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A WD Repeat Protein Controls the Cell Cycle and Differentiation by Negatively Regulating Cdc2/B-Type Cyclin Complexes

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In the fission yeast *Schizosaccharomyces pombe*, $p34^{cdc2}$ plays a central role controlling the cell cycle. We recently isolated a new gene named *srw1*$^+$, capable of encoding a WD repeat protein, as a multicopy suppressor of hyperactivated $p34^{cdc2}$. Cells lacking *srw1*$^+$ are sterile and defective in cell cycle controls. When starved for nitrogen source, they fail to effectively arrest in G_1 and die of accelerated mitotic catastrophe if regulation of $p34^{cdc2}$/Cdc13 by inhibitory tyrosine phosphorylation is compromised by partial inactivation of Wee1 kinase. Fertility is restored to the disruptant by deletion of Cig2 B-type cyclin or slight inactivation of $p34^{cdc2}$. *srw1*$^+$ shares functional similarity with *rum1*$^+$, having abilities to induce endoreplication and restore fertility to *rum1* disruptants. In the *srw1* disruptant, Cdc13 fails to be degraded when cells are starved for nitrogen. We conclude that Srw1 controls differentiation and cell cycling at least by negatively regulating Cig2- and Cdc13-associated $p34^{cdc2}$ and that one of its roles is to down-regulate the level of the mitotic cyclin particularly in nitrogen-poor environments.

INTRODUCTION

In virtually all eukaryotes, cyclin-dependent protein kinases (Cdk) play key roles controlling cell cycling (reviewed by Nurse, 1990; Nigg, 1995; Okayama *et al.*, 1996; Murakami and Okayama, 1997). In the fission yeast *Schizosaccharomyces pombe*, the single Cdk encoded by *cdc2*$^+$ ($p34^{cdc2}$) controls the onsets of both S phase and mitosis (Nurse and Bissett, 1981). During cell cycle progression the activity of $p34^{cdc2}$ is regulated positively and negatively at least by three kinds of biochemical events. One is association with a cyclin molecule, which is essential for kinase activity and strictly regulated in amount during cell cycling. The second is inhibitory phosphorylation at Tyr15 of $p34^{cdc2}$. The third is negative regulation by Cdk inhibitors.

Three kinds of B-type cyclins are known to associate with $p34^{cdc2}$. Cdc13 is a key cyclin essential for $p34^{cdc2}$ to perform mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989) and is also involved in the cell cycle "start" (Fisher and Nurse 1996; Mondesert *et al.*, 1996). Cig2 promotes the cell cycle start and negatively regulates differentiation (Obara-Ishihara and Okayama, 1994; Mondesert *et al.*, 1996). Cig1 is the third cyclin that is thought to act in G_2 or mitosis (Basi and Draetta, 1995). The protein levels of these cyclins are strictly regulated by transcription and ubiquitin-dependent proteolysis catalyzed by the 26S proteasome (Glotzer *et al.*, 1991; Gorden *et al.*, 1993; Funabiki *et al.*, 1996; Gorden *et al.*, 1996).

After passing through start in G_1, $p34^{cdc2}$/Cdc13 undergoes inhibitory phosphorylation at Tyr15, which is catalyzed by Wee1 and Mik1 kinases (Russell and Nurse, 1987; Gould and Nurse, 1989; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Hayles and Nurse, 1995). At the G_2–M boundary, the complex gets activated by Cdc25 and Pyp3 phosphatases-catalyzed dephosphorylation, which leads to the onset of mitosis (Russell and Nurse, 1986; Millar *et al.*, 1991, 1992).

One major Cdk inhibitor known in fission yeast is *rum1*$^+$, which was initially isolated as an inducer of

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multiple rounds of DNA replication without intervention by mitosis and subsequently shown to inhibit $p34^{cdc2}$/Cdc13 (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). Cells deleted for *rum1*$^+$ are sterile and defective in cell cycle control (Moreno and Nurse, 1994). The sterility but not the cell cycle defect is partially suppressed by deletion of Cig2 cyclin (Martin-Castellanos *et al.*, 1996), suggesting a link between this inhibitor and B-type cyclins. However, understanding the regulation of $p34^{cdc2}$ kinase as a key controller of cell cycling and differentiation is far from complete.

We recently launched an extensive search for factors regulating $p34^{cdc2}$ and screened a *S. pombe* cDNA library for multicopy suppressors of the *rad1–1 wee1–50* double mutant, a checkpoint mutant that dies of premature activation of $p34^{cdc2}$ at the restrictive temperature (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). Here we report the identification of *srw1*$^+$, a gene encoding a WD repeat protein that controls differentiation and cell cycling by negatively regulating $p34^{cdc2}$/B-type cyclin complexes.

MATERIALS AND METHODS

Strains, Media, Libraries, and Vectors

The strains of *S. pombe* used in this study are listed in Table 3. Media were prepared as described previously (Egel and Egel-Mitani, 1974; Gutz *et al.*, 1974; Moreno *et al.*, 1990; Okazaki *et al.*, 1990). The *S. pombe* cDNA library was constructed with mRNA from exponentially growing *S. pombe* cells by H. Tanaka. The vectors used have been described previously (Okazaki *et al.*, 1990; Igarashi *et al.*, 1991).

Isolation and Structural Analysis of the srw1$^+$ Gene

The *rad1–1 wee1–50 leu1–32* cells were cultured at 23°C to midlog phase in MB medium containing 0.15% leucine and transfected with the *S. pombe* cDNA library as described (Okazaki *et al.*, 1990). The cells were incubated at 23°C for 24 h on minimum medium agar (MMA) plates and then selected at 32.2°C for 4 d. Plasmid DNA was recovered from authentic transformants and cloned in *Escherichia coli*. DNA sequences were determined by the dideoxy method (Sanger *et al.*, 1977).

Gene Disruption

The *srw1*$^+$ gene was disrupted as follows. A genomic DNA fragment containing the *srw1*$^+$ gene was isolated from a *S. pombe Hin*dIII-genomic library by colony hybridization. The 2.2-kilobase (kb) *Eco*RI fragment containing 98% of the *srw1*$^+$-coding region was replaced with the 1.8-kb *Hin*dIII-excised *ura4*$^+$ gene. The linear fragment carrying the replaced gene was transfected into the *h*$^-$/*h*$^+$ *ade6-M210/ade6-M216 leu1–32/leu1–32 ura4-D18/ura4-D18* diploid strain, and stable *ura4*$^+$ cells were isolated. Disruptants were identified by Southern blot using the 0.7-kb *Hin*dIII–*Eco*RI fragment as a probe.

Assay for Conjugation

Mating frequencies were assayed as follows. The *h*$^-$ *leu1–32*, *h*$^-$ Δ*srw1 leu1–32*, *h*$^-$ Δ*cig2* Δ*srw1 leu1–32*, *h*$^{+s}$ *cdc2-M35* Δ*srw1 leu1–32* cells were grown in yeast extract liquid at 25°C, rinsed with water, and mixed with equivalent cultures of *h*$^{+s}$ *leu1–32* or *h*$^-$ *leu1–32* cells and incubated on malt extract agar plates for 2 d. Mating frequencies were calculated by dividing the number of zygotes by the number of total cells.

Flow Cytometry

Flow cytometry was performed as described previously (Tanaka *et al.*, 1992), by using the FACScan system and the CellFIT cell cycle analysis program with the software LYSIS (Becton Dickinson, San Jose, CA).

