Gid8p (Dcr1p) and Dcr2p Function in a Common Pathway To Promote START Completion in Saccharomyces cerevisiae

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How cells determine when to initiate DNA replication is poorly understood. Here we report that in Saccharomyces cerevisiae overexpression of the dosage-dependent cell cycle regulator genes DCR2 (YLR361C) and GID8 (DCR1/YMR135C) accelerates initiation of DNA replication. Cells lacking both GID8 and DCR2 delay initiation of DNA replication. Genetic analysis suggests that Gid8p functions upstream of Dcr2p to promote cell cycle progression. DCR2 is predicted to encode a gene product with phosphoesterase activity. Consistent with these predictions, a DCR2 allele carrying a His338 point mutation, which in known protein phosphatases prevents catalysis but allows substrate binding, antagonized the function of the wild-type DCR2 allele. Finally, we report genetic interactions involving GID8, DCR2, and CLN3 (which encodes a G1 cyclin) or SWI4 (which encodes a transcription factor of the G1/S transcription program). Our findings identify two gene products with a probable regulatory role in the timing of initiation of cell division.

MATERIALS AND METHODS

Media, strains, and plasmids. Media were prepared as described by Kaiser et al. (17), with the necessary biosynthetic requirements. All yeast molecular biology techniques were performed as described by Kaiser et al. (17), unless otherwise indicated. The strains used in this study are listed on Table 1. One-step gene replacements utilizing the hisMX and kanMX cassettes were done as described by Longtine et al. (19). For other gene replacements, URA3 was amplified by PCR with specific oligonucleotide primers carrying at their 5′ ends sequences that corresponded to flanking chromosomal sequences upstream and downstream of the open reading frame (ORF) that was replaced. The PCR products generated in this manner were then used directly in integrative transformations. The genotypes of all the strains were verified by PCR as described previously (12). The diploid strain coexpressing Gid8p-hemagglutinin (HA) and Dcr2p-Myc (SCMSP115) was obtained from a cross of DCR2-HA (SCMSP89) and GID8-MYC (SCMSP106) strains. The Cln3p-Pra strain (VAY27-1A) and its otherwise indicated. The strains used in this study are listed on Table 1. One-step gene replacements utilizing the hisMX and kanMX cassettes were done as described by Longtine et al. (19). For other gene replacements, URA3 was amplified by PCR with specific oligonucleotide primers carrying at their 5′ ends sequences that corresponded to flanking chromosomal sequences upstream and downstream of the open reading frame (ORF) that was replaced. The PCR products generated in this manner were then used directly in integrative transformations. The genotypes of all the strains were verified by PCR as described previously (12). The diploid strain coexpressing Gid8p-hemagglutinin (HA) and Dcr2p-Myc (SCMSP115) was obtained from a cross of DCR2-HA (SCMSP89) and GID8-MYC (SCMSP106) strains. The Cln3p-Pra strain (VAY27-1A) and its otherwise isogenic untagged counterpart (VAY27-1C) were gifts from F. Cross (7). To generate the strains shown on Fig. 8 and Table 4, we crossed a gid8 dcr2 strain (SCMSP112) with strains lacking CLN3 (10366), BCK2 (16163), or SWI4 (16109). The resulting diploids were sporulated, and the segregants were obtained by random spore analysis and tetrad dissection (17). The phenotypes reported for each strain were obtained after examining several independent transormants or segregants for the strain in question.

The CLN3-2CEN plasmid p205 (see Figs. 1 and 3) and the P<sub>Cut</sub>-CLN3 low-copy-number plasmid pW16 (see Fig. 2) were gifts from F. Cross (5). The high-copy-number plasmids described in this report were isolated from a yeast genomic DNA library (4) as we previously described (1). Standard molecular biology techniques (28) with reagents from New England Biolabs (Beverly, Mass.) were used to characterize the plasmids isolated from our enrichment procedure (1). The plasmid inserts were sequenced with vector-specific primers from both ends. Sequencing was performed at the Texas A&M University Genome Technologies Laboratory. We then digested the plasmids with the restriction endonucleases indicated in Table 2. The products were gel purified by DNA agarose gel elec-
was used to induce expression of the gene under induction. After elutriation, galactose (at 2%, wt/vol) was used to inhibit expression.

