A Survey of Essential Gene Function in the Yeast Cell Division Cycle[□]

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Mutations impacting specific stages of cell growth and division have provided a foundation for dissecting mechanisms that underlie cell cycle progression. We have undertaken an objective examination of the yeast cell cycle through flow cytometric analysis of DNA content in *TetO₇* promoter mutant strains representing 75% of all essential yeast genes. More than 65% of the strains displayed specific alterations in DNA content, suggesting that reduced function of an essential gene in most cases impairs progression through a specific stage of the cell cycle. Because of the large number of essential genes required for protein biosynthesis, G1 accumulation was the most common phenotype observed in our analysis. In contrast, relatively few mutants displayed S-phase delay, and most of these were defective in genes required for DNA replication or nucleotide metabolism. G2 accumulation appeared to arise from a variety of defects. In addition to providing a global view of the diversity of essential cellular processes that influence cell cycle progression, these data also provided predictions regarding the functions of individual genes: we identified four new genes involved in protein trafficking (*NUS1*, *PHS1*, *PGA2*, *PGA3*), and we found that *CSE1* and *SMC4* are important for DNA replication.

INTRODUCTION

Cell division is a fundamental biological process that in all organisms consists of a series of closely coordinated events. In the budding yeast *Saccharomyces cerevisiae*, the cell division cycle begins with an initial growth phase, G1, during which time the cell increases in mass and volume. The transition from G1 into S phase is marked by progression through Start (the point of commitment to cell division), initiation of nuclear DNA synthesis, and the emergence of a bud that will form the new daughter cell. S phase is followed by a second growth phase, G2, which is in turn followed by nuclear division, and then cell separation. A very similar series of events occurs in all eukaryotes, with the obvious exception of bud emergence. Because of the fundamental biological importance of cell cycle progression and its importance in both human development and diseases such as cancer, identification of the molecular determinants of specific stages of the eukaryotic cell cycle has been a subject of intense study for several decades.

The morphological landmarks of the cell cycle stages in budding yeast, most notably the size of the bud relative to the size of the mother cell, allows the identification of mutants blocked at specific stages of the cell cycle and thereby forms the basis for the classic cell cycle screens (Hartwell

et al., 1970; Culotti and Hartwell, 1971; Hartwell, 1971a, 1971b, 1973; Moir *et al.*, 1982). These genetic screens, using conditional temperature-sensitive mutants, identified more than 50 genes that are required for specific stages in the cell division cycle and so were termed *CDC* genes. On average 4.6 alleles were identified for each *CDC* gene in the original screens, suggesting the number of *cdc* mutants that could be identified by this approach had reached a plateau (Hartwell *et al.*, 1973). However, several lines of evidence suggested that additional genes with cell cycle stage-specific functions remained to be identified (Hartwell *et al.*, 1973; Hartwell, 1974; Pringle and Hartwell, 1981). Indeed, cell cycle screens in other model organisms identified additional *CDC* genes among the essential genes (Nurse, 1975; Nurse and Bisset, 1976; Nasmyth and Nurse, 1981), as have more recent screens using alternative strategies (Prendergast *et al.*, 1990; Stevenson *et al.*, 2001; Kanemaki *et al.*, 2003). Consistent with their roles in a biological process of fundamental importance, most of the *CDC* genes are conserved and appear to have conserved function in most eukaryotes, including humans.

Several approaches exist for studying the biological function of essential genes. Temperature-sensitive mutations have been used extensively in analysis of cell cycle genes, and many temperature-sensitive mutations lead to rapid depletion of the gene product being analyzed. However, temperature-sensitive mutations are difficult to construct in a systematic manner, and the molecular basis for temperature sensitivity is, in most cases, unknown. Induced proteolysis through the creation of gene fusions to sequences encoding the “N-degron,” a temperature-inducible proteolytic degradation signal (Dohmen *et al.*, 1994), has also been used to identify cell cycle genes (Kanemaki *et al.*, 2003). The N-degron can be applied systematically, and in many cases

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leads to rapid gene product depletion. However, in a recent study in which 104 essential genes were fused to the N-degron sequences, nearly 40% of essential genes fused to the N-degron did not result in inviability at the nonpermissive temperature (Kanemaki *et al.*, 2003), indicating that rapid protein depletion by the N-degron is not uniform across the proteome. Despite this limitation, functional information could be derived from strains in which depletion was incomplete (Aparicio, 2003; Kanemaki *et al.*, 2003). Essential gene function can also be studied systematically by gene product depletion using a repressible promoter. Promoter replacement alleles allow the systematic analysis of essential genes, although, like N-degron fusions, the degree of gene product depletion varies from gene to gene, depending on both mRNA and protein half-life. With promoter replacement alleles each open reading frame (ORF) remains intact, and repression conditions with minimal effects on cell physiology can be chosen. In a previous study (Mnaimneh *et al.*, 2004), we described the construction of tetracycline-regulatable promoter (*TetO₇* promoter) alleles of ~600 essential genes in *S. cerevisiae*. The *TetO₇* promoter collection has been used to probe essential gene function in cell size control, cell morphology, mitochondrial morphogenesis, and for gene expression and synthetic genetic interaction profiling (Mnaimneh *et al.*, 2004; Altmann and Westermann, 2005; Davierwala *et al.*, 2005). In the present study, we have expanded the *TetO₇* promoter collection to encompass 773 essential genes, almost 75% of the essential gene set, making it the most complete resource for systematic analysis of essential gene function in yeast.

Many genes involved in cell growth, cell division, and cell cycle progression are indispensable for these processes and are therefore included among the ~1050 yeast genes essential for viability (out of a total of ~5800 yeast genes). Here, we utilized the *TetO₇* promoter collection to analyze essential gene function in cell division and cell cycle progression, using flow cytometric analysis to measure cellular DNA content after promoter shut-off. More than 65% of the strains displayed an altered flow cytometry profile after promoter shut-off, allowing categorization of essential genes on the basis of cell cycle profile. Our systematic analysis is not only useful in illustrating widespread contribution of essential genes to individual stages of the cell cycle, but is also useful for elucidating the functions of uncharacterized genes.