Assay for Loss of Cell Viability Induced by the Expression of cdc2^{+F15} and cdc13$^+$

The coding regions of *cdc2*$^{+F15}$ and *cdc13*$^+$ were inserted into the pcL expression vector, which contains a LEU2 selection marker, a replication origin, and the SV40 promoter to drive the expression of the insert. The vector carrying the insert was then transfected into Δ*srw1 leu1–32*, *leu1–32*, Δ*srw1 wee1–50 leu1–32*, and *wee1–50 leu1–32* cells, and leu$^+$-transformed cells were selected. The ratios of leu$^+$ colonies formed with *cdc2*$^{+F15}$ and *cdc13*$^+$ to those formed with the empty vector were calculated and expressed as percent colony formation.

Northern Blot Analysis

Total RNA was prepared and Northern blot analysis was performed with the ^{32}P-labeled 1.3-kb fragment of *ste11*$^+$ as a probe as described previously (Nagata *et al.*, 1991; Kato *et al.*, 1996).

Western Blot Analysis

Cells ($2–5 \times 10^8$) were washed once with water, resuspended in 200 μl of 10% trichloroacetic acid, and disrupted by vortexing with glass beads. After washing with acetone five times, proteins were solubilized by boiling for 5 min in the extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% SDS) (Watanabe *et al.*, 1997). Cell extracts (20 μg per lane) were separated by 10% SDS-PAGE (Laemmli, 1970), transferred to Immobilon TM-P membrane (Millipore, Bedford, MA), and the desired protein was detected using ECL (Amersham). Immunoblot was carried out with 1:2000-diluted anti-Cdc13p rabbit antibodies (SP4), 1:2000-diluted anti-Cig2p affinity-purified antibody and 1:50,000-diluted anti-α-tubulin monoclonal antibody (Sigma T5168, Sigma Chemical, St. Louis, MO).

RESULTS

Isolation of the srw1$^+$ Gene

To isolate new elements negatively regulating $p34^{cdc2}$, we screened a *S. pombe* cDNA library for those that suppressed the lethality of the *rad1–1 wee1–50* double mutant as described previously (Okazaki *et al.*, 1990) and isolated several clones having such an activity. The *rad1–1 wee1–50* double mutant is defective in a S-G_2 checkpoint control and dies of mitotic catastrophe due to premature activation of $p34^{cdc2}$ upon shift to the nonpermissive temperature (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). Consequently, any one of *wee1*$^+$, *mik1*$^+$, and *rad1*$^+$ suppresses this mutant. One clone did not hybridize with any of them and was therefore characterized further.

The clone suppressed the *rad1–1 wee1–50* double mutant up to 34.5°C, 2°C above the restriction temperature. It also suppressed not only the *rad3–136 wee1–50* (Figure 1A), *rad9–192 wee1–50*, Δ*chk1*

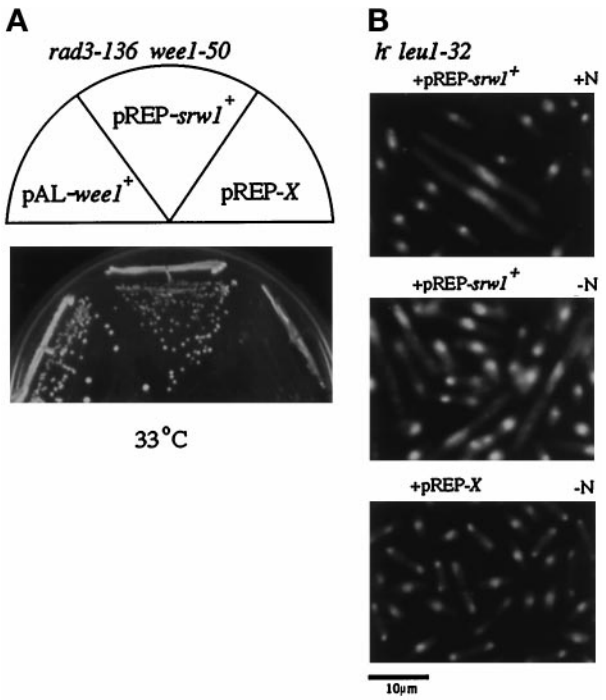

Figure 1. Overexpression of *srw1*$^+$ inhibits onset of mitosis. (A) Suppression of the *rad3–136 wee1–50* double mutant by *srw1*$^+$. The *rad3–136 wee1–50 leu1–32* strain (HM60) was transformed to leu$^+$ with pREP1-*srw1*$^+$, pAL-*wee1*$^+$, and empty pREP1-*X* (empty vector), and each transformant was spread on a minimum medium agar (MMA) plate and incubated at 33°C. (B) Morphology of wild-type cells overexpressing *srw1*$^+$. The *h*$^-$ *leu1–32* cells (ATCC38399) were transformed with pREP1-*srw1*$^+$, grown to midlog in PM+N medium with thiamin, then incubated in thiamin-free PM+N or PM-N medium for 15 h, fixed with 70% ethanol, and stained with DAPI. +N and −N denote nitrogen-rich and free. Control is the cells similarly transformed with pREP1-*X* and incubated in thiamin-free PM-N medium.

wee1–50 but also Δ*mik1 wee1–50* double mutants, all of which die of mitotic catastrophe at the nonpermissive temperature, suggesting that this new gene inhibits the activity of p34^{cdc2}/Cdc13 despite low levels of Tyr15 kinase activities. Consistently, overexpression of the clone in wild-type cells resulted in cell elongation, a typical phenotype of cell cycle retardation or arrest, although in a small population. Cell elongation became more evident in nitrogen-poor medium (Figure 1B), suggesting that the activity of this clone might be enhanced in nitrogen-starved environments. This gene was named *srw1*$^+$ (suppressor of *rad wee1*) and characterized more extensively.

srw1$^+$ *Encodes a WD Repeat Protein*

srw1$^+$ contains a contiguous open reading frame capable of encoding a 556-amino acid protein with seven WD repeats commonly present in the β-transducin family (Figure 2). It is significantly homologous (38% amino acid identity) with the hypothetical protein of *S. cerevisiae* Yg1003c identified by the Genome Sequence Project. In addition, Srw1 is also homologous with four distinct proteins, especially in the WD repeat domain. They are Fizzy (*Drosophila melanogaster*) (Dawson *et al.*, 1995), p55CDC (*Homo Sapiens*)(Weinstein *et al.*, 1994), CDC20 (*S. cerevisiae*)(Sethi *et al.*, 1991), and Slp1 (*S. pombe*)(Matsumoto, 1997)(Figure 2). Fizzy is reportedly required for the degradation of cyclins A and B during mitosis. Accordingly, *fizzy* mutations cause metaphase arrest accompanied by failure to degrade mitotic cyclins. p55CDC is expressed in dividing cells. CDC20 is required for microtubule-dependent processes, such as nuclear movements before anaphase, chromosome separation, and nuclear fusion during mating of G_1 cells, whereas Slp1 genetically interacts with Wee1 kinase or Cdc25 phosphatase, thereby promoting cells to restart the cell cycle after DNA repair is completed.

