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SCF^{Met30}-mediated control of the transcriptional activator Met4 is required for the G_1–S transition

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Progression through the cell cycle requires the coordination of basal metabolism with the cell cycle and growth machinery. Repression of the sulfur gene network is mediated by the ubiquitin ligase SCF^{Met30}, which targets the transcription factor Met4p for degradation. Met30p is an essential protein in yeast. We have found that a *met4*Δ*met30*Δ double mutant is viable, suggesting that the essential function of Met30p is to control Met4p. In support of this hypothesis, a Met4p mutant unable to activate transcription does not cause inviability in a *met30*Δ strain. Also, overexpression of an unregulated Met4p mutant is lethal in wild-type cells. Under non-permissive conditions, conditional *met30*Δ strains arrest as large, unbudded cells with 1N DNA content, at or shortly after the pheromone arrest point. *met30*Δ conditional mutants fail to accumulate *CLN1* and *CLN2*, but not *CLN3* mRNAs, even when *CLN1* and *CLN2* are expressed from strong heterologous promoters. One or more genes under the regulation of Met4p may delay the progression from G_1 into S phase through specific regulation of critical G_1 phase mRNAs.

Keywords: G1 cyclin/G1–S transition/methionine/SCF ubiquitin ligase

Introduction

Availability of nutrients in the environment is essential for cell growth and cell cycle progression. In the budding yeast *Saccharomyces cerevisiae*, nutrient deprivation can elicit different responses such as cell cycle arrest in G_1 phase, sporulation or pseudohyphal growth. In particular, sufficient sources of carbon, phosphate, nitrogen and sulfur are required for yeast cells to pass through the cell cycle commitment point called Start in late G_1 phase (reviewed in Pardee, 1989; Hartwell, 1994; Sherr, 1994; Polymenis and Schmidt, 1999).

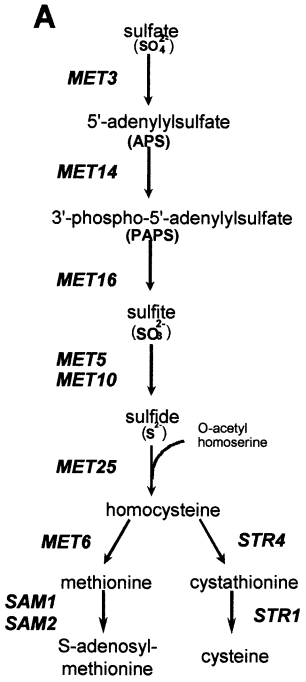
Budding yeast can satisfy their elemental sulfur requirement through the uptake and subsequent metabolism of a large number of either inorganic or organic sulfur compounds (reviewed in Thomas and Surdin-Kerjan, 1997). In addition to the biosynthesis of the two sulfur amino acids methionine and cysteine, reduced sulfur atoms are needed to synthesize *S*-adenosylmethionine (AdoMet), a molecule central to general metabolism. AdoMet is indeed next to ATP for the number of reactions in which a biological compound is used: AdoMet is the main donor of methyl groups for methylation of nucleic acids, proteins and lipids, serves as a precursor for the biosynthesis of the polyamines and is also the substrate for numerous reactions in vitamin biosynthesis and nucleotide modification.

Sulfur flux through the sulfate assimilation pathway and the metabolic arms specific for the methionine and cysteine syntheses is controlled by the intracellular content of AdoMet. A key component of this regulation is the *MET* gene transcriptional activator Met4p and its associated cofactors Cbf1p, Met28p, Met31p and Met32p (reviewed in Thomas and Surdin-Kerjan, 1997). Repression of Met4p-dependent transcription in response to increased intracellular AdoMet is mediated by the SCF^{Met30} complex, an E3 ubiquitin ligase (Patton *et al*., 1998; Rouillon *et al*., 2000). Met4p degradation and transcriptional activity are governed by an autoregulatory loop. When intracellular AdoMet is low, Met4p-containing transcription activation complexes drive expression of the *MET* gene network, including the *MET30* gene. Once expressed, Met30p targets Met4p for degradation via SCF^{Met30} and thereby limits expression of the *MET* genes in response to high intracellular AdoMet (Rouillon *et al*., 2000).

SCF ubiquitin ligase complexes target specific substrates for degradation, as determined by their F-box component (reviewed in Craig and Tyers, 1999). In addition to Met4p, SCF^{Met30} appears to target Swe1p, a Cdc28p inhibitory kinase necessary for the morphological checkpoint (Kaiser *et al*., 1998). SCF^{Met30} is one of three SCF complexes identified in yeast. SCF^{Cdc4} targets the cell cycle and transcriptional regulators Sic1p, Far1p, Gcn4p, Ctf13p and Cdc6p for degradation, while SCF^{Grr1} is responsible for the degradation of the G_1 cyclins, Cln1p and Cln2p, of the Cdc42p effector Gic1p, and regulation of the glucose repression pathway. Proteins similar to Met30p exist in other fungi, *Drosophila* and mammals, and are the SconB, Slimb and β-TrCP proteins, respectively (Kaiser *et al*., 1998; Margottin *et al*., 1998). Recently, the β-TrCP/Slimb proteins have been implicated in the ubiquitin-mediated degradation of protein targets in NF-κB, Wnt/Wingless and Hedgehog pathways (reviewed in Maniatis, 1999). Also, β-TrCP was shown to trigger the degradation of the lymphocyte receptor CD4 via the HIV protein Vpu (Margottin *et al*., 1998).

Regulation of the cell division cycle requires the activation of the essential cyclin-dependent kinase (CDK) Cdc28p by the G_1 cyclins Cln1p, 2p and 3p (reviewed in

A



B

genotype	phenotype	genotype	phenotype	crosses
met30Δ	lethal			
met3Δ	Met-	*met3Δ met30Δ*	lethal	CC781 = CD127-1CT x CD155 CC951 = CC906-13C x CC932-10C
str4Δ	Cys-	*str4Δ met30Δ*	lethal	CC782 = CD127-1CT x C180 CC952 = CC906-13C x CC932-10C
met6	Met-	*met6 met30Δ*	lethal	CC779 = CD127-1CT x CC650-8A CC953 = CC864-1B x CC932-8B
sam1Δ sam2Δ	AdoMet-	*sam1Δ sam2Δ met30Δ*	lethal	CC780 = CD127-1AT x CC435-6C CC921 = CC850-2B x W744-1A
met4Δ	Met-	*met4Δ met30Δ*	**Met-**	CC807 = CD127-1AT x CD106 CC808 = CC807-7D x W303-1A CC809 = CC807-7D x CD106

Fig. 1. (**A**) Simplified view of sulfur amino acid biosynthesis in yeast. (**B**) The *met4*Δ disruption mutation specifically suppresses the lethality induced by the loss-of-function mutation *met30*Δ. See Materials and methods for details of the crosses.

Nasmyth, 1996). Cyclin-CDK activity is rate-limiting for passage through Start, and control of cyclin-CDK activity has been reported at multiple levels including inhibition by cyclin inhibitors, transcription, translation and degradation (Hartwell, 1994; Sherr, 1994; Nasmyth, 1996; Polymenis and Schmidt, 1999). For example, non-degradable forms of the G_1 cyclins, such as Cln3-1, cause hyperactivation of Start and hence a decreased critical cell size (Nash *et al*., 1988). Repression of *CLN3* mRNA translation is required for cell cycle arrest in starvation and high iron conditions (Polymenis and Schmidt, 1997; Philpott *et al*., 1998).

While the *MET* network is only essential in the absence of sulfur amino acids, *MET30* is an essential gene under all nutrient conditions (Thomas *et al*., 1995). Here, we show that the essential function of Met30p is the regulation of Met4p transcriptional activity. Surprisingly, Met4p regulation is critical for progression through Start. Our results argue that the misregulation of genes under the control of Met4p inhibits passage through Start by preventing the accumulation of important G_1 phase transcripts, including those of the genes encoding the G_1 cyclins, Cln1p and Cln2p.

Results

Inactivation of MET4 suppresses met30Δ lethality

MET30 is an essential gene and encodes a negative regulator of the sulfur amino acid biosynthesis pathway (Thomas *et al*., 1995). The unregulated synthesis of a particular sulfur-containing metabolite might therefore be responsible for the lethality of a *met30*Δ strain. To test this hypothesis, we crossed a *met30::LEU2* strain harboring a *LexA-MET30, HIS3* plasmid with a battery of mutants that each specifically block one metabolic arm of the sulfur network (*met3*Δ, *met6*Δ, *str4*Δ and *sam1*Δ*sam2*Δ; Figure 1A; see Materials and methods). We also included a cross with a *met4*Δ mutant strain in our analysis as Met30p was shown to trigger Met4p degradation in response to an increase in intracellular AdoMet (Rouillon *et al*., 2000). Single deletions of *MET3*, *MET6* or *STR4* genes as well as the double deletion of the *SAM1* and *SAM2* genes did not alter the *met30*Δ lethality phenotype, indicating that the *met30*Δ lethality was not due to the accumulation of a sulfur-containing metabolite. In contrast, deletion of *MET4* bypassed the lethality of the *met30*Δ strain (Figure 1B), suggesting that the essential function of Met30p might be the regulation of Met4p.