MATERIALS AND METHODS

Strain Construction

TetO₇ strains were constructed as described previously (Mnaimneh *et al.*, 2004). The genotype of the wild-type *TetO₇* strain, R1158, is *MATa URA3::CMV-tTA his3Δ1 leu2Δ0 met15Δ0*. Primers for the PCR amplification for the DNA fragments containing the Tet-off promoter, Kan^R gene and partial sequences of the target genes and for confirmation of correct promoter replacement are available at http://hugheslab.med.utoronto.ca/Mnaimneh/data/Primers/Hughes_tet_promoter_primers_Supp.xls. Two strategies were used for PCR confirmation of integrations. The first utilized primers to genomic DNA outside the intended integration site, in which a correct integration yields a band of ~2.4 kb, an incorrect integration ~400 bases. The second confirmation strategy utilized one primer complimentary to the cassette and one to the genomic DNA, in which a band of ~700 bases represents an integration at the correct site. The *smc4-1* strain (*MATa smc4-1::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*) contains the *smc4-1* allele (Freeman *et al.*, 2000) integrated with a marker at the *SMC4* locus in the S288C strain background (Brachmann *et al.*, 1998).

Flow Cytometry

For screening the collection of *TetO₇* promoter alleles, cells were grown at 30°C in the absence or presence of 10 μg/ml doxycycline for 15 h, and ~1 × 10⁷ cells for each strain were harvested and fixed with 70% ethanol. Cells were typically harvested at an OD₆₀₀ of <1.0 and processed for flow cytometry as described (Davierwala *et al.*, 2005; Figure 1). The complete set of flow cytometry

histograms, ordered by position on the array plates of the *TetO₇* promoter collection, is available at: <http://biochemistry.utoronto.ca/brown/data.html>.

Manual Analysis of Flow Cytometry Profiles

Profiles were assigned to different classes according to the following criteria: Profiles with equal height for 1C and 2C peaks were assigned to the normal category; profiles with the 1C peak higher than the 2C peak were assigned to the 1C category; profiles with the 2C peak higher than the 1C peak were assigned to the 2C category; profiles with distinct peaks at 3C and/or 4C positions were assigned to the 3C/4C category; profiles with accumulation of DNA contents between 1C and 2C were assigned to the S-phase category; profiles with only a 2C and a 4C peak were classified as diploid; profiles with DNA contents less than 1C; and any profiles that did not fit any of the above criteria were classified as "other."

Computational Analysis of Flow Cytometry Profiles

Normalization. As a preprocessing step, we consecutively aligned the local maximum of all samples in each DNA content region. The alignment of flow cytometry data were done as follows: 1) select the rightmost channel left of the channel representing the DNA content region to align, such that most of the samples do not show DNA content at this channel (i.e., this channel is a sparse region of the flow cytometry matrix), and set this channel as the cutting point; 2) locate the local maximum in a fixed range (±50 channels) around the channel corresponding to the selected DNA content; 3) shift the vector of values starting at the cutting point per each sample so that its local maximum corresponds to the channel representing the selected DNA content; and 4) in case of left shifting discard any value left of the cutting point; otherwise, fill the created spaces with the value at the cutting point.

Classification. Profiles were assigned to six different abnormal categories according to the following criteria (thresholds were manually obtained based on examination of the data, i.e., the computational categorization was tuned to make the same decisions as manual inspection):

1. Other: Profiles whose local maximum before the 1C DNA content region is greater than 25.
2. 1C DNA contents: Profiles whose ratio of 1C DNA content to 2C DNA content is greater than 1.48.
3. S phase: Profiles whose local maximum between 1C DNA content and 2C DNA content regions is greater than 27; and, the ratio of this local maximum to the global maximum is greater than 0.23; and, the ratio of this local maximum to the mean value is greater than 2.8.
4. 2C DNA contents: Profiles whose ratio of 2C DNA content to 1C DNA content is greater than 1.4.
5. 3C/4C DNA contents: Profiles whose local maximum in the 3C or 4C DNA content regions is greater than 10.
6. Diploids: Profiles whose local maximum in the 1C DNA content region is <20, and, their ratio of 2C DNA content to 1C DNA content is greater than 1.4, and their local maximum in the 4C DNA content is greater than 30.

Cytometry histograms of the genes that did not fill any of the criteria above were inspected and classified as normal.

Functional Analysis. For analysis of the functional enrichment of each class, we used the annotations from the Biological Process hierarchy of the Gene Ontology (GO) in the YEAST annotation package 1.10 of Bioconductor (Gentleman *et al.*, 2004). Before the statistical analysis, GO annotations were up-propagated. GO Categories with a hypergeometric p value smaller than 0.001 were considered to be significantly overrepresented in a class, because this resulted in zero or one false-positive categories on average for each of 10,000 sets of randomly chosen genes of the same sizes as those analyzed here. For visualization, similar significant GO categories were joined together under their most specific common ancestor.

G1 Intragroup Functional Analysis. Genes in the G1 class were first ordered according to the ratio of 1C accumulation peak to 2C accumulation peak and then separated in four equally sized sets. The functional enrichment of each set of genes was determined as described in *Functional Analysis* above. p values for lower quartiles were calculated excluding genes in upper quartiles.

Clustering. Genes in each of the six DNA content classes shown in the heatmaps (Figures 2A and 3A) were separately clustered using Pearson correlation as a similarity measure and hierarchical clustering with the complete linkage method. Clustering and heatmaps were generated in R 2.2.0. The genes in the cell size heatmap (Figure 3A) are shown in the same order as in the DNA content figure (Figure 2A).