Cells Lacking srw1$^+$ *Are Sterile*

To investigate the biological role of *srw1*$^+$, a null allele of *srw1*$^+$ was constructed by one-step gene replacement. A genomic fragment containing *srw1*$^+$ was isolated by colony hybridization, and the almost entire open reading frame was replaced with the *ura4*$^+$ gene, followed by transfection into a diploid strain and by selection for stable ura$^+$ diploid cells. Successful disruptants were identified by Southern blot analysis. The diploid cells, in which one allele of *srw1*$^+$ was disrupted, were germinated to obtain haploid disruptants, which were then extensively backcrossed with wild-type cells to eliminate second mutations.

The *srw1* disruptants (Δ*srw1*) grew in the regular medium with no apparent growth defects. However, they showed severe sterility even if wild- type cells were used as a mating partner (Figure 3A and Table 1). We failed to find even a single conjugated cell or spore ascus after extensive search. Sterility was indeed caused by the inactivation of *srw1*$^+$ because it was effectively suppressed by the *srw1*$^+$ cDNA as well as genomic DNA.

Deletion of cig2$^+$ *or Inactivation of Cdc2 Partially Suppresses Sterility*

Cig2/Cyc17 cyclin negatively regulates sexual development as well as promotes the cell cycle start (Obara-Ishihara and Okayama, 1994), specifying p34^{cdc2} as a kinase partner (Martin-Castellanos *et al.*, 1996). Because *srw1*$^+$ was able to inhibit p34^{cdc2}/Cdc13 as described above, we speculated that the sterility of Δ*srw1* cells might have resulted, at least partly, from the hyperactivation of p34^{cdc2}/Cig2. This proved true. In a

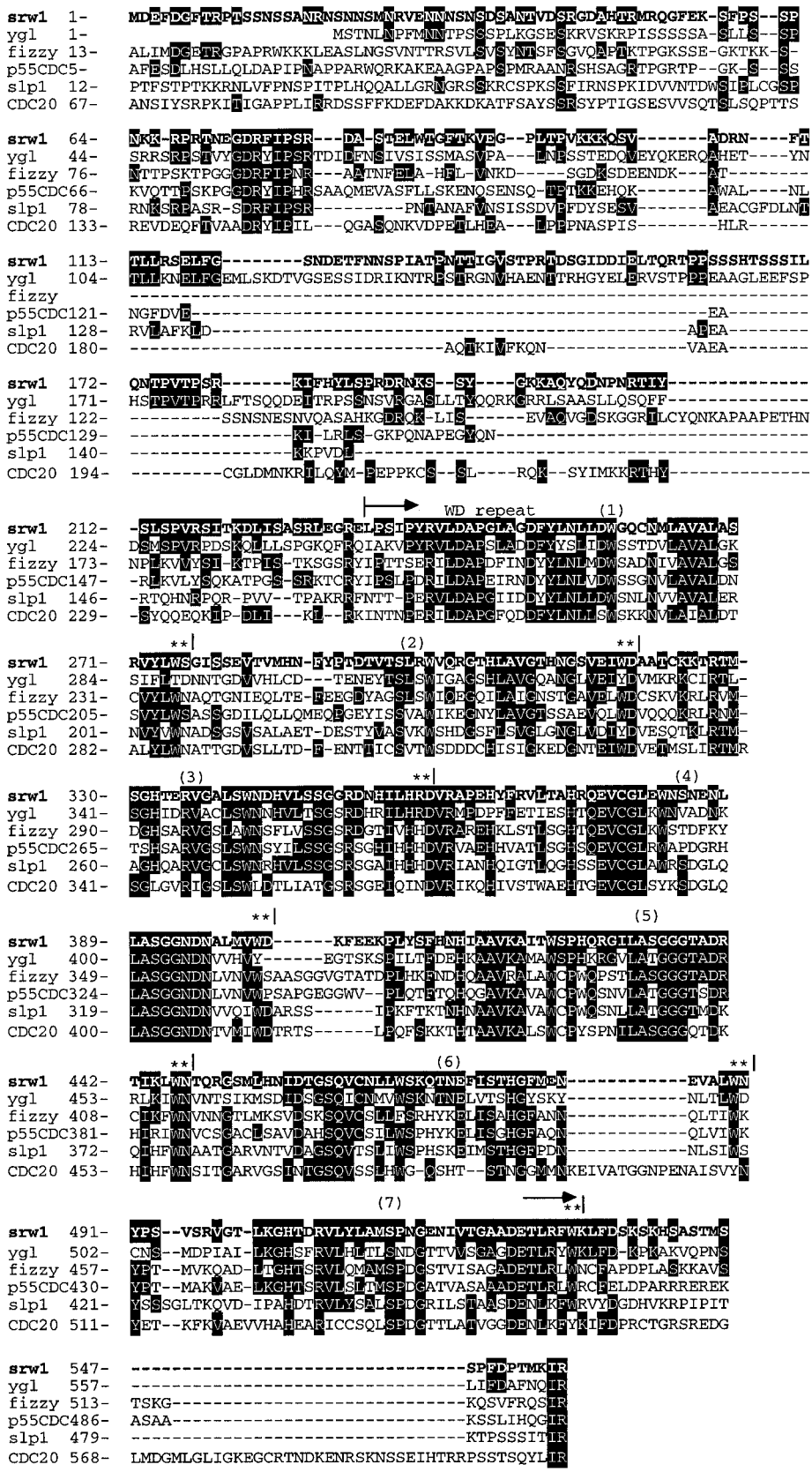

Figure 2. Sequence and structure of *srw1*$^{+}$. The predicted amino acid sequence of Srw1 is shown in single-letter code and compared with those of Yg1003c, Fizzy, p55CDC, Slp1, and CDC20. The amino acids identical to Srw1 are shaded. The positions of seven putative WD repeat motifs are indicated by bars with numbers, and WD by asterisks. The GenBank/EMBL/DDBJ accession number for *srw1*$^{+}$ is AB005589.