We made a conditionally viable *met30*Δ mutant by constructing strains that could express *MET4* under the *GAL1* promoter in the absence of *MET30*. As shown in Figure 2A, expression of *GAL1–MET4* in *met30*Δ cells induces lethality in minimal or complete galactose-containing medium. Also, repressive amounts of extracellular methionine or AdoMet, which have been shown to trigger the degradation of Met4p, did not prevent the detrimental effect of *MET4* expression in *met30*Δ mutants. Similar results were obtained with cells grown in sulfur-less B medium in the presence of homocysteine or methionine used as the sulfur source (data not shown). Altering the levels of *GAL1–MET4* expression by spotting the mutants on galactose concentrations ranging from 0.1 to 2.5% was equally effective in inducing lethality (data not shown).

The first functional analyses of Met30p were carried out in *MET30-1* and *MET30-2* strains, point mutants of *MET30* that are unable to fully repress *MET* gene expression in response to high levels of extracellular methionine (Thomas *et al*., 1995). The viable conditional *met30*Δ mutant provided us with the opportunity to verify that Met30p function is absolutely required for Met4p regulation. We analyzed the AdoMet-mediated repression of two structural genes from the sulfate assimilation pathway

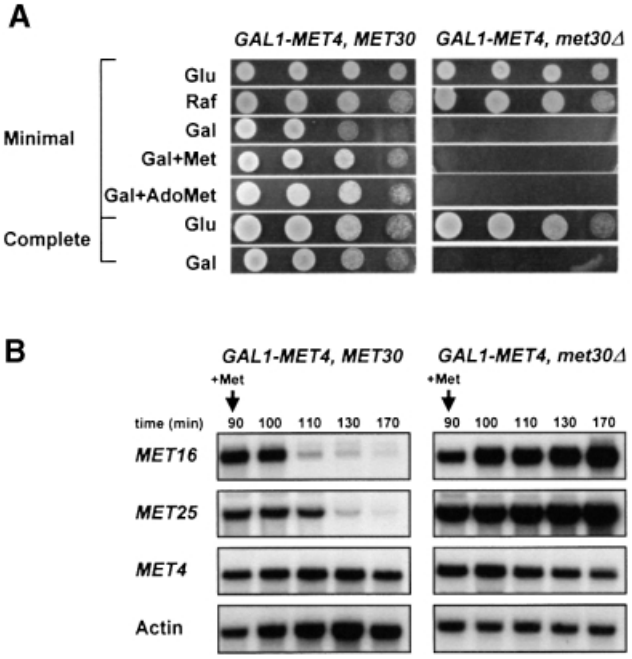


Fig. 2. Expression of Met4p from the *GAL1* promoter is lethal in *met30*Δ cells. (**A**) Serial dilutions of the CC932-6D (*met4::GAL1–MET4 MET30*) and CC932-8B (*met4::GAL1–MET4 met30::LEU2*) strains were plated on media containing 2% glucose (Glu), raffinose (Raf) or galactose (Gal) as carbon source in the absence or presence of 1 mM L-methionine (Met) or 0.2 mM AdoMet. (**B**) CC932-6D (*met4::GAL1–MET4 MET30*) and CC932-8B (*met4::GAL1–MET4 met30::LEU2*) cells were grown in raffinose medium and expression was induced by transferring the cells to a fresh galactose (2%) medium for 90 min. A repressing amount of L-Met (1 mM) was then added and total RNA was extracted at the times indicated. Expression of *MET16*, *MET25* and *GAL1–MET4* were determined by Northern analysis. The actin probe was used as a control of the amount of RNA loaded.

(*MET16* and *MET25*) in cells expressing *MET4* from the *GAL1* promoter in the presence or absence of *MET30*. The two corresponding strains (*met4::GAL1–MET4* and *met4::GAL1–MET4 met30*Δ) were first grown in the presence of raffinose for eight generations, transferred to a medium containing galactose to induce *GAL1–MET4* gene expression for 90 min before a repressive concentration of methionine was added, and samples extracted at regular time intervals after methionine addition. As shown in Figure 2B, Northern blot analysis demonstrated that repression of *MET16* and *MET25* transcription in response to high methionine was dependent on *MET30*. *MET16* and *MET25* genes were repressed with wild-type kinetics in *met4::GAL1–MET4* strains, but were insensitive to high extracellular methionine in *met4::GAL1–MET4 met30*Δ cells. Insensitivity of the *MET16* and *MET25* genes to high extracellular methionine in *met4::GAL1–MET4 met30*Δ cells is consistent with the above results showing that repressive amounts of extracellular methionine or AdoMet did not suppress the lethality induced by *MET4* expression in *met30*Δ cells. As a control, the transcription of the *GAL1–MET4* fusion gene was not affected by the presence of methionine. These results confirmed our previous findings that Met30p is required for repression of the *MET* gene network.

To function as a transcriptional activator, Met4p is recruited to the sequence-specific upstream regions of *MET* genes as a part of multiprotein complexes. Depending on the gene, different combinations of the Cbf1p, Met28p, Met31p and Met32p factors are assembled and tether Met4p to DNA (Kuras *et al*., 1997; Blaiseau and Thomas, 1998). To determine whether, in addition to Met4p, components of these complexes were required for the essential function of Met30p, we crossed a *met30::LEU2* strain harboring a *LexA-MET30, HIS3* plasmid with the single *cbf1*Δ or *met28*Δ mutants as well as with a *met31*Δ*met32*Δ double mutant. The results of these genetic tests showed that *met32*Δ *met30*Δ spores could be recovered while deletion of *CBF1*, *MET28* or *MET31* had no effect on the lethality of the *met30*Δ mutation (Table I). The essential function of Met30p might therefore be required to antagonize a specific Met4p–Met32p transcription complex.

Met4p regulation is essential for cell viability

We have recently reported the SCF^{Met30}-dependent degradation of Met4p in response to high extracellular methionine (Rouillon *et al*., 2000). Our genetic data, demonstrating that the essential function of Met30p could be bypassed by the deletion of *MET4* or *MET32*, suggested that the lethality of a *met30*Δ strain might be due to unregulated activity of Met4p, as Met32p lacks transcriptional activation function (Blaiseau *et al*., 1997). Detailed analysis of the functional organization of Met4p has identified one activation domain (AD), two regulatory regions required for the AdoMet response, called the inhibitory region (IR) and the auxiliary domain (AUX), and two domains required for tethering Met4p to DNA via protein–protein interactions with Met31p/Met32p and Met28p/Cbf1p, called the INT domain and the bZIP domain, respectively (Kuras and Thomas, 1995; Blaiseau and Thomas, 1998). To determine which particular aspect of Met4p function is required to induce lethality in the absence of Met30p, different mutant derivatives of Met4p were expressed from the *GAL1* promoter in *met4*Δ *met30*Δ cells and tested for viability. As shown in Figure 3A, deletion of the AD (pGalMet4Δ12) or INT (pGalMet4Δ38) domains of Met4p rescued the *GAL1–MET4*-induced lethality in *met4*Δ *met30*Δ cells. In contrast, deletion of the two AdoMet regulatory regions, the IR (pGalMet4Δ30) and AUX (pGalMet4Δ37) domains, did not prevent lethality. This is consistent with our findings that *met4*Δ and *met32*Δ, but not *cbf1*Δ or *met28*Δ, can bypass the *met30*Δ arrest, and reinforces the idea that an unregulated transcriptional activity of a DNA-bound complex containing Met4p and Met32p is lethal. This notion might be too simplified, however, because removal of the Met4p bZIP domain thought to bind Met28p and Cbf1p results in a partial rescue of *met30*Δ (pGalMet4Δ146; Figure 3A), while the simple deletion of *MET28* or *CBF1* is not sufficient to bypass the *met30*Δ lethality.