Cell Size Analysis. For the genes in the intersection of our manual and computational classifications we obtained the size bin where the cumulative sum of the cells that had size corresponding to that bin or a smaller size reaches 50% of the total number of cells measured per mutant in the cell size

Figure 1. A cell cycle screen of the essential genes in yeast. (A) Schematic diagram of the cell cycle screen. Strains were constructed by replacement of native promoters with a *TetO₇* cassette in the strain R1158, which contains sequences encoding the tet “off” activator tTA* integrated at *URA3*. *YFG1*, your favorite gene; *NGC1*, next gene on chromosome. The flow cytometry data are displayed as a histogram with fluorescence intensity (which is proportional to DNA content) plotted on the x-axis, and the number of cells with a given intensity plotted on the y-axis. The positions of cells with 1C, 2C, and 4C DNA contents are indicated. (B) Manual and computational scoring of the flow cytometry histograms. The number of *TetO₇* strains displaying each category of cell cycle profile is indicated for manual scoring and computational scoring. Strains that were placed in the same category by both methods are indicated as the overlap. (C) Flow cytometry histograms of mutants showing strong cell cycle effects in each category are shown, with the relevant gene indicated. The number of cells is indicated on the y-axis, and the DNA content is indicated on the x-axis.

data (Mnaimneh *et al.*, 2004). The box plot was generated using the box plot function available in R 2.2.0.

Raw and normalized flow cytometry data, as well as data used to generate the graphical displays in Figures 2, 3, and 4, and the complete functional enrichment data are available at: <http://biochemistry.utoronto.ca/brown/data.html>.

Western Blotting

Strains were grown in YPD in the absence or presence of 10 μ g/ml doxycycline for 15 h to an OD₆₀₀ of ~0.5. Cultures corresponding to 3 OD of cells were then collected, and the cell pellets were resuspended in 0.5 ml fresh YPD. Protein extracts were prepared by NaOH lysis and TCA precipitation, and western blotting with monoclonal anti-CPY or anti-ALP antibodies (Molecular Probes, Eugene, OR), or polyclonal anti-Gas1p antibody (kind gift from H. Riezman), as described (Davierwala *et al.*, 2005).

Chromatin Immunoprecipitation

Strains were grown in the presence of doxycycline for 5 h and synchronized with α -factor. G1-arrested cells were released into fresh media and ~20 OD of cells were harvested every 12 min. Cells were fixed and chromatin immunoprecipitates were prepared essentially as described (Kuras and Struhl, 1999), using IgG-agarose to immunoprecipitate TAP-tagged Mcm2 from wild-type and *TetO₇-SMC4* strains. Immunoprecipitated DNA was analyzed by PCR using four primer pairs for specific regions at and around ARS1 as described (Tanaka *et al.*, 1997). All PCR, gel electrophoresis, and visualization conditions were as described (Tanaka *et al.*, 1997), and the gel was quantified using ImageQuant.

RESULTS

A Collection of Titratable Promoter of Essential Genes

In an effort to create a complete collection of *TetO₇* promoter alleles for essential yeast genes, we attempted *TetO₇* promoter strain construction as described previously (Mnaimneh *et al.*, 2004) for all of the 1111 genes annotated as essential by *Saccharomyces* Genome Database (SGD), the Yeast Proteome Database (YPD), or the Munich Information Center for Protein Sequences (MIPS), as of June 2000 when this project was initiated. At least two attempts were made to construct a *TetO₇* promoter strain for each gene. For a total of 838 genes the resulting strains were subjected to PCR analysis to confirm correct integration of the *TetO₇* promoter and replacement of each wild-type promoter region (Mnaimneh *et al.*, 2004). Failure to obtain *TetO₇* promoter strains could be accounted for by errors in any step from genome sequencing to PCR confirmation, to the promoter being refractory to recombination, or to the promoter integration itself resulting in lethality (e.g., by mis-expressing the gene when the *TetO₇* promoter is “on”). All of the strains are available from Open Biosystems. Since June 2000, SGD has emerged as the single major source of public annotation of the yeast genome. Some genes have been removed from SGD because of resequencing

Figure 2. Enrichment of specific gene functions within the different flow cytometry categories. (A) Cluster diagram of flow cytometry histograms. Genes are positioned along the *y*-axis and DNA content is indicated on the *x*-axis. Genes are grouped into different categories according to the overlap between the computational and manual analysis and are clustered within the categories according to the similarity of the flow cytometry profiles. The number of cells with a given DNA content is indicated by color, with black representing the minimum and brown representing the maximum. The categories of flow cytometry profiles are indicated, with the category in gray being the diploid category. (B) GO-gram indicating statistically significant enrichments of gene ontology (GO) annotations from the biological process hierarchy of *TetO₇* alleles in each flow cytometry category. Red bars indicate that a particular gene is annotated with the given GO category *and* that the GO category is significantly overrepresented within the cell cycle category in which the gene was placed. Blue bars indicate that a particular gene is annotated with the given GO category and that the GO category is *not* significantly enriched within the given cell cycle category. Not all GO categories that displayed significant enrichment are shown.

and reannotation (mostly uncharacterized ORFs that are lethal when deleted, but overlap with essential genes that have now been characterized), and some genes categorized as essential in our previous analysis are no longer listed as being essential (presumably because slow-growing deletion mutants can be obtained). Thus, the collection encompasses 773 essential genes, out of a total of 1033 (75%) that are annotated currently by SGD.

A Cell Cycle Screen of the Titratable Promoter Alleles of the Essential Genes

We used the collection of *TetO₇* promoter alleles to systematically identify genes that cause a change in cell cycle profile after promoter shut-off. Because the nuclear DNA content of a cell reveals its position in the cell cycle, we measured the DNA contents of populations of cells using

flow cytometry, both before and after turning off transcription of each essential gene (Figure 1A). The resulting histograms can be used to determine the fraction of cells in a population that are at each cell cycle stage (G1, S, and G2), and accumulation of cells with a specific DNA content indicates a defect in progression through a particular cell cycle stage. In initial experiments with four of the *TetO₇* promoter replacement strains, samples were analyzed by flow cytometry at different times after the addition of doxycycline (Supplementary Figure 1). We found that for the strains analyzed, alterations in the flow cytometry histograms were first evident at varying times, from 2 h in the case of *CDC27*, to 8 h in the case of *SLD5*. Importantly, the same category of cell cycle accumulation was still evident at the latest time point, 15 h, and in the case of the S phase gene *SLD5* the accumulation became increasingly obvious and therefore

Figure 3. Comparison of cell cycle phenotypes and cell sizes. (A) Cluster diagram of DNA content flow cytometry histograms and cell size profiles. Genes are grouped on the y-axis as in Figure 2A. Cell size data from (Mnaimneh *et al.*, 2004) is presented alongside the flow cytometry data, with increasing cell sizes plotted on the x-axis, from left to right. The number of cells with a given cell size is indicated by color, with white representing the minimum and red representing the maximum. (B) Box plot of cell sizes in each cell cycle category. The horizontal black lines in the boxes indicate the median size bin where the cumulative sum of cells having a size corresponding to that bin, or a smaller size, reaches 50% of the total number of cells measured; the bottom and top of the boxes indicate the 25th and 75th percentiles, respectively. The vertical lines show the value range: the upper cap is drawn at the bin representing the largest size, and the lower cap is drawn at the bin representing the smallest size. The width of the boxes varies according to the number of genes per category.

easier to score at the later points. Thus, in order to detect changes in as many strains as possible, we chose to analyze the promoter replacement collection after 15 h of growth in the presence of doxycycline.