For the cell synchronization, the cells were cultured and collected at a pump speed of 40 ml/min and rotor speed of 2,400 rpm. Other techniques included immunoprecipitations for HA- and Myc-tagged proteins.

For fluorometry, the cultures were incubated with nocodazole (Sigma, St. Louis, Mo.) at 15 μg/ml for 4 h. The cell cycle was analyzed using cuComp software. The geometric mean is indicated in each case. For population doubling (generation) time measurements, we used the CellSyncer to obtain cell numbers (N) at multiple time points (t) during the exponential growth of the culture. From the slope of the line obtained after plotting ln N versus t, we obtained the specific growth rate constant of the culture (k).

Other techniques involved immunoprecipitation for HA- and Myc-tagged proteins, and for fluorescence microscopy, we followed the protocols of the Botstein laboratory, as described at http://genome-www.stanford.edu/group/botlab/protocols.html. DAPI (4',6'-diamidino-2-phenylindole) was used from Molecular Probes. All the secondary antibodies used in immunofluorescence microscopy, unless otherwise indicated, were from Molecular Probes.

### Table 1. Strains used in this study

| Strain          | Relevant genotype | Source              |
|-----------------|-------------------|---------------------|
| BY7471          | MATa his3Δ leu2Δ met15Δ ura3Δ | Res. Genetics* |
| BY7472          | MATa his3Δ leu2Δ met15Δ ura3Δ | Res. Genetics |
| BY7473          | BY7471/BY7472      | Res. Genetics |
| VAY27-1C        | MATa ade2 trp1 leu2 his3 ura3 can1 | F. Cross |
| VAY27-1A        | CLN3-PrA:HI33 (VAY27-1C otherwise) | F. Cross |
| SCMS1P07        | F<sub>cat</sub>-GID8::hisMX/GID8<sup>B</sup> (BY7473 otherwise) | This study |
| SCMS6776        | F<sub>cat</sub>-GID2::hisMX/DCR2<sup>B</sup> (BY7473 otherwise) | This study |
| RPY3            | F<sub>gal</sub>-DCR2::hisMX (BY7471 otherwise) | This study |
| SCMS1P112       | gid8Δ::URA3 dcr2Δ::hisMX (BY7471 otherwise) | This study |
| 6576            | gid8Δ::kanMX (BY7471 otherwise) | Res. Genetics |
| RPY1            | dcr2Δ::hisMX (BY7471 otherwise) | This study |
| SCMS89          | DCR2-3HA::kanMX (BY7472 otherwise) | This study |
| SCMS1P07        | DCR2-13MYC::hisMX (BY7471 otherwise) | This study |
| SCMS1P101       | GID8-3HA::kanMX (BY7472 otherwise) | This study |
| SCMS1P06        | GID8-13MYC::hisMX (BY7471 otherwise) | This study |
| SCMS1P115       | GID8-3HA::kanMX/GID8 DCR2-13MYC::hisMX/DCR2 (BY7473 otherwise) | This study |
| SCMS1P116       | gid8Δ::URA3 (BY7471 otherwise) | This study |
| SCMS1P31        | chn3Δ::kanMX gid8Δ::URA3 dcr2Δ::hisMX (BY7471 otherwise) | This study |
| SCMS1P23        | swi4Δ::kanMX gid8Δ::URA3 (BY7471 otherwise) | This study |
| SCMS1P24        | gid8Δ::URA3 (BY7471 otherwise) | This study |
| SCMS1P34        | bck2Δ::kanMX gid8Δ::URA3 (BY7471 otherwise) | This study |
| SCMS1P36        | bck2Δ::kanMX gid8Δ::URA3 dcr2Δ::hisMX (BY7471 otherwise) | This study |
| SCMS1P27        | chn3Δ::kanMX dcr2Δ::hisMX (BY7471 otherwise) | This study |
| SCMS1P35        | bck2Δ::kanMX dcr2Δ::hisMX (BY7471 otherwise) | This study |
| SCMS1P28        | swi4Δ::kanMX dcr2Δ::hisMX (BY7471 otherwise) | This study |
| SCMS3J78-7501699| SIC1-TAP::hisMX (BY7471 otherwise) | Open Biosystems |
| 10366           | chn3Δ::kanMX (BY7471 otherwise) | Res. Genetics |
| 16163           | bck2Δ::kanMX (BY7472 otherwise) | Res. Genetics |
| 16109           | swi4Δ::kanMX (BY7472 otherwise) | Res. Genetics |
| 36189           | bub2Δ::kanMX (BY7472 otherwise) | Res. Genetics |
| 31392           | mad2Δ::kanMX (BY7472 otherwise) | Res. Genetics |
| 36781           | mad2Δ::kanMX (BY7472 otherwise) | Res. Genetics |