Given the expectation that Met30p-mediated regulation of Met4p requires an interaction between the two factors, it could be predicted that mutations within Met4p should exist that by impairing the Met30p–Met4p interaction would cause lethality even in cells expressing *MET30*. We had previously mapped the IR domain as a region of Met4–Met30 protein interaction by two-hybrid analysis (Thomas *et al*., 1995); however, our IR deletion mutant was unable to induce lethality in the presence of Met30p. This suggested that the Met4 IR domain may include

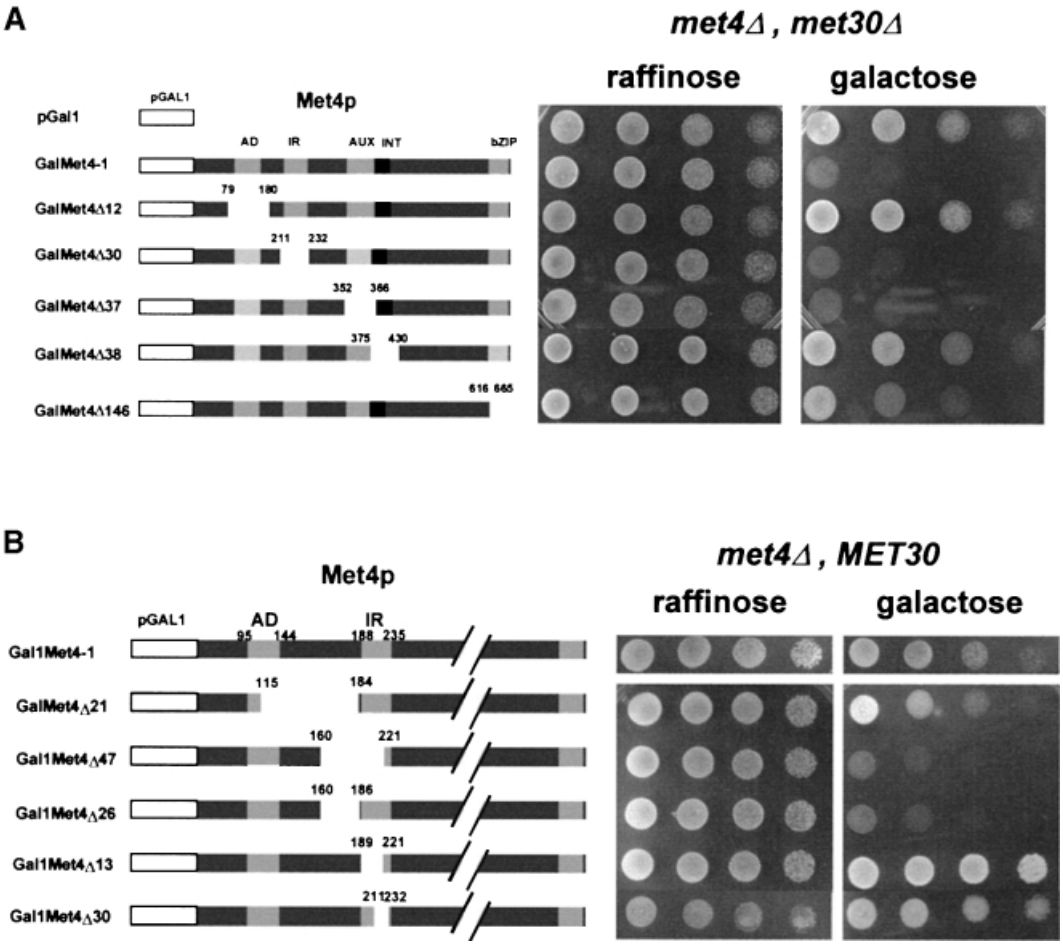


Fig. 3. (**A**) Expression of Met4p derivatives in *met4*Δ *met30*Δ cells. Schematic representations of the modified Met4p derivatives expressed from the *GAL1* promoter region are shown. Plasmids encoding the fusion genes were introduced into CC807-1C (*met4::TRP1 met30::LEU2*) cells. Serial dilutions of the resulting transformants were plated on media containing 2% raffinose or galactose as carbon source. (**B**) Expression of Met4p derivatives in *met4*Δ *MET30* cells. Details of the deletions are scaled up. Plasmids encoding the fusion genes were introduced into CD106 (*met4::TRP1*) cells. Serial dilutions of the resulting transformants were plated on media containing 2% raffinose or galactose as carbon source.

Table I. Suppression of *met30*Δ-induced lethality by inactivation of the *MET32* gene

Genotype	Phenotype	Genotype	Phenotype	Crosses
*met30*Δ	lethal			
*met4*Δ	Met^-	*met4*Δ *met30*Δ	Met^-	see Figure 1
*met28*Δ	Met^-	*met28*Δ *met30*Δ	lethal	CC958 = CD130-1A × CC850-2B
*cbf1*Δ	Met^-	*cbf1*Δ *met30*Δ	lethal	CC806 = CD127-1AT × R31-5C
*met31*Δ	Met^+	*met31*Δ *met30*Δ	lethal	CC867 = C847-1D × CD179
*met32*Δ	Met^+	*met32*Δ *met30*Δ	Met^+	CC867 = C847-1D × CD179

amino acid residues outside of 189–235, consistent with data reported by Omura and colleagues, who isolated a Met4p point mutant (F156S) that was unresponsive to high extracellular methionine (Omura *et al*., 1996). We thus constructed a new set of deletions around the IR region of *GAL1–MET4* and expressed them in *met4*Δ *MET30* cells. Two derivatives, Met4Δ47 (amino acids 160–221) and Met4Δ26 (amino acids 160–186), induced lethality in *met4*Δ *MET30* cells (Figures 3B and 4E), indicating that the Met30p-mediated regulation of Met4p was dependent on a larger IR domain than previously reported, and supporting the notion that unregulated Met4p activity might be responsible for the lethality of *met30*Δ mutants.

The essential function of Met30p is required for passage through G_1 phase

Conditional expression of the *MET4* gene from a *GAL1* promoter in *met4*Δ *met30*Δ double mutants allowed us to characterize the phenotype of *met30*Δ mutants. We performed growth curve analysis to determine the number of generations before arrest, fluorescence-activated cell sorter (FACS) analysis to measure DNA content, and determined the budding index. Early log phase cultures of *met4::GAL1–MET4 met30*Δ were grown in raffinose, split into two, galactose added to one culture, and cell number measured over time. Within one doubling the *GAL1–MET4 met30*Δ cells had arrested, while the raffinose culture continued to increase in cell number (Figure 4A).