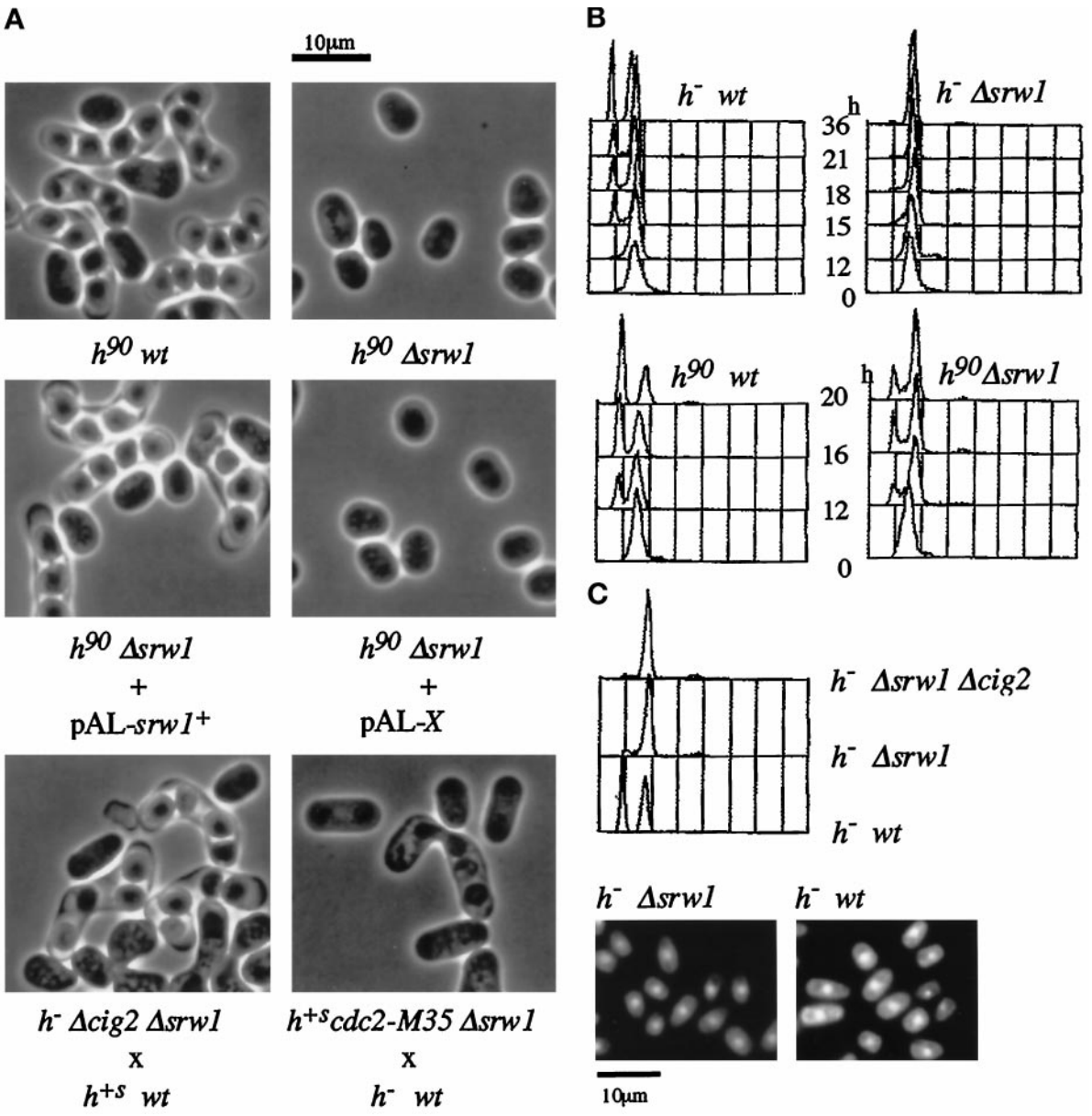

Figure 3. Phenotypes of *srw1* disruptant. (A) Δ*srw1* cells are sterile, but sterility is suppressed by deletion of Cig2 or partial inactivation of Cdc2. The h^{90} *leu1–32* (K153-B25) and h^{90} *srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY3) cells were grown to midlog phase in YEL and plated on MEA plates for conjugation at 27°C for 2 days. The h^{90} *srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY3) cells were transformed with pAL-*srw1*$^+$ or pAL-*X* (empty pAL vector) and plated for conjugation as above. The h^{+s} *leu1–32* (K150-A13) and h^- *srw1::ura4*$^+$ *cig2/cyc17::ura4*$^+$ *ura4-D18 leu1–32* (SY5) cells, or h^- *leu1–32* (ATCC38399) and h^{+s} *cdc2-M35 srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY6) cells, were mixed and plated for conjugation. (B) Δ*srw1* cells are unable to arrest or arrest weakly in G_1. The h^- *leu1–32* (ATCC38399), h^- *srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY1), h^{90} *leu1–32* (K153-B25), and h^{90} *srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY3) cells were grown in YEL to midlog phase, washed, and then incubated at 25°C in malt extract liquid (MEL) for the indicated times and analyzed by flow cytometry. (C) Deletion of Cig2 is unable to restore the G_1 arrest ability to Δ*srw1* cells. The h^- *leu1–32* (ATCC38399), h^- *srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY1), and h^- *srw1::ura4*$^+$ *cig2/cyc17::ura4*$^+$ *ura4-D18 leu1–32* (SY5) were grown in YEL to midlog phase, incubated at 25°C for 36 h in malt extract liquid (MEL) and analyzed by flow cytometry. The h^- *srw1::ura4*$^+$ *ura4-D18 leu1–32* (SY1) and h^- *leu1–32* (ATCC38399) cells were then stained with DAPI.

conjugation assay using wild-type cells as a mating partner, *cig2*$^+$ deletion restored fertility to the disruptant to more than one-half the ability of wild-type cells (Figure 3A and Table 1). The kinase partner of Cig2 for this mating inhibition is likely to be $p34^{cdc2}$ because partial inactivation of $p34^{cdc2}$ also restored mating ability to the disruptant, although only marginally. The temperature-sensitive *cdc2-M35* mutant

Table 1. Cells lacking *srw1*$^+$ are sterile but sterility is suppressed by deletion of *cig2*$^+$ or inactivation of *cdc2*$^+$

	% Mating frequencies	
Strain	27°C	25°C
h^- *leu1* × h^{+S} *leu1*	57.0	55.2
h^-Δ*srw1 leu1* × h^{+S} *leu1*	<0.01	<0.01
h^-Δ*cig2* Δ*srw1 leu1* × h^{+S} *leu1*	35.6	34.7
h^{+S} *cdc2-M35* Δ*srw1 leu1* × h^- *leu1*	0.44	0.02

Cells were grown to log phase in YEL, mixed and spotted on MEA plates followed by a 2-d incubation at the indicated temperature. Mating efficiencies were calculated as described in MATERIALS AND METHODS.

allele is partially inactivated at 25°C (Nurse and Thuriaux, 1980). At this temperature, the *cdc2-M35* Δ*srw1* double mutant was still sterile. However, when the temperature was raised to 27°C, a small fraction of the cells came to perform mating and sporulation (Figure 3A and Table 1). Further elevation in the temperature did not increase the mating frequencies perhaps because the cells tended to arrest in G_2 due to more inactivation of p34^{cdc2}. These results suggest that the *srw1*$^+$ gene product promotes sexual development at least by inactivating p34^{cdc2}/Cig2.

The transcriptional factor Ste11 is essential for the initiation of sexual development (Sugimoto *et al.*, 1991), and various differentiation signals including nitrogen starvation signal regulate the expression of *ste11*$^+$. Therefore, it was important to know whether *srw1*$^+$ influenced *ste11*$^+$ mRNA induction. Our unpublished observations show that in Δ*srw1* cells *ste11*$^+$ was expressed and induced to the same extent as wild-type cells upon nitrogen starvation. In addition, ectopic expression of *ste11*$^+$ driven by the SV40 promoter failed to restore fertility to Δ*srw1* cells, indicating that *srw1*$^+$ promotes sexual development but not via transcriptional regulation of *ste11*$^+$.