* Res. Genetics, Research Genetics.
cence were from Jackson ImmunoResearch (West Grove, Pa.). The samples were examined with a Nikon Eclipse TS100 inverted fluorescence microscope. For the phosphatase assays reported in Fig. 6, crude cell extracts were mixed with an equal volume of assay buffer containing 200 mM Tris-HCl (pH 7.8), 2 mM MgCl₂, 20 mM dithiothreitol, and 40 mM 4-nitrophenylphosphate, prepared fresh each time. The protein concentration of the crude cell extract in the supernatant was determined by the Bradford assay with reagents from Sigma, according to the manufacturer’s instructions. To obtain the enzymatic rates, the absorbance was measured at 405 nm every 5 s for 1 min with a Beckman DU 530 spectrophotometer.

RESULTS

GID8 and DCR2 alter cell cycle progression when overexpressed. We identified plasmids 5–18 and 2–6 in a screen for cell cycle regulators (1). Both plasmids significantly increased the fraction of budded cells (budding index) without altering the overall generation time in asynchronous cultures (Tables 2 and 3). Within the chromosomal insert of plasmid 5–18 there are the full-length ORFs of REC114, YMR134W, and YMR135C. Plasmid 2–6 carries VPS38, YLR361C, and YLR361C-A.

To identify the genes of interest, we disrupted individual ORFs by digestion with restriction endonucleases and religation and, in transformants carrying these plasmid derivatives, we looked for budding index values similar to that for the wild type. Removing a BamHI-BglII fragment from plasmid 5–18, which disrupts only YMR135C (Table 2), led to the loss of the high-budding-index phenotype of the cells carrying this plasmid derivative, implying that YMR135C was the gene of interest on plasmid 5–18. Likewise, digestion of plasmid 2–6 with BglII disrupted VPS38 and YLR361C, while digestion with KpnI and SalI disrupted YLR361C-A and YLR361C. In both cases the plasmid derivatives did not increase the budding index (Table 2), and, since the YLR361C ORF was the com-
GID8 and DCR2 overexpression on cell cycle progression in a synchronous population of cells obtained by elutriation (Fig. 1). Cells carrying GID8-2µ and DCR2-2µ had a shorter G1 phase based on budding index and DNA content measurements (Fig. 1A). For example, 45 min after elutriation 79% of wild-type cells were unbudded, compared to only 32% of cells overexpressing GID8 or DCR2 (Fig. 1A). GID8- and DCR2-overexpressing cells also appear to initiate DNA replication at a smaller size than wild-type cells (Fig. 1A; at 30 or 45 min after elutriation). These results suggest that synchronous cultures of cells containing GID8-2µ and DCR2-2µ complete START faster than wild-type cells, consistent with results obtained from asynchronous populations of cells where overexpression of these genes increased the budding index (Table 2 and results below).

We also monitored the levels of the Cdk inhibitor Sic1p in cultures released from a nocodazole arrest (Fig. 1B). In cells carrying GID8 or DCR2 on a high-copy-number plasmid, Sic1p disappeared sooner (~15 min), indicative of a shortened G1 phase (Fig. 1B). Finally, asynchronous populations of GID8- and DCR2-overexpressing cells were neither smaller overall nor pheromone resistant (Fig. 1C and D), in contrast to CLN3-overexpressing cells, which are smaller and resistant to pheromone (6, 22).