Scoring the Flow Cytometry Profiles

We scored the flow cytometry profiles using both manual inspection and computational analysis (see *Materials and*

Figure 4. Degree of G1 accumulation reflects gene function and growth defect. Genes in the 1C category are ordered from top to bottom by decreasing C1/C2 peak height ratio as shown. Quartiles are indicated at left. White dotted line indicates average wild-type C1/C2 ratio. The GO-gram indicates statistically significant enrichments of GO categories within the 1C flow cytometry category ($p < 0.001$). Red bars indicate that a particular gene is annotated with the given GO category *and* that the GO category is significantly over-represented within the cell cycle category in which the gene was placed. Blue bars indicate that a particular gene is annotated with the given GO category and that the GO category is *not* significantly enriched within the given cell cycle category. The growth scoring scheme is identical to that in (Mnaimneh *et al.*, 2004).

Methods). In both cases, the flow cytometry histograms were categorized according to the distribution of DNA contents (Figure 1B). The categories were as follows: strains that accumulate with 1C DNA content (and so are arrested or delayed in G1); strains that accumulate with DNA content between 1C and 2C (and so are delayed in S phase); strains that accumulate with 2C DNA content (and so are arrested or delayed in G2); strains that accumulate with 3C and/or 4C DNA content (an unexpected cell cycle profile, likely relating to defects in cell separation); and strains that were diploid; and strains exhibiting a normal cell cycle profile. In the manual analysis some profiles did not exhibit the characteristics of any of the categories, yet were clearly abnormal. These were grouped together as “other.” In the computational analysis the “other” category is made up of strains that showed accumulation of cells with less than 1C DNA contents. Strains that displayed accumulations at more than

one DNA content were placed in more than one category, and all strains were placed in at least one category.

Manual inspection indicated that of 838 *TetO₇* promoter mutants screened, 625 (or 75%) displayed an altered flow cytometry profile. Computational analysis (see *Materials and Methods* for details) indicated that 579 displayed an altered profile, and 563 mutants (or 67%) were scored as altered by both methods. On average, ~80% of assessments made by manual inspection were supported by computational analysis and vice versa. The fraction of genes in each category (supported by both manual and computational analysis) is shown in Figure 1B. Examples of the strongest cell cycle phenotypes exhibited by *TetO₇* promoter mutants are shown in Figure 1C. However, we scored even modest alterations in flow cytometry profile. For example, in the 1C accumulation category, modeling of the flow cytometry histograms indicated that the fraction of cells with a 1C DNA content ranged from 31 to 97% for mutants scored as 1C, compared with 28% for the wild-type control (see below).

In *TetO₇* promoter mutant strains the onset of growth cessation varies from gene to gene because of the time required for depletion of the transcript and protein. Even though we cannot be certain of the timing or degree of depletion of the different gene products after promoter shut-off for 15 h, we saw clear and specific effects on cell cycle progression with the majority of the essential genes. The accumulation of specific DNA contents suggests an intimate connection between essential gene function and cell cycle progression. The altered flow cytometry profiles did not appear to reflect cell debris, with the possible exception of some profiles classified as "other," indicating that our results were not artifacts due to simple slow growth or cell death. The flow cytometry profiles of the eight wild-type colonies we analyzed were scored as normal, and most of the *TetO₇* promoter strains with little or no growth defect on plates also had no perturbation of DNA content (112 of 190 *tet* promoter mutants with normal growth were scored by both methods as having normal flow cytometry profiles), indicating that our scoring of abnormal flow cytometry profiles was not overly liberal. Strains that did not show a growth defect in the presence of doxycycline likely represent strains in which gene product depletion is insufficient to cause a phenotype, perhaps because of long mRNA or protein half-life. However, 51 strains displayed normal growth and yet had altered cell cycle profiles in both manual and computational scoring (Supplementary Figure 2), indicating that in some instances the screen was sensitive enough to identify cell cycle delays in the absence of a growth defect. Conversely, of the *TetO₇* promoter strains with normal cell cycle profiles, almost half (82 of 194) exhibited growth defects in the presence of doxycycline. Therefore not all essential genes are linked to cell cycle processes; these 82 strains presumably cease growth without cell cycle stage specificity.

As a measure of the efficiency of the screen in identifying genes with roles in cell division cycle progression, we determined the fraction of known *CDC* genes that were scored as positive (Supplementary Figure 3). There are 67 *CDC* genes in the *Saccharomyces* Genome Database (SGD). Of these, 6 have not been mapped, and 16 are not present in the *TetO₇* promoter collection. Of the remaining 45, 89% displayed an altered cell cycle profile that was scored manually, computationally, or by both methods. The cell cycle arrest point for 38 of these *CDC* genes is known or can reasonably be inferred. In our screen, the majority (28 of 38; 74%) showed the expected cell cycle stage-specific accumulation. For example, *CDC6*, *CDC8*, *CDC9*, *CDC17*, *CDC21*, *CDC45*, *CDC47*, *CDC101*, *CDC102*, and *CDC105* all displayed accu-

mulation of S phase cells, in agreement with their known functions in DNA replication (Hartwell, 1971a, 1973, 1976; Johnston and Smayth, 1978; Piatti *et al.*, 1995; Zou *et al.*, 1997; Burgers, 1998; Kanemaki *et al.*, 2003). In addition, *CDC4*, *CDC25*, and *CDC64* are all required for G1 transit and accumulated cells with 1C DNA contents (Bedard *et al.*, 1981; Tripp and Pinon, 1986; Goh and Surana, 1999). We conclude that neither the extended period of gene product depletion nor the stringency of the categorization cutoffs precluded accurate categorization of the mutants and that the number of false negatives in the screen was low, because cell cycle defects were detected in most *TetO₇* promoter mutants of known *CDC* genes.