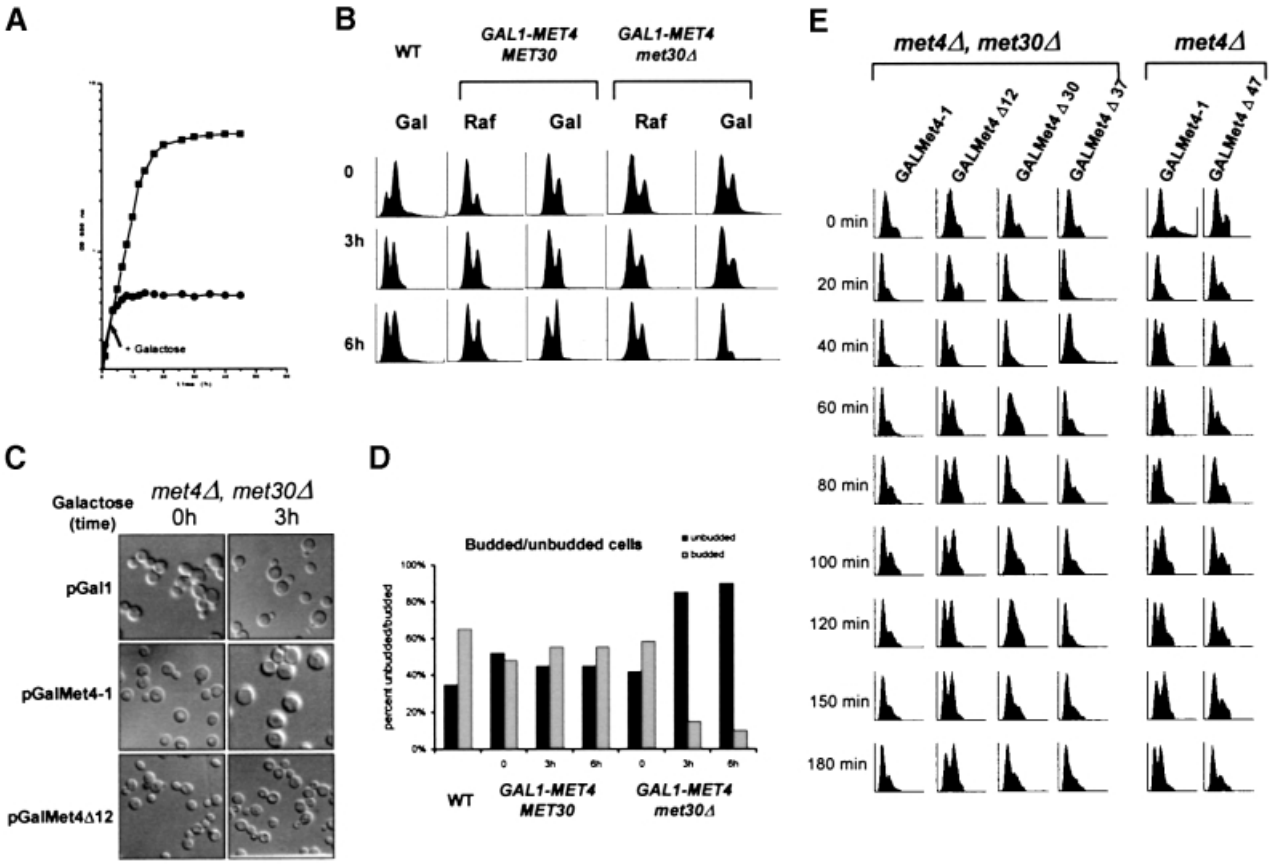


Fig. 4. Conditional *met30*Δ mutant cells arrest as large unbudded cells with 1N DNA. (**A**) CC932-6D (*met4::GAL1–MET4 MET30*) (■) and CC932-8B (*met4::GAL1–MET4 met30::LEU2*) (●) cells were grown in the presence of 2% raffinose as carbon source. At the time indicated by the arrow, 2% galactose was added to each culture and growth was followed by measuring the OD_{650} for the times indicated. (**B**) Wild type, *met4::GAL1–MET4 MET30* and *met4::GAL1–MET4 met30::LEU2* were grown in the presence of raffinose, cultures were divided into two, filtered and transferred to raffinose or galactose medium. FACS analyses were then performed at the times indicated. (**C**) CC807-1C (*met4::TRP1 met30::LEU2*) cells were transformed by the pGal316, pGalMet4-1 or pGalMet4Δ12 plasmids. Resulting transformants were grown in the presence of 2% raffinose as carbon source. Cultures were then filtered and transferred to a galactose (2%) medium. Photographs of the cells were then taken at the times indicated. (**D**) Budding index of the cells grown in (A). (**E**) CC807-1C (*met4::TRP1 met30::LEU2*) or CD106 (*met4::TRP1*) were transformed by the plasmids indicated. Resulting transformants were grown in raffinose medium to early log phase and arrested by α factor for 3 h in 2% galactose. The cells were released from α factor by washing with fresh galactose medium and analyzed for their DNA content by flow cytometry at the times indicated.

To examine whether the cells had any particular cell cycle arrest phenotype, we grew parallel cultures of *met4::GAL1–MET4* and *met4::GAL1–MET4 met30*Δ strains in raffinose and galactose, and took samples at 0, 3 and 6 h. As shown in Figure 4B, expression of *MET4* in the absence of *MET30* causes the cells to accumulate in G_1 with unreplicated DNA. Microscopic observation showed that >90% of the *GAL1–MET4 met30*Δ cells remain unbudded 6 h after the shift to the galactose medium (Figure 4C and D).

The G_1 arrest phenotype of *met4::GAL1–MET4 met30*Δ cells correlated with the requirement for the Met4p activation domain to induce lethality in these strains. Expression of *GAL1–MET4*Δ*AD* produced little alteration of the FACS profile, while expression of *GAL1–MET4* in *met4*Δ *met30*Δ cells caused a G_1 arrest (Figure 4E). Also, expression of *GAL1–MET4*Δ*IR*(160–221), which had been shown to induce lethality in the presence of *MET30*, also arrested the cells in G_1 phase. Expression of other *GAL1–MET4* derivatives, described above, showed FACS profiles similar to wild type. Thus, unregulated activity of Met4p, either by lack of the IR(160–221) domain or by deletion of *MET30*, is sufficient to cause a G_1 arrest.

There are multiple biological pathways that can cause an unbudded G_1 phenotype when disrupted (Nasmyth, 1996). To gain insight into the mechanism responsible for the G_1 arrest observed in the *GAL1–MET4 met30*Δ cells, we undertook a detailed study of the characteristics of the *GAL1–MET4 met30*Δ arrest. To map the arrest position of the *met4::GAL1–MET4 met30*Δ mutant, we arrested an asynchronous culture growing in raffinose with mating pheromone for 2 h, split the culture into two, added raffinose to one half and galactose to the other half for 20 min, released the culture into pheromone-free medium, and took samples at 10 min intervals to measure DNA content and budding morphology. Cells in raffinose proceeded through the cell cycle with a similar FACS profile to wild type (Figure 5A), demonstrating that in the absence of Met4p, the deletion of *MET30* has only a marginal effect on cell cycle progression. In contrast, the *met4::GAL1–MET4 met30*Δ mutants remained in G_1 phase without undergoing DNA replication (Figure 5A) or initiating budding (Figure 5B). When *GAL1–MET4* was induced in the *met4*Δ *met30*Δ mutants after the release from pheromone, the cells progressed through the cell cycle before accumulating in G_1 phase (data not shown),

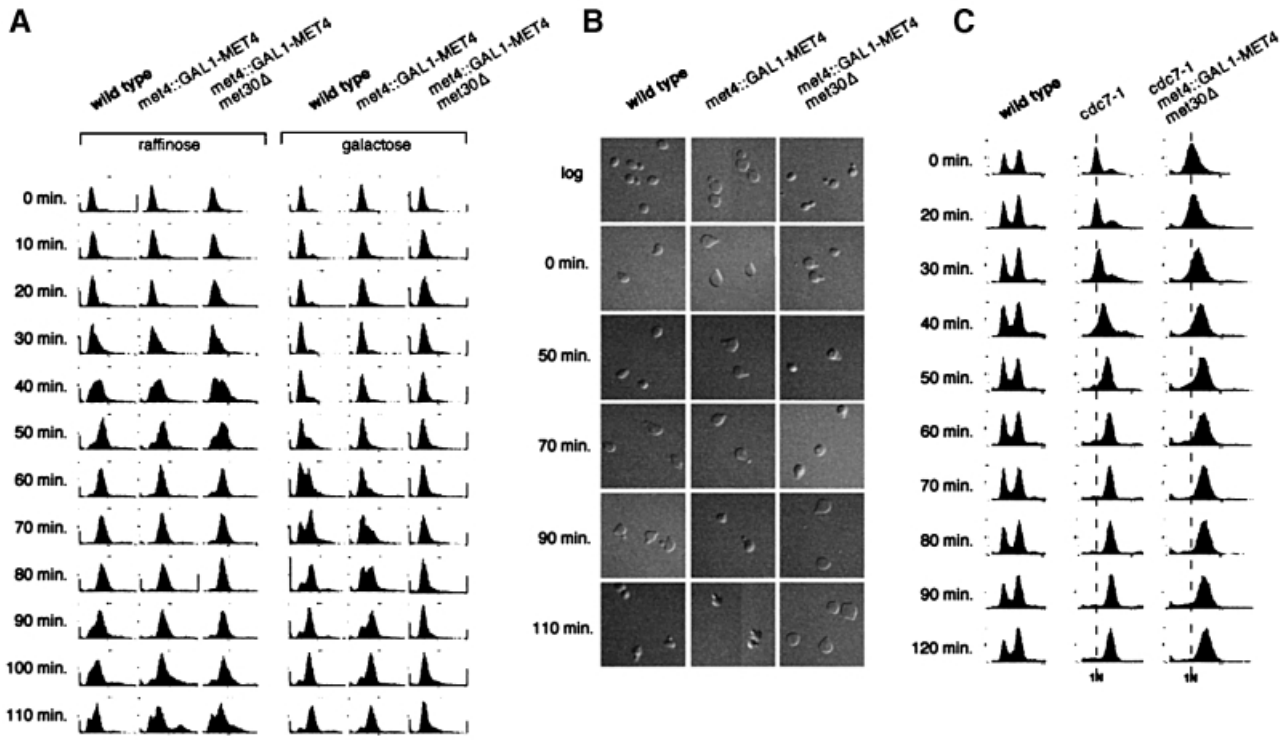


Fig. 5. The essential function of *MET30* is required at or after the pheromone arrest position, but prior to the initiation of budding or DNA replication. (**A**) Wild-type, *met4::GAL1–MET4* and *met4::GAL1–MET4 met30*Δ cells were grown in rich raffinose medium to early log phase and arrested with α factor for 2 h. The cells were then split into two cultures and raffinose added to one, while galactose was added to another for 20 min. The cells were released from α factor by washing with fresh medium and time points taken at the designated intervals. (**B**) Photographs of the cells in (A) demonstrate that the *met4::GAL1–MET4 met30*Δ arrest as large unbudded cells. The tear shape of the cells is due to effects of the pheromone. (**C**) *MET30* is required before the *cdc7-1* arrest point. Wild-type, *cdc7-1* and *cdc7-1 met4::GAL1–MET4 met30*Δ cultures were incubated at 37°C for 2 h, had galactose added for 30 min, and were then shifted to 25°C and samples taken at the time indicated.

suggesting that the effects of *GAL1–MET4* are specific to the G_1 phase of the cell cycle. Unregulated Met4p might, therefore, function at or shortly after the pheromone arrest point to inhibit passage through Start. Consistent with this hypothesis, overexpression of *MET4*, still under the control of endogenous levels of Met30p, induces a 20 min delay through Start (Figure 5A). Met30p was originally identified as a mutant that was defective for repression of *MET* gene transcription (Thomas *et al*., 1995). Given that overexpression of *MET4* could delay passage through G_1 of the cell cycle by 20 min, we examined the progression of *MET30-1* mutant cells through the cell cycle by FACS analysis. *MET30-1* cells were delayed for passage into S phase by 10 min (data not shown), indicating that non-lethal mutations in *MET30* that are partially defective for Met4p regulation are also capable of producing a G_1–S phase delay.