Gid8p and Dcr2p affect cell cycle progression by regulating START. GID8 and DCR2 overexpression may alter cell cycle progression either by directly shortening the G1 phase, which leads to a high budding index due to a compensatory expansion of subsequent cell cycle phases, or by simply delaying mitotic progression (34). A mitotic delay can lead to a shorter G1 phase in the next cell cycle, presumably because it allows the cells to grow and reach the critical size for initiation in the next division faster. This is usually accompanied by an increase in the doubling time and cell size of the culture (20), as we have recently shown for SIK1 overexpression (1), which we identified in the same screen that yielded GID8 and DCR2. However, GID8- and DCR2-overexpressing cells were not larger than wild-type cells (Fig. 1A and C), and they proliferated at the same rate as wild-type cells (94 ± 3, 91 ± 1, and 95 ± 3 min for vector-2µ, GID8-2µ, and DCR2-2µ, respectively, at 30°C in SC-glucose media).

We then used heterozygous diploid cells where one copy of GID8 or DCR2 was under the control of a galactose-inducible promoter while the other was under the control of its native promoter. The cells were grown in raffinose-containing media before elutriation so that gene overexpression was not induced. Postelutriation, the cells were shifted to galactose-containing media to induce the GAL promoter and overexpress the gene of interest. Budding index as well as flow cytometry data indicated that, in the presence of galactose, the transition from the G1 to S phase was accelerated in PGAL-GID8’/GID8+ (GAL-GID8) or PGAL-DCR2’/DCR2 (GAL-DCR2) or carrying a PGAL-CLN3-CEN plasmid (GAL-CLN3), were grown and elutriated in raffinose-containing media to obtain a synchronous early G1 population of cells in each case. Galactose was then added, and progression through the cell cycle was evaluated as for Fig. 1. All the strains were in the BY4743 background. (B) The percentages of cells in G1 from the flow cytometry panels in panel A were calculated from the DNA histograms with the ModFit software (Verity Software House, Topsham, Maine).

mon ORF disrupted in these two cases, we concluded that it might be the gene of interest. We reserved the names DCR1 and DCR2 (dosage-dependent cell cycle regulators 1 and 2) for YMR135C and YLR361C, respectively, with the Saccharomyces Genome Database, according to their guidelines (http://www.yeastgenome.org/gene_guidelines.shtml). In the meantime, another group implicated YMR135C in proteasome-mediated degradation of fructose-1,6-bisphosphatase and down regulation of gluconeogenesis and named it GID8 (27). Hereafter, we refer to plasmids 5–18 and 2–6 as GID8-2µ and DCR2-2µ, respectively. Based on reverse transcription-PCR experiments, cells carrying the high-copy-number plasmids overexpress GID8 about 10-fold and overexpress DCR2 about 2-fold (data not shown).

Next we examined the effect of GID8 and DCR2 overexpres-
containing media). Type (94)

The mean and standard deviation of the relative budding index, compared to those for the wild type, are shown. The numbers on either sides of the full insert denote their respective chromosomal positions.

TABLE 3. Genetic interactions between GID8 and DCR2

| Strain* | Relative Bl\(b\) |
|---------|------------------|
| GID8\(\Delta\) DCR2\(\Delta\) (vector-2\(\mu\)) | 1 ± 0.12 (19; 1) |
| GID8\(\Delta\) DCR2\(\Delta\) (GID8-2\(\mu\)) | 1.29 ± 0.10 (18; 3 × 10\(^{-10}\)) |
| GID8\(\Delta\) DCR2\(\Delta\) (DCR2-2\(\mu\)) | 1.14 ± 0.12 (20; 5 × 10\(^{-4}\)) |
| GID8\(\Delta\) dcr2\(\Delta\) (GID8-2\(\mu\)) | 1.05 ± 0.09 (18; 0.1) |
| gid8\(\Delta\) DCR2\(\Delta\) (DCR2-2\(\mu\)) | 1.11 ± 0.07 (19; 1 × 10\(^{-3}\)) |
| gid8\(\Delta\) DCR2\(\Delta\) (vector-2\(\mu\)) | 1.03 ± 0.12 (20; 0.5) |
| GID8\(\Delta\) dcr2\(\Delta\) (vector-2\(\mu\)) | 1.02 ± 0.08 (19; 0.5) |
| gid8\(\Delta\) dcr2\(\Delta\) (vector-2\(\mu\)) | 1.01 ± 0.1 (32; 0.8) |
| GID8\(\Delta\) DCR2\(\Delta\) (vector-2\(\mu\)) | 1 ± 0.05 (30) |
| GID8\(\Delta\) DCR2\(\Delta\) (GID8-2\(\mu\)) | 1.17 ± 0.05 (30; 7 × 10\(^{-11}\)) |
| GID8\(\Delta\) P\(_{GAL}\)-DCR2 (vector-2\(\mu\)) | 1.23 ± 0.10 (30; 8 × 10\(^{-7}\)) |
| GID8\(\Delta\) P\(_{GAL}\)-DCR2 (GID8-2\(\mu\)) | 1.22 ± 0.08 (30; 3 × 10\(^{-10}\)) |