Overall, our analysis indicated that most essential genes were linked to a specific stage of cell cycle progression. We next asked whether there are relationships between categories of DNA content and other gene properties, and if so, whether they are illuminating with regard to either the nature of the cell cycle or the functions of the individual genes.

G1 Phase

For visualization, flow cytometry histograms from the overlap between manual and computational scoring methods were grouped and subjected to hierarchical clustering (Figure 2A). We then asked if any GO functional annotations were enriched in any of the flow cytometry categories. These data are displayed as a "GO-gram" (Figure 2B), in which the annotations of genes within the categories are displayed; red bars indicate that a given GO annotation is significantly enriched in a given flow cytometry category. The 1C accumulation category, which at 270 genes was the largest, showed a dramatic enrichment for genes involved in protein synthesis (Figure 2B). This included genes involved in ribosome biogenesis, translation, tRNA metabolism, tRNA charging, and RNA polymerase III transcription (GO:0042254, ribosome biogenesis and assembly, p value 2.79e-23; GO:0043037, translation, 1.47 e-08; GO:0006399, tRNA metabolism, 6.70e-07; GO:0043038, amino acid activation, 3.60e-06; and GO:0006383, transcription from RNA polymerase III promoter, 0.0002). Although ribosome biogenesis is not cell cycle regulated in yeast (Bernstein and Baserga, 2004), there is some prior evidence that overexpression or depletion of ribosomal proteins and factors involved in ribosome biogenesis causes cell cycle defects. For example, depletion of SSU processome proteins leads to G1 arrest due to lack of ribosomes (Bernstein and Baserga, 2004), as does depletion of the 20S pre-rRNA maturation factor Rio1 (Angermayr *et al.*, 2002). Similarly, tRNA charging has long been linked to G1 transit as a number of mutants in tRNA synthetases arrest in G1 (Unger and Hartwell, 1976; Bedard *et al.*, 1981). Consistent with existing links between protein synthesis and G1 transit, our data indicate that the most dramatic restriction on a cell's ability to progress past the cell cycle commitment point in G1 (known as Start in yeast) is the capacity for protein synthesis, as inhibiting protein synthesis by a range of different means caused accumulation of cells in G1. This observation is reminiscent of previous analyses of cell size, which indicated that cells mutated in ribosome biogenesis factors tend to be small and unbudded (Jorgensen *et al.*, 2002; Mnaimneh *et al.*, 2004). However, there does not appear to be a direct relationship between cell size and our categorized flow cytometry profiles. When we grouped the cell size data from Mnaimneh *et al.* (2004) according to the assigned flow cytometry categories (Figure 3), we found that although strains in the 1C category tended to be slightly smaller than normal strains, there was not a strong predic-

tive correspondence between the flow cytometry data and the cell size data. Thus, cell cycle accumulation in G1 was far from an absolute predictor of cell size (and vice versa).

Eleven unannotated genes gave rise to G1 arrest after promoter shut-off, suggesting they might be involved in some aspect of ribosome biogenesis or protein biosynthesis. Indeed, high-throughput data suggest several of these genes perform such functions: *YPR169W* encodes a nucleolar protein of unknown function, which interacts with Tpt1p, a protein involved in tRNA splicing (Hazbun *et al.*, 2003; Huh *et al.*, 2003); *Ynl313cp* is localized to both cytoplasm and nucleus and interacts with proteins involved in RNA processing, ribosomal biogenesis import, and protein metabolism (Gavin *et al.*, 2002; Huh *et al.*, 2003; Krogan *et al.*, 2004); *Ynl310cp* encodes a protein that interacts with proteins involved in RNA processing (Gavin *et al.*, 2002; Ho *et al.*, 2002; Hazbun *et al.*, 2003); and *Yhr020wp* is inferred to have tRNA aminoacylation and ligation activity and also affinity-precipitates with Utp13, which is part of the SSU processome involved in pre-18S rRNA processing (Tatusov *et al.*, 2000; Ho *et al.*, 2002). Finally, *Ynr046wp* has been recently identified as subunit of tRNA methyltransferase (Purushothaman *et al.*, 2005), and *Ynr054cp*, which encodes a nucleolar protein that interacts with proteins involved in RNA processing, has been recently characterized as a component of the SSU processome (Gavin *et al.*, 2002; Ho *et al.*, 2002; Huh *et al.*, 2003; Hoang *et al.*, 2005). Thus at least 6 of the 11 unannotated ORFs in the G1 arrest category are likely to function in ribosome biogenesis, tRNA metabolism, or protein metabolism.

Histograms for mutants in the 1C accumulation category revealed that the category encompassed a range of degrees of 1C DNA content accumulation, from 31 to 97% 1C (compared with an average wild-type value of 28% 1C). Although this threshold was not exceeded by any wild-type isolates analyzed (unpublished data), we nonetheless considered that our threshold might have been too lenient, in which case there should be little relationship between growth effect or functional category of the mutated gene for those with relatively low 1C accumulation. We therefore ordered the mutants in the 1C category according to the relative 1C accumulation and asked whether severity of phenotype (in this case 1C accumulation) was related to particular gene functions and growth defect (Figure 4). We found that in the top quartile there was a significant enrichment for annotations related to tRNA synthesis and activation, and translation, whereas the second and third quartiles were enriched for functions related to ribosome biogenesis. The fourth quartile was significantly populated by genes in the broader RNA metabolism category. Additionally, there was a tendency for the mutants with highest 1C accumulation to display a more prominent growth defect. This suggests that given additional phenotypic data, we may be able to understand why some of the tet-promoter mutants display greater growth phenotypes than others; we had previously not been able to draw strong conclusions in this regard (Mnaimneh *et al.*, 2004). In summary, functional information could be derived from a single flow cytometry parameter (1C:2C ratio) in addition to the information contained in the different categories. Moreover, our choice of threshold was not overly liberal, because there was functional information contained even in the lowest quartile.