The shift from a pheromone-induced arrest to a *met30*Δ arrest demonstrated that the *met30*Δ arrest phenotype was at or after the pheromone arrest point but prior to the DNA replication and budding initiation point. In the converse experiment, i.e. arresting the *met4::GAL1–MET4 met30*Δ cells in G_1 phase in galactose and releasing them into glucose and pheromone medium, the cells were unable to recover from the prolonged *GAL1–MET4* arrest. As an alternative, we constructed a *met4::GAL1–MET4 met30*Δ *cdc7-1* mutant, arrested the cells at 37°C and released them into a galactose-containing medium at 25°C. The Cdc7p kinase is required for the initiation of DNA replication, but not for budding (Yoon *et al.*, 1993). *cdc7-1* mutants arrested at 37°C with small buds and 1N DNA content (Figure 5C), and progressed through S and G_2 phase upon shift to 25°C. Similarly, *met4::GAL1–MET4 met30*Δ *cdc7-1* mutants arrested in G_1 phase at 37°C, and progressed through S and G_2 phase when shifted to 25°C in galactose, placing the *met30*Δ arrest position after the pheromone block but before the Cdc7p requirement point in the cell cycle.

G_1-phase-arrested met30Δ mutants are compromised for efficient growth and translation

Start mutants can be categorized into those affecting growth such as mutations in the cAMP pathway or the translational machinery, and those affecting division such as mutations in *CDC28* or the G_1 cyclins (reviewed in Hartwell, 1994). To determine which component of Start was altered in the conditional *met30*Δ mutants, we monitored the rate of growth in small, unbudded *met4::GAL1–MET4 met30*Δ G_1 cells collected by centrifugal elutriation and then grown in raffinose or galactose (Figure 6A). As expected, in raffinose medium cells grew steadily over time and passed through Start (25% budded) at a cell volume of 50 fl. These cells progressed through S phase and into G_2–M phase, as indicated by the increase in bud index and DNA content (Figure 6B and C). In contrast, cells grown in galactose medium did not initiate budding (Figure 6B) and maintained a 1N DNA content (Figure 6C), but did, however, accumulate mass over time (Figure

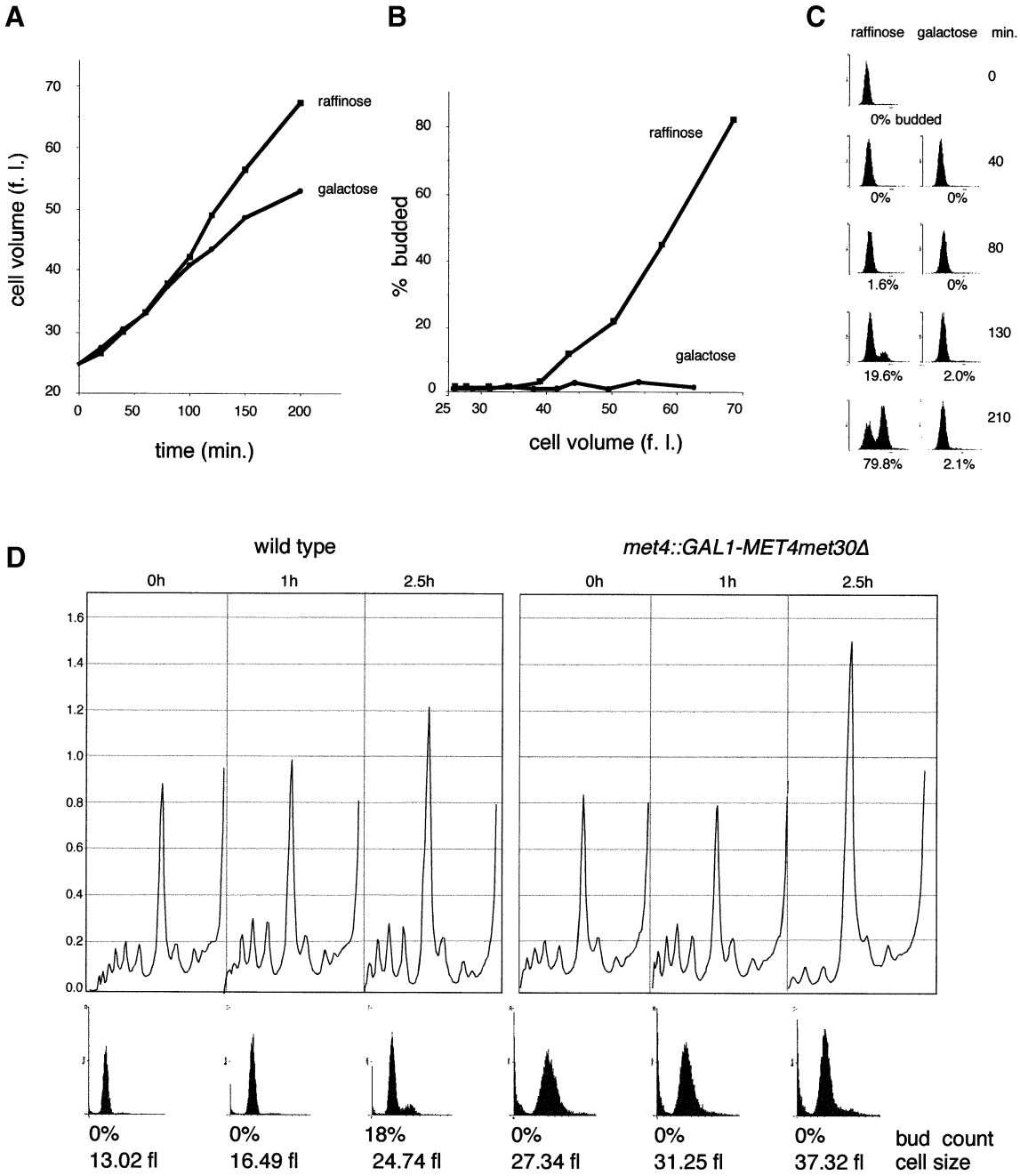


Fig. 6. Conditional G_1-arrested *met30*Δ mutants are slowed for cell growth and translation. Cultures of small G_1 *met4::GAL1–MET4 met30*Δ cells grown in raffinose were collected by centrifugal elutriation, divided into two, and raffinose added to one culture and galactose to the other. Samples were taken at the times indicated to measure (**A**) cell volume, (**B**) budding index and (**C**) DNA content by flow cytometry. (**D**) Polysome profiles of wild-type or *met4::GAL1–MET4 met30*Δ cells through the Start position. G_1 cells were collected by centrifugal elutriation in a rich, raffinose medium, galactose was added at time 0, and samples were taken at the times indicated for polysome profiles, flow cytometry and budding analysis.

6A). Initially, the conditional *met30*Δ mutants grown in galactose medium accumulated mass at a rate equal to those in raffinose medium, but after 100 min the rate of growth was slowed somewhat.

To determine whether the decreased rate of mass accumulation in the *met30*Δ mutants might be due to defects in translation, we measured the incorporation of [^{35}S]methionine/cysteine into total protein, and examined the polysome profiles of the conditional *met30*Δ mutants. [^{35}S]methionine/cysteine incorporation was decreased by 20% in the conditional *met30*Δ mutants after 2.5 h in galactose. Similarly, the number of discrete polysome peaks and UV absorbance of the polysome peaks were slightly reduced in the *met30*Δ mutants after 2.5 h (data not shown). To perform a more detailed analysis, we used synchronized G_1 cells isolated by centrifugal elutriation and examined polysome profiles of wild-type and *met30*Δ cells as a function of time. In comparison with wild-type cells, the extent of polyribosome formation was reduced and, correspondingly, the 80S ribosome peak was increased in the *met30*Δ mutants at 2.5 h, by which time wild-type cells had passed through Start (Figure 6D). It therefore

appears that *met30*Δ cells are able to reach the critical cell size required for passage through Start but gradually become impaired in translation.