* The cells (all in the BY4741 background) were grown in SC media, at 30°C, with glucose or galactose (*) as the carbon source. In these growth conditions, the generation times of all strains were indistinguishable from those of the wild type (94 ± 3 min in glucose-containing media and 165 ± 5 min in galactose-containing media).

b The mean and standard deviation of the relative budding index, compared to those for the wild type, are shown in each case. The numbers of individual cultures evaluated (n) and the probabilities associated with Student’s t test when the budding indices are compared to that for the wild type are shown in parentheses.

Overall, all our data thus far suggest that GID8 and DCR2 have a positive role in G₁ and the timing of START.

**Gid8p AND Dcr2p PROMOTE START**

VOL. 3, 2004

Gid8p and Dcr2p functionally interact to regulate the G₁/S transition. We next examined if the GID8 and DCR2 gene products may function in a common pathway to regulate the completion of START. We overexpressed one gene product in the absence of the other to see if it resulted in the loss of the high-budding-index phenotype associated with the overexpression of the former gene product. Note that overexpression of Gid8p does not affect Dcr2p levels and vice versa (see Fig. 7). Interestingly, overexpression of GID8 did not increase the budding index of dcr2\(\Delta\) cells (Table 3), indicating that Gid8p requires the function of Dcr2p to accelerate the G₁/S transi-
The viability of cells lacking mitotic checkpoint genes. Growth of copy-number plasmid. The simplest interpretation of our data effect (Table 3). Similar results (see Fig. 6) were also observed cells in the presence of galactose, did not produce an additive effect (Table 3). Since Gid8p requires the presence of functional Dcr2p (Table 3), the epitope-tagged Dcr2p probably retains function. Based on the granular staining pattern by immunofluorescence of the HA- or Myc-tagged Gid8p or Dcr2p, we conclude that Gid8p requires the presence of functional Dcr2p to accelerate the G1/S transition. This conclusion is further supported by additional experiments that we describe below, based again on budding index measurements (see Fig. 6). However, from the cell cycle profiles (Fig. 4) and additional experiments we describe below (see Fig. 8), combined loss of Gid8p and Dcr2p had the strongest phenotypic consequences, arguing against an exclusive linear pathway for these two gene products.

**Subcellular localization of Dcr2p.** Localization data for Gid8p are available from a genome-wide database (15) (Gid8p was present in both the nucleus and the cytoplasm), but there is no record for Dcr2p’s subcellular localization in any database. Consequently, we epitope tagged Gid8p and Dcr2p with HA and c-Myc epitope tags (19). In both cases proteins of the expected size were detected from cell extracts after immunoprecipitations and immunoblotting with anti-HA and anti-Myc antibodies (Fig. 5). Cells carrying the epitope-tagged proteins were indistinguishable from the wild type, based on generation time, cell size, and budding index measurements (data not shown). Overexpression of GID8 in strains carrying a epitope-tagged DCR2 allele still increased the budding index (data not shown). Since Gid8p is predicted to encode a 578-amino-acid protein of 66,463 Da. Motif searches suggested that Dcr2p may belong to a family of calcineurin-like metal-containing phosphoesterases (E value = 1e-5, from CDART [11]), which includes protein phosphoserine phosphatases, nucleotidases, nucleases, sphingomyelin