S Phase

The S phase accumulation class, which comprises only 27 genes (Figure 5), showed a dramatic enrichment for genes involved in DNA replication (GO:0006260, DNA replication, *p* value 4.02e-17; GO:0045005, maintenance of fidelity during

Figure 5. Flow cytometry histograms for the S phase category. The flow cytometry histograms for wild type and each of the 27 genes scored as having S phase accumulation are shown. Cultures were sampled for flow cytometry 15 h after the addition of doxycycline. Genes with known functions in DNA replication (red) and nucleotide metabolism (blue) are indicated, as are genes with no previously described connection to S phase (green). The positions of 1C and 2C DNA contents are also indicated.

DNA-dependent DNA replication, 9.97e-05; GO:0006281, and DNA repair, 1.20e-07; Figure 2B). These include *CDC6*, *SLD5*, *PSF2*, *POL1*, *POL2*, *PRI2*, *POL30*, *POL12*, *DPB11*, *DPB2*, *PSF1*, and *CDC45*, which all have direct roles in the initiation and/or the elongation of DNA replication. Several of the genes in this class, although not directly involved in replication, have known roles in nucleotide metabolism (*CDC8*, *CDC21*, *DFR1*, *FOL2*, and *DUT1*). We explored the possibility that *CSE1* and *SMC4* could play roles in regulating DNA replication. *CSE1* is responsible for the nuclear shuttling of the nuclear transporter importin α (Hood and Silver, 1998; Kunzler and Hurt, 1998; Solsbacher *et al.*, 1998) and a role for *CSE1* in mitosis (but not in S phase) has been described (Xiao *et al.*, 1993; Schroeder *et al.*, 1999). We synchronized cells in G1, released them into the cell cycle after shut-off of *TetO₇-CSE1*, and measured the DNA contents of the cells by flow cytometry (Figure 6A). Cells depleted of *CSE1* failed to undergo DNA replication, with 70% of cells still containing 1C DNA contents 90 min after release from G1. We examined whether these cells had passed Start by measuring the fraction of cells that had elicited buds, a marker of cell cycle progression through the G1/S boundary

Figure 6. Depletion of *CSE1* or *SMC4* caused defects in S phase progression. (A) Flow cytometry histograms measuring DNA contents of WT, *TetO₇-CSE1*, and *TetO₇-SMC4* cells in asynchronous culture (asy), arrested in G1 (α) and at the indicated times after release from G1 in the presence of doxycycline. Nocodazole was added at 60 min to prevent return to G1. The percent of cells in G1 at each time is indicated. (B) Flow cytometry histograms measuring DNA contents of WT and *smc4-1* cells in asynchronous culture (asy), arrested in G1 (α) and at the indicated times after release from G1 at 30°C. The percent of cells in G1 at each time is indicated. (C) The percent of cells with no bud (□), small bud (▨), and large bud (■) was measured at the indicated times after release from G1.

that is independent of the initiation of DNA replication (Figure 6C). At 90 min almost 90% of the *TetO₇-CSE1* cells had budded (51% had a large bud, and 38% had a small bud), indicating that most cells had passed Start. These data indicate that categorization of *TetO₇-CSE1* in the S phase accumulation class in the initial screen accurately predicted a role for *CSE1* in S phase, either in the initiation of DNA replication or after initiation but before significant DNA synthesis has occurred. Additionally, the depletion of Cse1p was robust enough to observe a clear phenotype in the first cell cycle. Thus, *CSE1* is required for DNA replication, suggesting that an essential DNA replication protein is an importin α cargo.

SMC4 encodes a "structural maintenance of chromosomes" protein, is a subunit of condensin and has clear roles in chromosome condensation and mitosis (Freeman *et al.*, 2000). In synchronous progression from G1 into S phase, we found that *SMC4* was required for normal S phase progression, indicating a previously unrecognized role of Smc4p (Figure 6A). When *TetO₇-SMC4* was shut-off, almost 40% of cells failed to enter S phase at times when S phase was complete in the wild-type control (50–60 min after release), as indicated by their failure to progress from a 1C to a 2C DNA content. This failure to enter S phase was not due to cell death or failure to pass Start, as *TetO₇-SMC4* cells elicited buds with kinetics and efficiency similar to that of wild type (Figure 6C). Additionally, the *TetO₇-SMC4* strain arrested in G1 with efficiency that was similar to wild type (86% of *TetO₇-SMC4* cells in α -factor displayed a shmoo morphology vs. 98% of wild-type cells), suggesting that the 33% of *TetO₇-SMC4* cells that did not enter S phase during

the course of the experiment were not cells that had failed to arrest in G1. Finally, we observed a similar phenotype with the temperature-sensitive *smc4-1* strain, in which 35% of cells failed to enter S phase (Figure 6B). As with *CSE1*, categorization of *SMC4* in the initial cell cycle screen accurately predicted a role for *SMC4* in entry into S phase.

We explored the role of *SMC4* in DNA replication by observing the association of the Mcm2 protein with replication origins and replication forks during S phase, using chromatin immunoprecipitation (ChIP; Figure 7, A and B). In wild-type cells, Mcm2p was preferentially associated with the replication origin *ARS1* in G1. As cells progressed into S-phase the association of Mcm2 with the origin decreased and the association of Mcm2p with origin-distal DNA fragments increased. This pattern implies association with the replication fork, as has been previously described (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). We found that when *TetO₇-SMC4* was shut off, Mcm2p binding to *ARS1* decreased as cells progressed into S phase, but Mcm2p failed to then associate with origin-distal regions. Thus, our data indicate that Smc4p depletion affected the binding of Mcm2p to the regions flanking *ARS1* during S phase and suggest a role for Smc4p in regulating DNA replication, perhaps in the transition from initiation to elongation. Previous work has hinted at a role for condensin in S phase, including the hydroxyurea sensitivity of condensin mutants in fission yeast (Aono *et al.*, 2002) and the presence of condensin at the rDNA locus during S phase (Johzuka *et al.*, 2006). Additionally, genome-wide protein-DNA interaction data suggests that Smc4p is excluded from replication origins (Wang *et al.*, 2005), although this analysis was not performed during