Δ*srw1 Cells Are Partially Defective in Nitrogen Starvation-Induced G_1 Arrest*

The ability to arrest in G_1 in response to both nitrogen starvation and mating pheromones is considered to be critical for cells to perform conjugation. We therefore examined the ability of the disruptant to arrest in G_1 in response to nitrogen starvation. In conjugation-inducing malt extract medium, which contains a limited amount of nitrogen, Δ*srw1* cells proliferated as rapidly as wild-type cells, and both ceased proliferation as nitrogen source was exhausted. At this point, approximately 50% of heterothallic wild-type cells arrested in G_1 whereas virtually none of the Δ*srw1* cells arrested in G_1 (Figure 3B). In homothallic cells, in which mat-

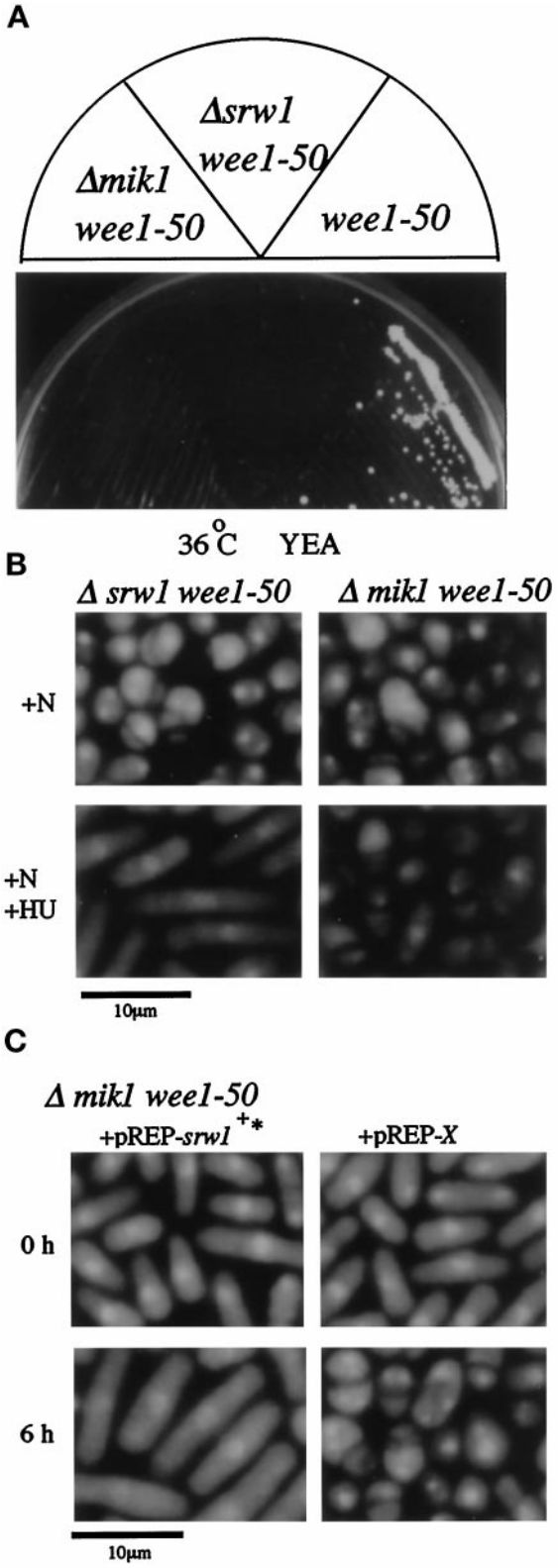

Figure 4A–C.