Various G_1 cyclin RNA transcripts are reduced in met30Δ mutants

As the conditional *met30*Δ strain was only slightly compromised for translation, we reasoned that other components of the cell division machinery might be altered in these cells. We therefore tested whether overexpression of the G_1 cyclins could rescue the *met30*Δ phenotype. Expression of the genes encoding the G_1 cyclins, *CLN1*, *CLN2*, *CLN3*, *CLN3-1*, *PCL1* or *PCL2*, and the S and G_2 cyclins, *CLB2* or *CLB5*, from the *GAL1* promoter in *met4::GAL1–MET4 met30*Δ mutants was unable to bypass the lethal phenotype (data not shown). Intriguingly, even if *CLN1* and *CLN2* genes were expressed from the *GAL1* promoter for 3 h, Cln1 or Cln2-Cdc28 kinase activity could not be detected in the *met4::GAL1–MET4 met30*Δ mutant strain. Similarly, Western analysis revealed that Cln1p and Cln2p were barely detectable (data not shown). To ensure that the decrease in protein level was not due to repression of the *GAL1* promoter, we also examined the levels of Cln2p expressed from the endogenous promoter or from the constitutive *ADH1* promoter. In each case, Cln2p was undetectable in the *met4::GAL1–MET4 met30*Δ cells (data not shown). We next performed Northern analysis with total RNA extracted from *met4::GAL1–MET4 met30*Δ cells grown in the presence of galactose and found that *CLN2* transcripts were undetectable, while in contrast, *ACT1* mRNA levels were clearly detectable, although slightly reduced (Figure 7A). As the protein level had suggested, *CLN1* transcripts were also undetectable, as were *PCL2* transcripts, while *CLN3*, *WHI3* and *WHI4* transcripts appeared normal (data not shown).

It was important to determine whether *CLN1* and *CLN2* mRNAs failed to accumulate during G_1 phase in the *met30*Δ mutant cells, or whether the lack of detectable transcripts was a secondary consequence of the prolonged G_1 arrest. We therefore collected small, unbudded, G_1 *met4::GAL1–MET4 met30*Δ cells by centrifugal elutriation, added raffinose to one half of the culture and galactose to the other, and collected samples for Northern, FACS and budding analysis. The cells grown in raffinose medium expressed *CLN1*, *CLN2* and *CLN3* transcripts appropriately, grew in cell size, initiated budding and replicated their DNA (Figure 7B and C). In contrast, the cells grown in galactose medium failed to accumulate *CLN1* and *CLN2* mRNAs but did have wild-type levels of *CLN3* mRNAs (Figure 7B). As before, the cells did not initiate budding or DNA replication (Figure 7C). The inability of *met30*Δ cells to accumulate *CLN1/2* and *PCL1/2* mRNAs is sufficient to account for the arrest at Start (Espinoza *et al.*, 1994; Measday *et al.*, 1994). However, we cannot rule out the possibility that other G_1 phase transcripts are also affected.

Discussion

Met4p and passage through the G_1 phase of the cell cycle

In this study, we demonstrate that components of the *MET* gene network are critical for passage through Start in budding yeast. Our genetic analysis shows that the essential function of *MET30* is bypassed by deletion of *MET4*, the gene that encodes the main transcriptional activator of the sulfur amino acid biosynthesis pathway, and that unregulated Met4p activity is lethal. The creation of a *met4::GAL1–MET4 met30*Δ conditional mutant has allowed us to confirm the function of Met30p in regulating Met4p function (Figure 2B) and to characterize the arrest phenotype of *met30*Δ cells (Figures 3 and 4). Surprisingly, *met30*Δ conditional mutants arrest as large, unbudded cells with unreplicated DNA. Genetic analysis has allowed us to determine that the G_1 arrest phenotype of the *met30*Δ conditional mutant is most likely not a result of an unbalanced synthesis of a sulfur metabolite because mutations in structural genes of the *MET* pathway do not alter the lethal phenotype of *met30*Δ strains (Figure 1). The G_1 arrest phenotype is dependent on the transcriptional activity of Met4p, because Met4p mutants without a transcriptional activation domain (Met4ΔAD), or that are incapable of binding the cofactors Met31p and Met32p (Met4ΔINT), are not lethal in a *met30*Δ background (Figure 3A). Consistent with these results, the *met32*Δ mutation is also capable of bypassing the *met30*Δ lethal phenotype (Figure 1B). The fact that the *met32*Δ mutation, but not the *met31*Δ mutation, was specifically capable of bypassing the *met30*Δ mutation was somewhat unexpected since Met31p and Met32p, which are two highly homologous zinc finger factors, are thought to have redundant functions (Blaiseau *et al.*, 1997). For instance, only the double *met31*Δ *met32*Δ mutant is a methionine auxotroph, while single *met31*Δ and *met32*Δ mutants are not (Blaiseau *et al.*, 1997). Likewise, mobility shift assays revealed no difference between the Met4–Met28–Met31 and Met4–Met28–Met32 complexes assembled on the *MET3* and *MET28* promoter regions (Blaiseau and Thomas, 1998). Our results suggest, however, that either the precise function of Met31p and Met32p, or at least the amount of the two proteins required for the assembly of the Met4p-containing complexes, might differ.

Regulation of Met4p by Met30p is dependent on a Met30p binding region called the IR (Thomas *et al.*, 1995). Accordingly, a Met4p derivative lacking the IR region (amino acids 189–235) was recently shown to be less sensitive to SCF^{Met30}-triggered degradation in response to high extracellular methionine (Rouillon *et al.*, 2000). However, during our analysis of the regions of Met4p required for the G_1 arrest in *met30*Δ mutants, we found that the Met30p–Met4p interaction region previously defined by two-hybrid analysis did not entirely correlate with the Met4p region required for inhibition by Met30p because deletion of the former was not sufficient to induce a G_1 arrest in cells carrying *MET30*. Deletion of a slightly greater domain (amino acids 160–220, Met4Δ47) was, in contrast, capable of inducing a G_1 arrest in a *MET30* background. We suggest that our previously defined IR domain is required for the Met30p–Met4p two-hybrid interaction, yet full Met30p repression of Met4p requires amino acids 160–220 of Met4p.

Conditional *met4::GAL1–MET4 met30*Δ mutants arrest in G_1 phase within the first cell cycle after a shift to a galactose medium (Figures 4A and 5). The essential function of Met30p is required at or after the pheromone arrest point, but before budding or DNA replication has