**TABLE 4. Proliferation parameters of CLN3, BCK2, SWI4, GID8, and DCR2 mutants**

| Strain                  | \( \text{g}^b \) | Cell size (\( \mu \text{m}^2 \)) |
|-------------------------|-----------------|---------------------------------|
| WT                      |                 |                                 |
| gid8\( \Delta \)        | 1.01 ± 0.01     | 37.1 ± 1.6                      |
| dcr2\( \Delta \)        | 1.01 ± 0.02     | 37.0 ± 1.6                      |
| gid8\( \Delta \) dcr2\( \Delta \) | 1.03 ± 0.03  | 42.0 ± 1.6                      |
| bck\( \Delta \)         | 1.02 ± 0.02     | 45.6 ± 1.7                      |
| bck\( \Delta \) gid8\( \Delta \) dcr2\( \Delta \) | 1.02 ± 0.02 | 49.5 ± 1.7                      |
| chs\( \Delta \)         | 1.05 ± 0.03     | 49.2 ± 1.7                      |
| chs\( \Delta \) gid8\( \Delta \) dcr2\( \Delta \) | 1.05 ± 0.02 | 54.3 ± 1.8                      |
| swi4\( \Delta \)        | 1.08 ± 0.01     | 48.4 ± 1.7                      |
| swi4\( \Delta \) gid8\( \Delta \) dcr2\( \Delta \) | 1.07 ± 0.01 | 56.8 ± 1.9                      |

*Cell numbers and cell sizes were obtained with a Coulter counter as described in Materials and Methods. The average and standard deviation from three independent liquid cultures in rich yeast extract-peptone-dextrose media are shown in each case. All the strains were in the haploid BY4741 background.

The generation times (g) of the strains shown are relative to that of wild type, which was 94 ± 1.9 min.

WT, wild type.
phosphodiesterases, and 2'-3' cyclic AMP phosphodiesterases. Within the conserved \( \beta \alpha \beta \alpha \beta \) phosphoesterase structure there are sequence “signatures” common to these proteins. Among them is a GNHD/E sequence motif, thought to be important for the hydrolysis of phosphate esters in the active-site dinuclear metal center (36). Mutational analysis suggested that the His of the GNHD motif probably affects catalysis but not substrate binding in a Ser/Thr phosphatase and calcineurin (21, 36).

To test the possibility that Dcr2p may function as a phosphoesterase, we introduced an H338A mutation in the GNHD motif of Dcr2p (Fig. 6A). The presence of this \( \text{DCR2-H338A} \) allele does not alter the endogenous levels of Dcr2p or Gid8p (Fig. 7). In phosphatase assays with 4-nitrophenylphosphate as a substrate, crude extracts from cells lacking \( \text{DCR2} \) or carrying the \( \text{DCR2-H338A} \) allele had significantly lower (~20%) phosphatase activity than extracts from wild-type cells (Fig. 6B). However, in the same assays extracts from cells overexpressing \( \text{DCR2} \) had only minimally increased (~5%) phosphatase activity (Fig. 6B). This could be due to the high background of this crude assay.

FIG. 4. Loss of \( GID8 \) and \( DCR2 \) delays completion of START. (A) Wild-type (WT) haploid cells and \( \text{gid8} \Delta, \text{dcr2} \Delta, \) and \( \text{gid8} \Delta \text{dcr2} \Delta \) cells were grown and elutriated in SC-glucose-containing media. All the strains were in the BY4741 background. At the indicated time points the DNA content was evaluated by flow cytometry. Cell numbers are plotted on the y axis, and the x axis indicates fluorescence. Cell size was measured with a Channelizer. The percentage of \( G_1 \) cells was calculated from the DNA histograms with ModFit software (Verity Software House). (B) Cell cycle progression was also monitored by determining the percentage of budded cells, from the samples shown in panel A.
We then examined the ability of the DCR2-H338A allele to interfere with the two phenotypic attributes of the wild-type DCR2: first, overexpression of DCR2 increases the budding index; second, DCR2 is necessary for GID8 overexpression to increase the budding index. However, in cells carrying DCR2-H338A, overexpression of wild-type DCR2 (Fig. 6B) or GID8 (Fig. 6C) did not increase the budding index. Therefore, DCR2-H338A is an antimorph or dominant negative, presumably because it encodes a mutant protein capable of antagonizing the wild-type DCR2 gene product. These results are consistent with a putative role for Dcr2p as a phosphoesterase.

