–161
14. Lew, D. J., and Reed, S. I. (1993) *J. Cell Biol.* **120,** 1305–1320
15. Scolnick, D. M., and Halazonetis, T. D. (2000) *Nature* **406,** 430–435
16. Mizuno, K., Osada, H., Konishi, H., Tatematsu, Y., Yatabe, Y., Mitsudomi, T., Fujii, Y., and Takahashi, T. (2002) *Oncogene* **21,** 2328–2333
17. Shibata, Y., Haruki, N., Kuwabara, Y., Ishiguro, H., Shinoda, N., Sato, A., Kimura, M., Koyama, H., Toyama, T., Nishiwaki, T., Kudo, J., Terashita, Y., Konishi, S., Sugiura, H., and Fujii, Y. (2002) *Carcinogenesis* **23,** 1695–1699
18. Corn, P. G., Summers, M. K., Fogt, F., Virmani, A. K., Gazdar, A. F., Halazonetis, T. D., and El-Deiry, W. S. (2003) *Carcinogenesis* **24,** 47–51
19. Toyota, M., Sasaki, Y., Satoh, A., Ogi, K., Kikuchi, T., Suzuki, H., Mita, H., Tanaka, N., Itoh, F., Issa, J. P., Jair, K. W., Schuebel, K. E., Imai, K., and Tokino, T. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7818–7823
20. Mariatos, G., Bothos, J., Zacharatos, P., Summers, M. K., Scolnick, D. M., Kittas, C., Halazonetis, T. D., and Gorgoulis, V. G. (2003) *Cancer Res.* **63,**

7185–7189
21. Satoh, A., Toyota, M., Itoh, F., Sasaki, Y., Suzuki, H., Ogi, K., Kikuchi, T., Mita, H., Yamashita, T., Kojima, T., Kusano, M., Fujita, M., Hosokawa, M., Endo, T., Tokino, T., and Imai, K. (2003) *Cancer Res.* **63,** 8606–8613
22. Harashima, S., and Hinnebusch, A. G. (1986) *Mol. Cell. Biol.* **6,** 3990–3998
23. Hannig, E. M., Cigan, A. M., Freeman, B. A., and Kinzy, T. G. (1993) *Mol. Cell. Biol.* **13,** 506–520
24. Dorris, D. R., Erickson, F. L., and Hannig, E. M. (1995) *EMBO J.* **14,** 2239–2249
25. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* **10,** 1793–1808
26. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Elledge, S. J., Mulligan, J. T., Ramer, S. W., Spottswood, M., and Davis, R. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1731–1735
28. Bai, C., and Elledge, S. J. (1996) *Methods Enzymol.* **273,** 331–347
29. Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields, S., and Rothberg, J. M. (2000) *Nature* **403,** 623–627
30. Guarente, L. (1983) *Methods Enzymol.* **101,** 181–191
31. Fortuna, M., Sousa, M. J., Corte-Real, M., Leao, C., Salvador, A., and Sangonetty, F. (2000) in *Current Protocols in Cytometry* (Robinson, J. P., Darzynkiewicz, Z., Dean, P., Orfao, A., Rabinovitch, P., Stewart, C. C., Tanke, H., and Wheeless, L., eds) pp. 11.13.1–11.13.9, John Wiley & Sons, Inc., New York
32. Okuda, A., and Kimura, G. (1996) *Exp. Cell Res.* **223,** 242–249
33. Okuda, A., Ohtsu, M., and Kimura, G. (1999) *Cell Struct. Funct.* **24,** 443–449
34. Okuda, A., Ohtsu, M., and Kimura, G. (2001) *Cell Struct. Funct.* **26,** 205–214
35. Onisto, M., Zeilante, P., Scannapieco, P., Pellati, D., Pozza, M., Caenazzo, C., Negro, A., and Garbisa, S. (1998) *Exp. Cell Res.* **242,** 451–459
36. Thuret, J. Y., Valay, J. G., Faye, G., and Mann, C. (1996) *Cell* **86,** 565–576
37. Espinoza, F. H., Farrell, A., Erdjument Bromage, H., Tempst, P., and Morgan, D. O. (1996) *Science* **273,** 1714–1717
38. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) *Methods Enzymol.* **154,** 164–175
39. Reed, S. I. (1980) *Genetics* **95,** 561–577
40. Kang, D., Chen, J., Wong, J., and Fang, G. (2002) *J. Cell Biol.* **156,** 249–259
41. Chaturvedi, P., Sudakin, V., Bobiak, M. L., Fisher, P. W., Mattern, M. R., Jablonski, S. A., Hurle, M. R., Zhu, Y., Yen, T. J., and Zhou, B. B. (2002) *Cancer Res.* **62,** 1797–1801
42. Bothos, J., Summers, M. K., Venere, M., Scolnick, D. M., and Halazonetis, T. D. (2003) *Oncogene* **22,** 7101–7107
43. Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott, S. L., Jackson, S. P., Smerdon, S. J., and Yaffe, M. B. (2000) *Mol. Cell* **6,** 1169–1182
44. Li, J., Lee, G. I., Van Doren, S. R., and Walker, J. C. (2000) *J. Cell Sci.* **113,** 4143–4149
45. Skowyra, D., Koepp, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999) *Science* **284,** 662–665
46. Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999) *Science* **284,** 657–661
47. Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K., and Deshaies, R. J. (1999) *Genes Dev.* **13,** 1614–1626
48. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999) *Science* **286,** 309–312
49. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11364–11369
50. Xie, Y., and Varshavsky, A. (1999) *EMBO J.* **18,** 6832–6844
51. Stavridi, E. S., Huyen, Y., Loreto, I. R., Scolnick, D. M., Halazonetis, T. D., Pavletich, N. P., and Jeffrey, P. D. (2002) *Structure* (*Lond.*) **10,** 891–899
52. Li, J., Smith, G. P., and Walker, J. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7821–7826
53. Roll-Mecak, A., Shin, B. S., Dever, T. E., and Burley, S. K. (2001) *Trends Biochem. Sci.* **26,** 705–709
54. Laurino, J. P., Thompson, G. M., Pacheco, E., and Castilho, B. A. (1999) *Mol. Cell. Biol.* **19,** 173–181
55. Hinnebusch, A. G., and Natarajan, K. (2002) *Eukaryot. Cell* **1,** 22–32
56. Cherkasova, V. A., and Hinnebusch, A. G. (2003) *Genes Dev.* **17,** 859–872