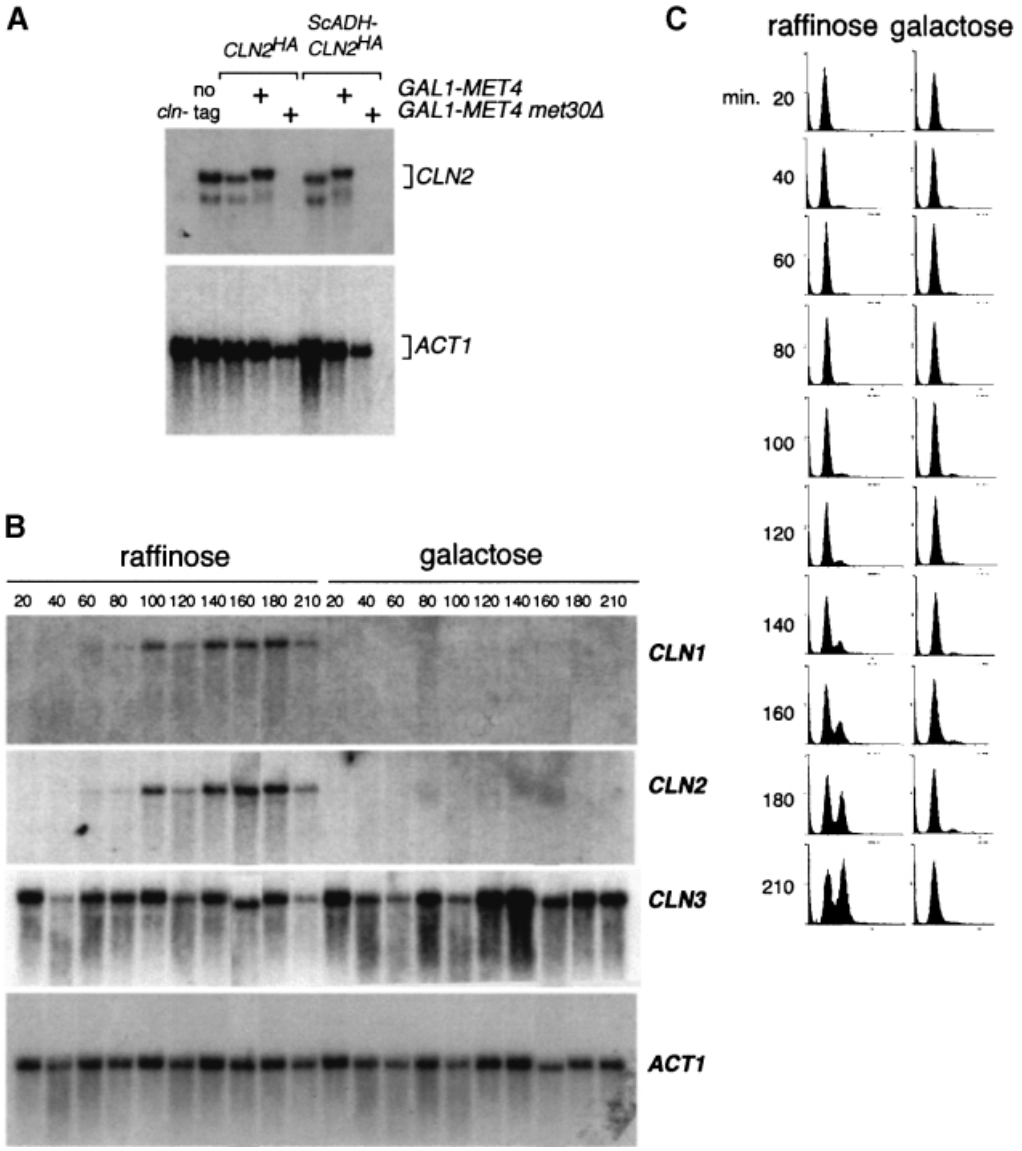


Fig. 7. Conditional *met30*Δ mutants lack *CLN1* and *CLN2* mRNA transcripts. (**A**) Northern analysis of *CLN* mRNA transcripts demonstrates a lack of *CLN2* mRNA expressed from the endogenous or the *ADH1* promoter in galactose-grown *met4::GAL1–MET4 met30*Δ cells. (**B**) *CLN1* and *CLN2*, but not *CLN3* mRNAs, fail to accumulate in elutriated G_1 *met4::GAL1–MET4 met30*Δ cells grown in galactose, but accumulate normally in *met4::GAL1–MET4 met30*Δ cells grown in raffinose. Cells were grown in a rich, raffinose medium, the culture split into two, and raffinose added to one culture and galactose to the other. Samples were taken for (B) Northern analysis and (**C**) flow cytometry.

been initiated. Overexpression of *MET4* alone does not cause a G_1 arrest, but it is sufficient to cause a slight delay from G_1 into S phase, suggesting that overexpression of Met4p can transiently overcome a limiting amount of repression by SCF^{Met30}. Indeed, the *MET30-1* mutation, which is partially defective in Met4p repression in response to high extracellular methionine (Thomas *et al*., 1995), also exhibits a delay in passage through G_1 phase. That the *MET30-1* mutation is not lethal may indicate that while some excess Met4p activity is admissible in the cell, a complete lack of regulation, such as in *met30*Δ, is intolerable.

Models for Met4p function in the G_1 phase

We are currently exploring two models for the manifestation of the *met30*Δ G_1 arrest phenotype. In the first one, a specific transcription activation complex comprising Met4p and Met32p regulates the expression of a gene or series of genes that inhibits passage though G_1 phase. Since the G_1 arrest is dependent upon the Met4p activation domain, transcriptional activity in this model would arise specifically from Met4p while Met32p would act by recruiting Met4p to DNA, as Met32p does not appear to have transcriptional activity (Blaiseau *et al*., 1997). Transcription of this G_1 phase 'inhibitor' gene might not depend on the known Met4p transcription complexes that are assembled upstream of the classical *MET* genes because deletion of *CBF1* and *MET28* does not suppress the lethality of the *met30*Δ mutation (Figure 1B). Likewise, the 'inhibitor' gene(s) might not be required for methionine biosynthesis because the *met32*Δ *met30*Δ cells are methionine prototrophs. Thus, Met4p might be responsible for the regulation of multiple gene networks and assemble into numerous transcriptional activation complexes. An alternative model for the *met30*Δ G_1 arrest might be that, due to a lack of Met4p degradation, increased protein

Table II. Yeast strains

Strain	Genotype	Reference
C180	*MAT***a**, *ade2, his3, leu2, ura3, str4::URA3*	Chérest and Surdin-Kerjan (1992)
CD127-1AT	*MAT***a**, *ade2, his3, leu2, ura3, met30::LEU2*, pLexMet30-4	Thomas *et al*. (1995)
CD127-1CT	*MAT***a**, *ade2, his3, leu2, ura3, met30::URA3*, pLexMet30-4	Thomas *et al*. (1995)
CD106	*MAT***a**, *ade2, his3, leu2, ura3, met4::TRP1*	Thomas *et al*. (1992)
CD130-1A	*MAT***a**, *ade2, his3, leu2, ura3, met28::LEU2*	Kuras *et al*. (1996)
CD155	*MAT***a**, *ade2, his3, leu2, ura3, met3::URA3*	this study
CD171	*MAT***a**, *ade2, his3, leu2, ura3, met4::URA3, met30::LEU2*	this study
CD179	*MAT***a**, *ade2, his3, leu2, ura3, met31::LEU2, met32::TRP1*	Blaiseau *et al*. (1997)
CC435-6C	*MAT***a**, *ade2, his3, leu2, ura3, sam1::URA3, sam2::URA3*	Thomas *et al*. (1988)
CC807-1C	*MAT***a**, *ade2, his3, leu2, ura3, met4::TRP1, met30::LEU2*	this study
CC807-7D	*MAT***a**, *ade2, his3, leu2, ura3, met4::TRP1, met30::LEU2*	this study
CC847-1C	*MAT***a**, *ade2, his3, leu2, ura3, met30::URA3*, pLexMet30-4	this study
CC849-8A	*MAT***a**, *ade2, his3, leu2, ura3, met4::TRP1*	this study
CC850-2B	*MAT***a**, *ade2, his3, leu2, ura3, met4::TRP1, met30::URA3*	this study
CC864-1B	*MAT***a**, *ade2, his3, leu2, ura3, met6*	this study
CC867-1B	*MAT***a**, *ade2, his3, leu2, ura3, met30::URA3, met31::LEU2, met32::TRP1*	this study
CC867-1D	*MAT***a**, *ade2, his3, leu2, ura3, met30::URA3, met32::TRP1*	this study
CC874-18D	*MAT***a**, *ade2, his3, leu2, ura3, str4::URA3*	this study
CC906-13C	*MAT***a**, *ade2, his3, leu2, ura3, met3::URA3*	this study
CC932-6D	*MAT***a**, *ade2, his3, leu2, ura3, met4::GAL1–MET4*	this study
CC932-8B	*MAT***a**, *ade2, his3, leu2, ura3, met4::GAL1–MET4, met30::LEU2*	this study
CC954-10A	*MAT***a**, *leu2, ura3, cdc7-1*	this study
CC954-10C	*MAT***a**, *his3, leu2, ura3, cdc7-1, met4::GAL1–MET4, met30::LEU2*	this study
CC970-1A	*MAT***a**, *ade2, his3, leu2, ura3, cdc7-1, met4::GAL1–MET4*	this study
R31-5C	*MAT***a**, *ade2, his3, leu2, lys2, ura3, cbf1::TRP1*	O'Connell and Baker (1992)
W303-1A	*MAT***a**, *ade2, his3, leu2, trp1, ura3*,	R.Rothstein
W744-1A	*MAT***a**, *ade2, his3, leu2, ura3, sam1::LEU2, sam2::HIS3*	Bailis and Rothstein (1990)

Table III. Plasmid list

Plasmid	Relevant characteristics	Source
pGalMet4-1	*pGal1-MET4*$^{15–666}$, *URA3*, CEN	this study
pGalMet4Δ12	*pGal1-MET4*$^{\Delta 79–180}$, *URA3*, CEN	this study
pGalMet4Δ13	*pGal1-MET4*$^{\Delta 189–221}$, *URA3*, CEN	this study
pGalMet4Δ21	*pGal1-MET4*$^{\Delta 115–184}$, *URA3*, CEN	this study
pGalMet4Δ26	*pGal1-MET4*$^{\Delta 160–186}$, *URA3*, CEN	this study
pGalMet4Δ30	*pGal1-MET4*$^{\Delta 211–232}$, *URA3*, CEN	this study
pGalMet4Δ37	*pGal1-MET4*$^{\Delta 352–366}$, *URA3*, CEN	this study
pGalMet4Δ38	*pGal1-MET4*$^{\Delta 375–430}$, *URA3*, CEN	this study
pGalMet4Δ47	*pGal1-MET4*$^{\Delta 160–221}$, *URA3*, CEN	this study
pGalMet4Δ146	*pGal1-MET4*$^{\Delta 616–666}$, *URA3*, CEN	this study
pMT291	*CLN2*^{HA}, *LEU2*, CEN	Tyers laboratory
pMT1241	*CLN1*^{HA}, *LEU2*, CEN	Tyers laboratory
pMT485	*pGAL1-CLN1*^{HA}, *LEU2*, *CEN*	G.Tokiwa
pMT634	*pGAL1-CLN2*^{HA}, *LEU2*, *URA3*, *CEN*	Tyers laboratory
pCB1317	*Sc-pADH1-CLN2*^{HA}, *LEU2*, *CEN*	K.Arndt

levels of Met4p inhibit passage through G_1 phase by associating specifically or non-specifically with other transcriptional complexes. However, the specificity of the *met32*Δ mutation to bypass the G_1 arrest, as well as the altered *CLN1* and *CLN2* but not *CLN3* transcript levels, argue for more than Met4p non-specifically sequestering the general transcription machinery away from promoters of G_1-specific genes.