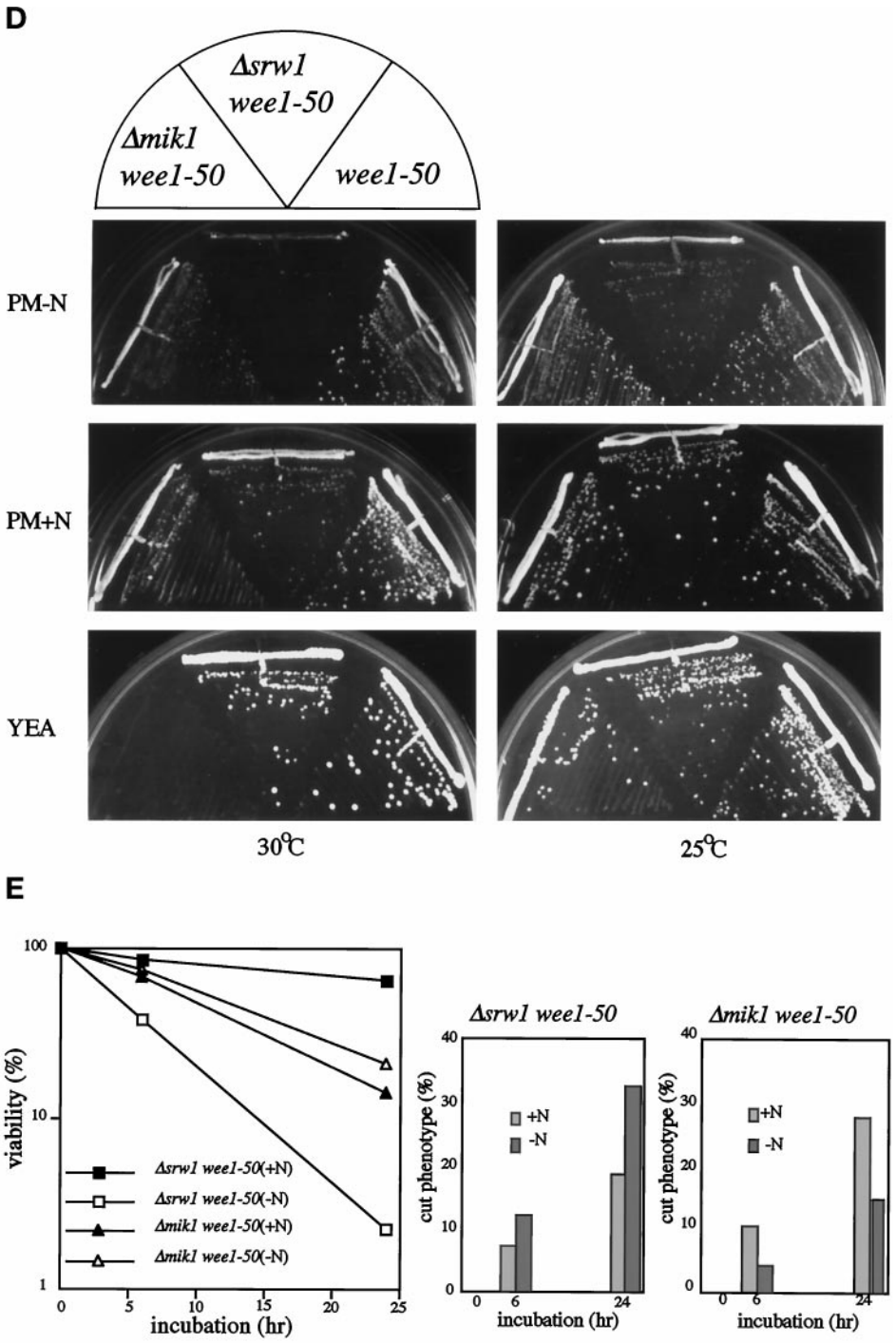

Figure 4D and E (A–C on facing page). Δ*srw1 wee1–50* cells die of mitotic catastrophe. (A) Δ*srw1 wee1–50* cells are unable to grow on YEA plate at 36°C. (B) Δ*srw1 wee1–50* cells die of mitotic catastrophe, which is blocked by hydroxyurea. The h^{+s} *srw1::ura4*$^+$ *wee1–50 ura4-D18 leu1–32* (SY4) cells were grown to midlog phase in PM+N medium at 25°C and incubated at 36°C for 6 h in PM+N medium with or without 12 mM hydroxyurea. Cells were fixed with 70% ethanol and stained with DAPI. (C) Overexpression of *srw1*$^+$ rescues Δ*mik1 wee1–50* cells from mitotic catastrophe. The h^- *mik1::ura4*$^+$ *wee1–50 ura4–294 leu1–32* (HM43) cells were transformed with pREP1-*srw1*$^{+*}$ (deleted for G-tail) or pREP1-X(empty vector), grown to midlog phase at 25°C in PM+N medium, and then shifted to 36°C. Cells were fixed with 70% ethanol and stained with DAPI. (D)Δ*srw1 wee1–50* cells are unable to grow even at 30°C on PM-N plate. The h^{+s} *srw1::ura4*$^+$ *wee1–50 ura4-D18 leu1–32* (SY4), *wee1–50 leu1–32* (HM65), and h^- *mik1::ura4*$^+$ *wee1–50 ura4–294 leu1–32* (HM43) cells were streaked on PM-N, PM+N, and YEA plates and incubated at 30°C and 25°C. (E) Δ*srw1 wee1–50* cells lose viability in nitrogen-poor medium due to mitotic catastrophe. The h^{+s} *srw1::ura4*$^+$ *wee1–50 ura4-D18 leu1–32* (SY4) and h^- *mik1::ura4*$^+$ *wee1–50 ura4–294 leu1–32* (HM43) cells were grown to midlog phase in PM+N medium, incubated in PM+N or PM-N at 25°C for 6 h, and transferred to fresh PM+N or PM-N medium at 32°C followed by further incubation for the indicated time. The percent cell viability was calculated by dividing the number of colonies formed on YEA plates at 25°C by that of 0 h after appropriate dilution. The number of cells in cut phenotype was counted under the microscope after staining with DAPI.

ing pheromone signaling is activated, *srw1*$^+$ was not essential but still required for full G_1 arrest. Contrary to our expectation, unlike fertility, G_1 arrest ability failed to be restored by the deletion of *cig2*$^+$ (Figure 3C). Needless to say, lack of G_1-arrested cells in the disruptant was not caused by a failure of cell separation before arrest. The growth-arrested Δ*srw1* cells became small, and each cell had a single nucleus just like wild-type cells (Figure 3C). These results indicate that there are other target molecule(s) for *srw1*$^+$ that are specifically required for G_1 arrest.

Nevertheless, heterothallic Δ*srw1* cells were not totally defective in G_1 arrest ability. Abrupt removal of nitrogen source, such as shift to nitrogen-free mini-

mum medium, induced G_1 arrest to the disruptant although the G_1 arrest was partial and significantly delayed (see below).

Δsrw1 wee1–50^{ts} Double Mutant Dies of Mitotic Catastrophe

As shown already, overexpression of *srw1*$^+$ inhibited the activity of p34^{cdc2}/Cdc13. This does not necessarily mean that endogenous Srw1 is involved in mitotic control. We therefore investigated this point. As aforementioned, Δ*srw1* cells showed no apparent defects in mitotic control in the regular growth conditions. However, *srw1*$^+$ was absolutely required for proper mitotic control when the negative regulation of Cdc2/Cdc13 by tyrosine phosphorylation was compromised by inactivation of Wee1 kinase. Despite inactivation of Wee1, the temperature-sensitive *wee1–50* mutant grows at 36°C because of the presence of the functionally redundant Mik1 kinase (Nurse, 1975; Thuriaux *et al.*, 1978; Lundgren *et al.*, 1991). However, the Δ*srw1 wee1–50* double mutant was unable to grow at this temperature even on nutritionally rich YEA plates (Figure 4A). This resulted from massive entry into mitotic catastrophe, which was characterized by anucleated cells and cells with the nucleus divided by septum ("cut" phenotype) (Figure 4B). These results indicate that *srw1*$^+$ plays a role negatively controlling the activity of p34^{cdc2}/Cdc13 in cell cycling.

The next question we addressed is how *srw1*$^+$ regulates the activity of p34^{cdc2}/Cdc13. Inhibition of DNA synthesis by hydroxyurea activates a S-G_2 checkpoint control, which prevents the activation of p34^{cdc2}/Cdc13 predominantly via inhibiting Tyr15 dephosphorylation (Enoch *et al.*, 1992). Hydroxyurea treatment effectively blocked entry of Δ*srw1 wee1–50* cells into mitotic catastrophe that occurred upon shift to the nonpermissive temperature, and induced cell elongation (Figure 4B). This is in sharp contrast to Δ*mik1 wee1–50* cells, which failed to arrest by hydroxyurea and prematurely entered mitosis with massive catastrophic cell death. This result suggests that *srw1*$^+$ is likely to negatively regulate the p34^{cdc2}/Cdc13 activity mainly by a mechanism independent of tyrosine 15 phosphorylation.

To further investigate the relationship between *srw1*$^+$ and Tyr15 regulation, we used Δ*mik1 wee1–50* strain and Cdc2^{F15}. As noted earlier, overexpressed *srw1*$^+$ rescued not only the *rad1 wee1–50* but also Δ*mik1 wee1–50* strain. As shown in Figure 4C, 4,6-diamidino-2-phenylindole (DAPI) staining revealed that overexpressed *srw1*$^+$ effectively suppressed the mitotic catastrophe of the Δ*mik1 wee1–50* strain. This result supports our initial observation that the action of *srw1*$^+$ is mostly, if not entirely, independent of tyrosine 15 phosphorylation. To further confirm and extend this observation, we tested the effect on cell viability of the expression of constitutively active Cdc2^{F15}, in which tyrosine 15 was replaced with unphosphorylatable phenylalanine to completely eliminate the regulation by Tyr15 phosphorylation. The coding region of *cdc2*$^{+F15}$ was inserted into the pcL expression vector driven by the SV40 promoter and containing the *leu*$^+$ marker gene. If the cells transfected with the vector would lose viability by the expression of the insert, less leu$^+$ colonies would be formed. Expression of pcL-*cdc2*$^{+F15}$ was not highly toxic to wild type cells, and leu$^+$ colonies were formed at 42% the efficiency of the empty vector (Table 2). On the contrary, pcL-*cdc2*$^{+F15}$ was extremely toxic to Δ*srw1* cells, and its colony-forming efficiency was reduced more than 200-fold. Thus, the combination of Cdc2^{F15} expression and inactivation of *srw1*$^+$ seemed lethal to cells, reinforcing our initial observation.

Our observation suggested that the function of *srw1*$^+$ seemed to be enhanced during nitrogen starvation. To investigate this possibility, the effect of nitrogen starvation on the growth ability of Δ*srw1 wee1–50* cells was studied. Both *wee1–50* and Δ*mik1 wee1–50* cells were used as controls. At 30°C on PM+N plates, all these mutants grew without noticeable difficulties. But on nitrogen-poor PM-N plates or in such medium, unlike *wee1–50* single and Δ*mik1 wee1–50* double mutants, Δ*srw1 wee1–50* cells failed to grow (Figure 4D) and died of increased mitotic catastrophe (Figure 4E). By contrast, Δ*mik1 wee1–50* cells were unable to grow on nutritionally rich YEA plates or in nitrogen-rich PM+N medium and died of increased mitotic catastrophe. These results confirm the initial observation and strongly indicate that the biological role of Srw1 is signified in nitrogen-poor environments.