Functional interactions with other START regulators. Cells carrying GID8 or DCR2 high-copy-number plasmids do not have altered Cln3p levels (Fig. 8A), consistent with the fact that, for these cells, size and resistance to pheromone are similar to those for wild-type cells (Fig. 1). We then overexpressed GID8 and DCR2 in cells lacking CLN3. Interestingly, in the absence of CLN3 GID8-2a and DCR2-2a did not increase the budding index, suggesting that Gid8p and Dcr2p may regulate cell cycle progression via Cln3p (Fig. 8B). Thus, a role for Gid8p and Dcr2p in G1 might require Cln3p, but it does not lead to higher Cln3p levels.

We then deleted GID8 and/or DCR2 in cells lacking CLN3, BCK2, or SWI4. Bck2p activates START in a Cln3p-independent manner (33), while Swi4p is a G1/S transcription factor (2). Cells lacking CLN3, BCK2, or SWI4 proliferate at almost the same rate as wild-type cells in rich media, but these mutants are larger than wild-type cells (Table 4). Interestingly, mutants with deletions of CLN3, BCK2, or SWI4 as well as GID8 and DCR2 were even larger (Table 4). The growth rate of the triple mutants was similar to those of cells with a single CLN3, BCK2, or SWI4 deletion in rich liquid (Table 4) or solid (Fig. 8C) media. Surprisingly, in the presence of high salt concentrations there were clear effects, with the triple cln3 gid8 dcr2 and swi4 gid8 dcr2 mutants growing very poorly (Fig. 8C). Cells with double mutations in GID8 or DCR2 and CLN3 or SWI4 proliferated normally, suggesting that GID8 and DCR2 might have synergistic functions under these conditions. Slightly poorer growth was also evident in bck2 gid8 dcr2 cells, but the effect was not as pronounced (Fig. 8C). Therefore, Gid8p and Dcr2p are required for normal rates of cell proliferation under high salt concentrations and in the absence of Cln3p or Swi4p.

**DISCUSSION**

In this study we have shown that Gid8p and Dcr2p affect cell cycle progression by regulating the completion of START. We discuss these findings in the general context of START control. The **FIG. 5. Subcellular localization of Gid8p and Dcr2p. (A and B, top) Immunoblots showing HA- or Myc-tagged Gid8p and Dcr2p, immuno-precipitated from cell extracts of the corresponding strains. (Bottom) Cells carrying a single epitope-tagged copy of the product of GID8 or DCR2, expressed from its native chromosomal location, or untagged controls (BY4742 for the HA-tagged strains or BY4741 for the Myc-tagged strains) were photographed through phase optics (left) and by fluorescence microscopy. The nuclei (middle) were visualized by DAPI staining. Epitope-tagged Gid8p or Dcr2p (right) were visualized by immunofluorescence. (C) Cells coexpressing Gid8p-HA and Dcr2p-Myc were processed as described for panels A and B and compared to the untagged control strain (BY4743). The merged colored image was produced by false coloring the Gid8p-HA image green and the Dcr2p-Myc image red.**
Why have \textit{GID8} and \textit{DCR2} not been previously identified in various screens for START regulators? Since \textit{GID8} and \textit{DCR2} are not essential, they were not targeted by the classic \textit{cdc} mutant screen done by Hartwell and colleagues, which focused on essential genes (13). Cells overexpressing \textit{GID8} and \textit{DCR2} seem to initiate START at a smaller size than wild-type cells (Fig. 1A and 2A), similar to \textit{CLN3}-overexpressing cells (Fig. 2A). However, unlike overexpression of \textit{CLN3}, overexpression of \textit{GID8} or \textit{DCR2} does not change the overall size of the population (Fig. 1C), probably because these cells continue