Potential mechanisms for the conditional met30Δ G_1 arrest

A variety of G_1 mutants that arrest as unbudded cells with unreplicated DNA have been identified in *S.cerevisiae*, including cell division mutants (such as *cdc28* and *cln* mutants) and cell growth and translation mutants (such as the Ras-cAMP and *tor*$^-$ mutants; Hartwell, 1994; Nasmyth, 1996; Polymenis and Schmidt, 1999). Because our *GAL1–MET4 met30*Δ mutants were unable to pass through Start, we examined both possibilities as the potential route to the G_1 arrest phenotype. We used synchronized cells to determine whether the *met30*Δ mutants were capable of growth in G_1 phase, and to determine whether translation was compromised (Figure 6). While the *met30*Δ mutant did continue to accumulate mass in G_1 phase after a prolonged growth arrest, translation was slightly slowed in comparison with *MET30* cells, and polysome profiles showed a corresponding defect in translational apparatus. The delayed kinetics of the protein synthesis response compared with the first cycle G_1 arrest suggest that the effect on translation is a secondary consequence of the arrest. In contrast, we unexpectedly found that *CLN1*, *CLN2* and *PCL2* transcripts were entirely absent from the *met30*Δ cells, while *CLN3*, *WHI3* and *WHI4* transcripts were unaffected. Since the loss of G_1 cyclin transcripts occurs well before any effects on protein synthesis, we suggest that the effect on G_1 transcripts might directly result from unregulated Met4p activity. Because *cln1 cln2 pcl1 pcl2* mutants arrest at Start, the absence of these transcripts may account for the phenotype of *met30*Δ cells. As *CLN* transcripts are very unstable even in wild-type cells, unregulated Met4p activity in the *met30*Δ mutant may hyperactivate a G_1 cyclin RNA degradation mechanism. Our results further indicate that any such pathway would presumably not affect *CLN3* mRNA. In possible comparison, an SCF-like complex called VCB–CUL2 (comprised of VHL–ElonginB/C–CUL2) is thought to regulate, either directly or indirectly, accumulation of hypoxia-inducible mRNAs by targeting a protein for degradation in hypoxic conditions (reviewed in Kaelin and Maher, 1998; Tyers and Rottapel, 1999). Nutrient-dependent modifications of RNA stability have also been

identified as a mechanism of glucose repression (Lombardo *et al*., 1992; Cereghino and Scheffler, 1996). While control over transcription, translation and protein stability has been explored as a means to control G_1 cyclin levels, to our knowledge, our results constitute the first suggestion of a control at the level of G_1 cyclin mRNA stability.

Methionine and the cell cycle

Connections between the cell cycle and methionine were first identified by Unger and Hartwell (1976) with the study of *met* mutants that arrested in G_1 phase in the absence of methionine, and of methionine tRNA synthase mutants that arrested in G_1 phase even in the presence of methionine. They predicted that a 'signal' allowed the cell to arrest specifically in G_1 phase and acted at the level of protein biosynthesis. Whether the *met30*Δ G_1 arrest is similar to the arrest described by Unger and Hartwell (1976) has yet to be determined. DNA microarray analysis has also revealed that the expression of most of the *MET* genes is specifically activated during S phase (Spellman *et al*., 1998), suggesting that cell cycle and sulfur amino acid metabolism are closely linked. Our study reinforces this connection by linking the regulation of the main regulator of the *MET* network, Met4p, with the cell cycle. Important questions that arise from this work are whether and how regulation of Met4p might couple methionine with passage through G_1 phase. Cell size experiments and the rate of passage through Start in synchronized, small G_1 cells suggest that in the presence of high extracellular methionine, cells reset their critical cell size to a larger volume and delayed passage through Start (E.E.Patton and M.Tyers, unpublished data). This initial finding is superficially at odds with the clear inhibition of Start by excessive Met4p activity. However, as the *met30*Δ *met32*Δ mutant is a methionine prototroph, it is possible that a specific Met4–Met32 transcription complex responds to a different signal than an increase of intracellular AdoMet.

Finally, the sulfur amino acid biosynthesis pathway is highly conserved in eukaryotes (Griffith, 1987). Connections between methionine metabolism and cell cycle control have already been noted in mammals. Both *in vitro* and *in vivo* studies have demonstrated the methionine dependence of numerous transformed cells and tumors (Hoffman, 1997). More recently, it was shown that athymic mice that are grafted with human cancers and fed a methionine-free diet are greatly reduced in their tumor burden (Kokkinakis *et al*., 1997). The results reported here emphasize the importance of future studies on the coordination of nutrients, such as methionine, with the cell cycle.

Materials and methods

Yeast strains and media

Saccharomyces cerevisiae strains used in this work are listed in Table II. They are all isogenic to the W303 background. Standard yeast media were prepared as described by Chérest and Surdin-Kerjan (1992). *Saccharomyces cerevisiae* was transformed after acetate chloride treatment as described by Gietz *et al*. (1992). Genetic crosses, sporulation, dissection and scoring of nutritional markers were as described by Sherman *et al*. (1979).

For crosses as shown in Figure 1, diploids were selected and grown for several generations in the presence of histidine to induce plasmid loss. Diploids were sporulated, and the progeny of each cross were analyzed (20 tetrads each, Figure 1B). For the crosses involving the *met3*Δ, *met6*Δ, *str4*Δ or the double *sam1*Δ*sam2*Δ mutants with the *met30*Δ *LexA-MET30*, *HIS3* strain, the progeny tetrads contained two or three viable spores, and those spores that were Leu^+ (i.e. *met30*Δ) were also His^+ (i.e. *LexA-Met30*). The progeny of the cross involving the *met4*Δ mutation showed a high number of tetrads giving rise to four viable spores (13 for a total of 20 tested tetrads) and, of 67 viable spores, 17 were found to be leucine prototrophs (i.e. *met30*Δ) and histidine auxotrophs (i.e. they did not harbor the pLexM30-4 plasmid). In addition, all these 17 spores were found to be tryptophan prototrophs and methionine auxotrophs, proving that they were all *met4*Δ. Conversely, no leucine prototroph spores that were both histidine and tryptophan auxotrophs were recovered. These results therefore suggested that *met30*Δ spores could be recovered if they contain the *met4*Δ disruption mutation. To confirm this result, a *met4*Δ *met30*Δ spore was backcrossed to a wild-type strain or to a *met4*Δ *MET30* strain. In the former cross (CC808), only 25% of the recovered spores were leucine prototroph spores and all these spores were tryptophan prototrophs, while in the latter cross (CC809) the leucine character segregated perfectly 2+/2–.

Plasmids

To express the different Met4p derivatives from the *GAL1* promoter region from an autonomous replicating plasmid, we used the pGal316 and pGal313 plasmids (Rouillon *et al.*, 2000). They were cleaved by *Eco*RI and *Bam*HI and ligated with different *Eco*RI–*Bam*HI fragments encoding deleted derivatives of Met4p from the set of pLexMet4 plasmids described in Kuras and Thomas (1995). New Met4p derivatives were constructed as described in Kuras and Thomas (1995) except that the ligations were made directly in the pGal313 or pGal316 plasmids cleaved by *Eco*RI and *Bam*HI (Table III).

To integrate the *GAL1–MET4* fusion gene into the genome, we first cloned the *Stu*I–*Eco*RI fragment, blunt ended with Klenow fragment from plasmid pM4-1 (Thomas *et al.*, 1992) into pGalM4-1, cleaved by *Asp*718, blunt ended with Klenow fragment and dephosphorylated. Since correct ligation events restored one *Asp*718 site, the resulting plasmid contained one *Asp*718–*Bam*HI fragment corresponding to the *GAL1–MET4* gene fusion flanked by 5′ and 3′ regions of the *MET4* gene. Owing to the one-step gene disruption method (Rothstein, 1983), this fragment was used to replace the *met4::TRP1* locus of strain CC849-8A by the *GAL1–MET4* fusion gene by selecting for Met^+ transformants in galactose medium. Correct integration events were verified by Southern blot analyses.

Northern blot analyses

Northern blotting was performed as described by Thomas (1980), with total cellular RNA extracted from yeast as described by Schmitt *et al.* (1990) and oligolabeled probes (Hodgson and Fisk, 1987).

Flow cytometry

For flow cytometry analyses, cells were harvested, fixed in 70% ethanol and stained with propidium iodide as described (Mann *et al.*, 1992).

Polysome profiles

Polysomes were isolated as described (Zhong and Arndt, 1993). Briefly, cells were grown to log phase, and 60 μg/ml cycloheximide was added for 5 min before collecting the cells. Cells were lysed in a Tris-based buffer containing heparin, and extracts were loaded onto sucrose gradients ranging from 7 to 47% sucrose, spun at 35 000 r.p.m. in an SV41 rotor for 2 h, and read at A_{254} via continuous drip.

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