Figure 7. Depletion of SMC4 caused replication fork defects. (A) Chromatin immunoprecipitation analysis of Mcm2 binding to *ARS1* and adjacent regions in WT and *TetO₇-SMC4* cells. Cells were arrested in G1 and released into S phase in the presence of doxycycline. Sample were taken for ChIP analysis at the indicated times. DNA was amplified by PCR before (input) or after (IP) immunoprecipitation of Mcm2. Ethidium bromide-stained agarose gels of the products of multiplex PCR reactions to amplify *ARS1*, regions 4 kb upstream (+4 kb), 4 kb downstream (-4 kb), and 8 kb downstream (-8 kb) of *ARS1* are shown. (B) The PCR products from three independent ChIP experiments were quantified and the average amount of DNA in each band relative to the amount in the 12-min sample is plotted. Error bars span one SD. The quantification of the PCR products for each region amplified (*ARS1*, +4 kb, -4 kb, and -8 kb) is plotted for WT (□) and for *TetO₇-SMC4* (■).

synchronous progression through S phase and therefore may not have detected the relevant localization. Perhaps Smc4p exerts its effect on DNA replication by creating a chromatin environment that facilitates progression of replication forks away from replication origins. We were unable to detect S phase defects for the other condensin subunit genes in the *TetO₇*-promoter collection, *SMC2*, *YCG1*, and *YCS4*. It will be of great interest to determine whether this S phase function is a property of Smc4p or of the condensin complex.

G2 Phase

Processes enriched in this category included chromosome segregation, protein transport and secretion, DNA replication, cell cycle, and metabolism (GO:0007059, chromosome segregation, Pvalue 6.19e-05; GO:0016192, vesicle-mediated transport, 1.61e-05; GO:0046903, secretion, 3.73e-06; GO:0007049, cell cycle, 0.0007; and GO:0019222, regulation of metabolism, 0.0009; Figure 2B). The diversity of gene functions represented in the 2C accumulation category (there are 26 significant GO categories in this group; $p < 0.001$) suggested that transit through G2 phase of the cell cycle is dependent on a large number of different cellular processes.

Figure 8. Protein trafficking defects in the 3C/4C category. (A) Micrographs of four strains from the 3C and 4C accumulation category. Promoters were shut off by the addition of doxycycline, and cells were fixed after 10 h. Cells were stained with DAPI, which stains the nuclei, and cells were visualized by fluorescence and phase-contrast microscopy. Merged images are shown. (B) Immunoblots of carboxypeptidase Y (CPY), alkaline phosphatase (ALP), and Gas1 in the indicated *TetO₇* strains before and after the addition of doxycycline for 15 h. The positions of processing and trafficking intermediates of each protein are indicated (pCPY, ER/Golgi modified proCPY; uCPY, underglycosylated CPY; mCPY, mature CPY; proALP, ER/Golgi modified proALP; sALP, soluble ALP; mALP, mature ALP; proGas1, ER glycosylated proGas1; mGas1, mature Gas1). Tubulin is shown as a loading control.

3C/4C

The 3C/4C accumulation flow cytometry category showed a clear enrichment for genes associated with protein transport, protein secretion, and glycoprotein metabolism (GO:0016192, vesicle-mediated transport, P value 3.38e-08; GO:0046903, secretion, 1.91e-10; and GO:0009100, glycoprotein metabolism, 4.04e-08; Figure 2B and unpublished data). An enrichment of similar functions was also evident in the category of mutants exhibiting multiple buds in our earlier morphological analysis of the *TetO₇* promoter collection (Mnaimneh *et al.*, 2004), although far fewer genes were scored in this category. Four uncharacterized genes, YJL097W/*PHS1*, YDL193W/*NUS1*, YNL149C, and YML125C, were included in the 3C/4C category, suggesting they might be involved in some aspect of protein trafficking. The proteins encoded by these four genes all localize to the ER or the ER and vacuole (Huh *et al.*, 2003) and the YJL097W/*PHS1* gene was recently found to be a regulator of sphingosine levels (Schuldiner *et al.*, 2005). We examined the cellular morphology of these strains after promoter shut-off and found that all exhibited a multiple-budded or large-budded phenotype with normal postmitotic nuclei (Figure 8A), indicating a defect in cytoti-

nesis or in cell separation. Normal nuclear division accompanied by a cytokinesis or cell separation defect likely resulted in the appearance of events with apparent 3C and 4C DNA contents in the flow cytometry analysis. We also examined the *TetO₇* alleles of *YJL097W/PHS1*, *YDL193W/NUS1*, *YNL149C*, and *YML125C* for defects in the processing and trafficking of three different glycosylated proteins (Figure 8B). *TetO₇-YJL097W* did not show detectable defects in carboxypeptidase Y (CPY) processing, indicating that it is not essential for ER-to-Golgi transport, N-linked glycosylation, or vacuolar signal peptide cleavage; however, it displayed abnormal processing of alkaline phosphatase (ALP), accumulating the soluble form of ALP. The presence of soluble form ALP is reminiscent of *apl5Δ* mutants (Cowles *et al.*, 1997) and of depletion of the recently described *PGA1* and suggests a defect in vacuolar transport. Additionally, *TetO₇-YJL097W* showed defects in the accumulation of mature Gas1p. *TetO₇-YDL193W* accumulated hypoglycosylated forms of CPY, much like *TetO₇-SEC59*, suggesting a role in glycosylation in the ER. *TetO₇-YDL193W* also accumulated immature ER forms of Gas1p. *TetO₇-YNL149C* and *TetO₇-YML125C* both displayed normal CPY processing, but accumulated increased levels of abnormal soluble ALP and slightly decreased levels of mature Gas1p. Together these results indicate roles for *YJL097W*, *YDL193W*, *YNL149C*, and *YML125C* in protein trafficking. On the basis of these observations we have named the *YNL149C* and *YML125C* genes *PGA2* and *PGA3*, for processing of Gas1p and ALP.