Overexpression of srw1$^+$ *Induces Endoreplication*

As one might have noticed previously, there is a striking functional similarity between *srw1*$^+$ and *rum1*$^+$.

Table 2. The viability of cells expressing Cdc^{F15} and Cdc13

	% Leu$^+$		
Strain	pcL-X	pcL-*cdc2*$^{+F15}$	pcL-*cdc13*$^+$
h$^-$ *leu1*	100	41.7 ± 7.9	38.4 ± 7.6
h$^-$ Δ*srw1 leu1*	100	0.157 ± 0.076	20.3 ± 5.66
h$^-$ *wee1-50 leu1*	100	ND	48.7 ± 20.6
h^{+S} Δ*srw1 wee1-50 leu1*	100	ND	1.35 ± 0.11

Cells were transfected with the indicated plasmids and selected for leu$^+$ phenotype at 30°C for the *h*$^-$ *leu1-32* and *h*$^-$ Δ*srw1 leu1-32* cells and at 25°C for the *h*$^-$ *wee1-50 leu1-32* and *h*$^{+S}$ Δ*srw1 wee1-50 leu1-32* cells, both temperatures being permissive for these strains. The ratios of leu$^+$ colonies formed with the indicated plamids to those formed with the empty vector are expressed as % leu$^+$. Each experiment was performed three times and values of mean ± SD are shown.

Both genes inhibit the onset of mitosis, both gene disruptants are phenotypically similar, being sterile and defective in nitrogen starvation-induced G_1 arrest and in mitotic control, and their sterility is suppressed by deletion of Cig2 (Moreno and Nurse, 1994; Martin-Castellanos *et al.*, 1996). To further investigate the similarity, we examined the ability of *srw1*$^+$ to induce endoreplication. To obtain high expression of *srw1*$^+$, the G-tail of the *srw1*$^+$ cDNA was deleted, reinserted into the thiamin-repressible pREP1 vector (pREP-*srw1*$^{+*}$), and expressed in wild-type cells. Upon removal of thiamin, many cells showed enlarged nuclei brightly stained with DAPI (Figure 5A). FACS analysis revealed that some cell population contained 4C (4 DNA content) or more DNA highly indicative of endoreplication (Figure 5B). Interestingly, a considerable fraction of cells arrested in 1C (1 DNA content), suggesting that Srw1 has the ability to block the onsets of both S phase and mitosis. Partial block of S phase onset was also observed with wild-type cells harboring an integrated copy of pREP2-*srw1*$^{+*}$. In addition, overexpression of *srw1*$^+$ restored fertility to Δ*rum1* cells (Figure 5C). With wild-type cells as a mating partner, *srw1*$^+$-overexpressing Δ*rum1* cells conjugated at about 20% the frequency of control wild-type cells. By contrast, overexpression of *rum1*$^+$ failed to restore fertility to Δ*srw1* cells. These results establish functional similarities between Srw1 and Rum1 although the molecular mechanisms by which they inhibit p34^{cdc2}/B-type cyclin are unlikely to be the same.

Srw1 Is Involved in Regulation of the Amount of Cdc13

To gain further insights into the function of *srw1*$^+$, we examined the effect of Cdc13 overproduction on the viability of Δ*srw1* cells. Overexpression of *cdc13*$^+$ in Δ*srw1* cells had little effect but decreased colony formation slightly. However, when the disruptant was slightly inactivated for Wee1 kinase, Cdc13 overproduction had a dramatic effect. Even at a highly permissive temperature of 25°C for *wee1–50*, overexpressed *cdc13*$^+$ was highly toxic to Δ*srw1 wee1–50* cells (Table 2), and they died of mitotic catastrophe. These results, combined with the independence of Srw1 function from Tyr15 phosphorylation and its structural similarity to Fizzy reportedly required for cyclin degradation in *Drosophila* (Dawson *et al.*, 1995), suggest that *srw1*$^+$ inhibits the p34^{cdc2}/Cdc13 activity possibly by modulating the amount of Cdc13.

We therefore investigated the protein level of Cdc13 and also Cig2 B-type cyclins in Δ*srw1* cells by Western blot analysis. As already noted, when transferred to nitrogen-free minimum medium, Δ*srw1* cells could arrest in G_1 despite a significant delay. At 24 h post-transfer, more than one-half of wild-type cells arrested in G_1 with marked reduction in the level of Cdc13

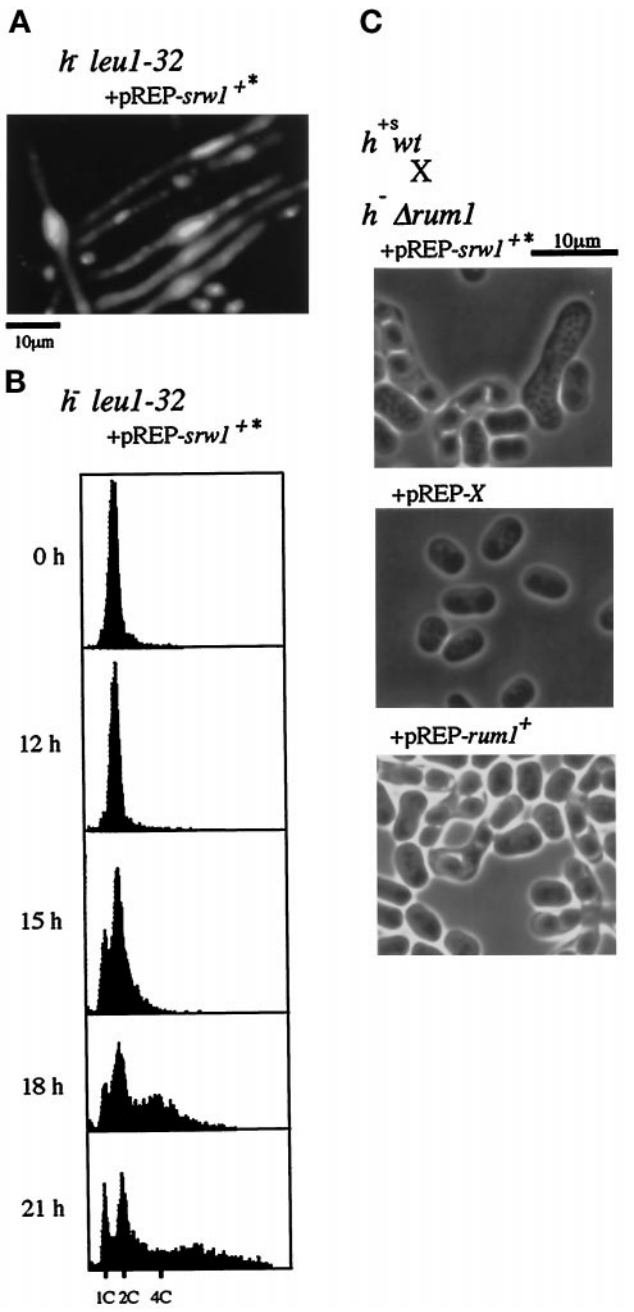

Figure 5. Overexpression of *srw1*$^+$ can induce endoreplication and restores fertility to Δ*rum1* cells. (A) DAPI staining and (B) Flow cytometry (FACS) analysis of wild-type cells expressing *srw1*$^+$. The *srw1*$^+$ cDNA was deleted for G-tail, reinserted into the pREP1 vector (pREP1-*srw1*$^{+*}$) and transformed into wild-type cells. Cells were grown first in PM+N medium with thiamin and then washed and transferred to PM+N medium without thiamin (0 h) and incubated for the indicated times. (C) Overexpression of *srw1*$^+$ restores fertility to Δ*rum1* cells. The *h*$^-$ *rum1::ura4*$^+$ *ura4-D18 leu1–32* (SY100) cells transformed with pREP1-*srw1*$^{+*}$, pREP1-*rum1*$^+$ or pREP1-X were mixed with *h*$^{+s}$ *leu1–32* (K150-A13) cells and spotted on MEA plates and incubated at 27°C for 2 d.