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**FIG. 6.** Dcr2p might function as a phosphoesterase. (A) \textit{DCR2} is predicted to encode a polypeptide with a metallophosphoesterase (metallophos) domain. Numbers indicate amino acid positions of the predicted Dcr2p polypeptide. (B) Relative phosphatase specific activity from crude cell extracts (means $\pm$ standard deviations; $n \geq 3$) from haploid cells. Where indicated, the strains were transformed with a high-copy-number plasmid carrying \textit{DCR2} (\textit{DCR2}-2\mu, \textit{DCR2-H338A} (\textit{DCR2-H338A-2\mu}), or the empty high-copy-number vector (vector-2\mu). (C) Relative budding indices (BI) of \textit{DCR2} and \textit{P}_{\text{cyc}}^{-}\textit{DCR2} cells (in the BY4741 background) carrying the indicated plasmids. The averages and standard deviations from at least eight independent transformants in each case are indicated. (D) Relative budding indices of \textit{GID8}\textsuperscript{-}/\textit{GID8}\textsuperscript{-} and \textit{P}_{\text{cyc}}^{-}\textit{GID8}\textsuperscript{-}/\textit{GID8}\textsuperscript{-} cells (in the BY4743 background) carrying the indicated plasmids. The averages and standard deviations from at least eight independent transformants in each case are indicated.
growing to the same size as wild-type cells in subsequent phases of the cell cycle after START completion. They also retain sensitivity to the antimitogenic properties of pheromone (Fig. 1D). Consequently, they would have been missed by previous approaches that relied on overall changes in cell size or resistance to pheromone for the identification of START regulators (6, 8, 16, 24, 26, 30, 35). These properties of GID8 and DCR2 mutants are important because they suggest that the list of START regulators may be larger than previously thought.

At this point, we can only speculate about the possible role(s) of GID8 and DCR2 in START control. Neither GID8 nor DCR2 mRNA levels are cell cycle regulated (29). Gid8p is predicted to contain LisH and CTLH domains (27). These domains have been previously associated with cytoskeletal functions (9). Recently, the mammalian cyclin E/Cdk2 substrate p220 (NPAT) was shown to regulate G1/S histone transcription through its LisH domain (32). It is important, however, that no clear function can be deduced from the presence

FIG. 7. Steady state levels of Myc-tagged Gid8p and Dcr2p in cells carrying the indicated high-copy-number plasmids or the untagged control strain are shown on an immunoblot produced with an anti-Myc antibody. The corresponding levels of Pgk1p are shown as a loading control. All the cells were in the haploid BY4741 background.

FIG. 8. Functional interactions with other START regulators. (A) The steady-state levels of Cln3p-PrA are shown on an immunoblot, from cells carrying the indicated plasmids (in the VAY27-1A background) and the untagged control strain (VAY27-1C). The corresponding levels of Pgk1p are shown as a loading control. (B) Relative budding indices (BI) of CLN3/H11001/CLN3/H11001 and cln3/H9004/cln3/H9004 cells (in the BY4743) background) carrying the indicated plasmids. The averages and standard deviations from at least eight independent transformants in each case are shown. The probability associated with a Student’s t test when the indicated samples were compared is shown. (C) Growth of the indicated strains was evaluated by spotting 10-fold serial dilutions of the cultures on solid rich media (yeast extract-peptone-dextrose [YPD]). The plates were incubated at 30°C and photographed after 2 (YPD) or 4 to 5 (YPD plus 1.2 M NaCl) days.
of these domains. Gid8p does not appear to colocalize with the 
cytoskeleton based on genome-wide localization data (15) and 
our own observations (Fig. 5). It was also recently suggested 
that Gid8p is involved in proteasome-mediated catabolite deg-
radation of fructose-1,6-biphosphatase when cells are trans-
ferred from a nonfermentable carbon source to glucose (27).
However, since all our experiments did not involve such media 
changes and since the GID8 overexpression phenotype was 
evident in steady-state conditions in glucose-rich media, it is 
iclare what role (if any) this activity might play in the 
regulation of START.

Based on genetic evidence, Gid8p and Dcr2p may function through a common pathway, with Dcr2p being downstream of 
Gid8p (Table 3 and Fig. 6), to positively control the timing of 
START. It is also clear that Gid8p's effects on overall cell 
growth when their combined loss produces more-severe cell size 
changes and since the 

GID8

and 

DCR2

point to a phosphoesterase activity of Dcr2p (Fig. 6). This 
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