DISCUSSION

Several major conclusions can be drawn from the results presented here. First, we expected at the outset that the major proportion of the strains would be scored as normal, indicating that they ceased division at random positions of the normal cell cycle. However, most of the strains we analyzed (65%) displayed a specific defect in cell division or cell cycle progression. One trivial explanation for the large number of genes that displayed cell cycle defects after depletion is that the rate of false positives could be high. However, in the S phase category 20 of 27 genes identified have known roles in DNA replication or nucleotide metabolism, and at least two more were found to have S phase defects when analyzed in synchronous culture (Figure 6), indicating that the false positive rate in our screen was not high. Similarly, the majority of genes in the 1C category have roles in ribosome biogenesis and translation and so likely represent true positives. Further analysis of the 1C category revealed functional enrichment even within the quartile with the most modest 1C accumulation (Figure 4), indicating that our scoring was not overly liberal. The most striking result of our analysis is therefore that the majority of strains appear to have stage-specific cell cycle defects and thus the function of most essential genes is most important at a specific time in the cell cycle.

It is important to note that the present screen differs from the classic cell cycle screens in that we did not require arrest of a strain at a particular cell cycle stage, but instead scored any significant cell cycle stage-specific accumulation. In this respect our screen is conceptually similar to a recent cell cycle screen in *Drosophila* cells using gene product depletion by RNA-mediated interference (RNAi; Bjorklund *et al.*, 2006). One reasonable explanation for the observation that in some strains the cell cycle phenotype is a delay rather than an arrest and that in some strains <100% of cells accumulate at the relevant cell cycle stage is that promoter shut-off will not uniformly result in protein depletion within the time-

frame of the experiment. Although this might be perceived as a limitation, we were able to detect cell cycle defects in 89% of known CDC genes as *TetO₇* alleles and correctly categorize 74% of these, suggesting that the false negative rate in our screen was low. Additionally, the functional enrichment within the 1C and S phase categories was consistent with published work: the 1C category displayed a massive enrichment for genes involved in tRNA and ribosome biogenesis, and the S phase category displayed a massive enrichment for genes involved in DNA replication. Finally, one of the hallmarks of cell cycle arrest is continued cell growth in the absence of cell cycle progression. Strains in the S-phase, 2C, and 3C/4C categories all exhibited larger than normal cell size (Figure 3), consistent with cell growth despite cell cycle arrest. By contrast, strains in the normal cell cycle category exhibited wild-type cell sizes, despite almost half of the strains in the category having clear growth defects after promoter shut-off. Thus we conclude that measuring DNA contents after promoter shut-off of essential genes represents a sensitive and accurate means of identifying cell cycle mutants.

A second major conclusion is that down-regulation of genes with diverse functions in regulating and maintaining the protein biosynthetic capacity of the cell causes arrest in G1. The intimate relationship between protein biosynthesis and cell cycle progression through G1 that we observed is consistent with previous observations linking protein synthesis with G1 transit (Unger and Hartwell, 1976; Bedard *et al.*, 1981; Moreno and Nurse, 1994), with connections between ribosome biogenesis and cell size regulation at Start (Jorgensen *et al.*, 2002, 2004), and with links between translation rate and G1 (Polymenis and Schmidt, 1997) and provides a global view of cellular processes that contribute to passage through the cell cycle commitment point in G1, Start. There is concordance between our G1 data and that derived from cell cycle screening of *Drosophila* cells after gene product depletion by RNAi (Bjorklund *et al.*, 2006) in that both screens show clear involvement of protein biosynthesis pathways in G1 transit. However, in the *Drosophila* screen the genes identified were largely ribosomal subunit genes, whereas our data also illustrate the roles of ribosome assembly factors, and tRNA synthesis, processing, and charging proteins in regulating G1. Given that down-regulation of ribosome biogenesis can cause cell cycle arrest in G1 in mouse and human cells (Strezoska *et al.*, 2000; Volarevic *et al.*, 2000; Pestov *et al.*, 2001; Oliver *et al.*, 2004), our identification of essential genes that regulate G1 in yeast could be useful in dissecting connections between ribosome biogenesis and cell cycle progression in mammalian cells.

A third conclusion is that sorting genes into different categories on the basis of the flow cytometry profiles allowed prediction of gene function. We derived information about gene function within the flow cytometry categories by identifying biological processes that are enriched within each category. Although in the present screen the cell cycle accumulation stage may not necessarily reflect the execution point or the point of action of the genes, we identified significant functional enrichments within each cell cycle category and so were able to make predictions about the biological function of uncharacterized essential genes. In particular, we found that accumulation of 3C and 4C DNA content reliably identified components of protein trafficking, modification, and secretion pathways. Four uncharacterized genes in this category, *NUS1*, *PHS1*, *PGA2*, and *PGA3*, had detectable changes in the modifications of one or more of the markers of protein secretion and modification analyzed, indicating roles in protein trafficking. The 1C category pre-

dicted function in protein biosynthesis: 6 of the 11 unannotated genes in the G1 accumulation category have connections to protein biosynthesis suggested by other functional genomic data, including protein–protein interaction and protein localization data. Additionally, we found that depletion of the condensin subunit Smc4p or the importin α export receptor Cse1p resulted in defective DNA replication and therefore that accumulation of S phase DNA contents accurately predicted function in DNA synthesis. Thus, despite the possibility that some of the observed cell cycle delays could have been due to indirect effects of protein depletion, this comprehensive data set was useful for prediction of essential gene function.

Our study complements several decades of cell cycle experimentation, and provides a systematic survey of essential gene function in the cell division cycle. Regulation of proliferation is of primary importance in the study of human development and in the study of many human diseases. We anticipate that many of the connections between essential cellular processes and cell division cycle progression illustrated here will be conserved in humans. Indeed, a number of pathways that we find are important for cell cycle progression in yeast are also important for cell cycle progression in *Drosophila* (Bjorklund *et al.*, 2006), encouraging the view that this global analysis of the connections between essential genes and the cell cycle will be useful in dissecting the network of processes that impinge on progression through the cell cycle in metazoans.

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