Table 3. Strain list

Strain	Genotype
DP2	*h*$^{-}$/*h*$^{+}$ *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*
ATCC38399	*h*$^{-}$ *leu1-32*
K150-A13	*h*$^{+S}$ *leu1-32*
K153-B25	*h*^{90} *leu1-32*
HM332	*h*$^{-}$ *ura4-294 leu1-32*
HM43	*h*$^{-}$ *mik1::ura4*$^{+}$ *wee1-50 ura4-294 leu1-32*
HM65	*h*$^{-}$ *wee1-50 leu1-32* (from P. Nurse)
HM58	*h*$^{+N}$ *rad1-1 wee1-50 leu1-32*
HM60	*h*$^{+N}$ *rad3-136 wee1-50 leu1-32*
HM61	*h*$^{+N}$ *rad9-192 wee1-50 leu1-32*
HM64	*h*$^{-S}$ *chk1::ura4*$^{+}$ *wee1-50 ura4-D18 leu1-32*
TI101	*h*$^{-}$ *cig2::ura4*$^{+}$ *ura4-D18 ade6-M210 leu1-32*
HM270	*h*$^{-}$ *cdc2-M35 ura4-D18 leu1-32* (from P. Nurse)
HM1000	*cdc13::ura4*$^{+}$ int pREP41::*cdc13*$^{+}$ *ura4-D18 leu1-32 ade6-M216* (from P. Nurse, described in Fisher and Nurse, 1996)
SY1	*h*$^{-}$ *srw1::ura4*$^{+}$ *ura4-D18 leu1-32*
SY2	*h*$^{+S}$ *srw1::ura4*$^{+}$ *ura4-D18 leu1-32*
SY3	*h*^{90} *srw1::ura4*$^{+}$ *ura4-D18 leu1-32*
SY4	*h*$^{+S}$ *srw1::ura4*$^{+}$ *wee1-50 ura4-D18 leu1-32*
SY5	*h*$^{-}$ *srw1::ura4*$^{+}$ *cig2/cyc17::ura4*$^{+}$ *ura4-D18 leu1-32*
SY6	*h*$^{+S}$ *cdc2-M35 srw1::ura4*$^{+}$ *ura4-D18 leu1-32*
SY7	*h*$^{-}$ *int pREP2::srw1*$^{+}$ *ura4-294 leu1-32*
SY100	*h*$^{-}$ *rum1::ura4*$^{+}$ *ura4-D18 leu1-32* (from S. Moreno)

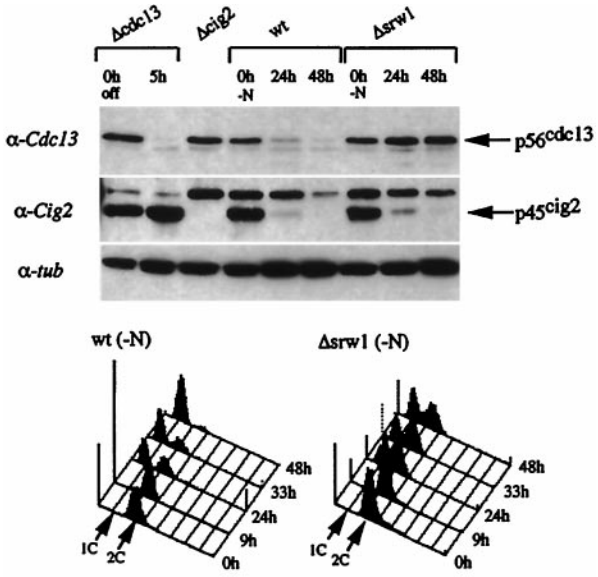

Figure 6. Srw1 is required for nitrogen starvation-induced decrease of the amount of Cdc13 but not Cig2. The *h*$^{-}$ *leu1–32* (wt, ATCC38399) and *h*$^{-}$ *srw1::ura4*$^{+}$ *ura4-D18 leu1–32* (Δsrw1, SY1) cells were grown in minimal medium to log-phase at 25°C and then incubated in nitrogen-free minimum medium for the indicated times. The *h*$^{-}$ *cig2::ura4*$^{+}$ *ura4-D18 ade6-M210 leu1–32* (Δcig2, TI101) and *cdc13::ura4*$^{+}$ int pREP41::*cdc13*$^{+}$ *ura4-D18 leu1–32 ade6-M216* (Δcdc13, HM1000) strains were used as controls. For Δ*cdc13* cells, 5 μg/ml thiamin were added to the culture to shut off cdc13 expression. Extracts were prepared and separated by SDS-PAGE, and immunoblotted with anti-Cdc13, anti-Cig2, and anti-α-tubulin antibodies as probes. Flow cytometric analysis was performed. The 1 N and 2 N DNA contents are indicated by arrows.

(Figure 6). At 48 h, more than 90% of the cells were in G_1 with a nearly undetectable level of Cdc13. By contrast, in Δ*srw1* cells, the amount of Cdc13 did not decrease, but tended to rather slightly increase, at 48 h despite that more than one-half the cells arrested in G_1. This result shows that Srw1 is essential for decrease of Cdc13 upon nitrogen starvation. By contrast, Cig2 cyclin behaved differently. In both wild-type and Δ*srw1* cells, Cig2 disappeared upon nitrogen starvation, suggesting that Srw1 inhibits p34^{cdc2}/Cig2 by a different mechanism and that the action of Srw1 to p34^{cdc2}/Cig2 might be early in nitrogen starvation.

DISCUSSION

How cell differentiation and the cell cycle are coordinately regulated is one critical question that remains to be addressed. Our data show that the newly identified *srw1*$^{+}$ gene is involved in this regulation. Cells lacking this gene are defective in both cell differentiation and cell cycle controls. Their most apparent phenotypes are sterility and defects in G_1 and mitotic controls. Sterility was so severe that no conjugated cells were detected throughout this series of experiments (Table 1). The defect in G_1 control mainly involves the cell's inability to arrest or slow down before the onset of S phase. However, the disruptant is not completely defective in G_1 arrest ability. When mating pheromone signaling was activated or cells were rapidly starved for nitrogen, they could arrest in G_1 at least partly. By contrast, their defect in mitotic control is dormant until the regulation of Cdc2 by tyrosine phosphorylation is slightly compromised by partial inactivation of Wee1 kinase. In such a situation, Srw1 is absolutely required for blocking premature mitosis and such a role of Srw1 is particularly evident in nitrogen-poor environments.

All the genetic and functional analysis data led us to conclude that, particularly responding to nitrogen starvation, Srw1 promotes differentiation and inhibits the onset of mitosis via inhibiting at least p34^{cdc2}/Cig2 and p34^{cdc2}/Cdc13, respectively (Figure 7). Cig2 cyclin plays a dual role inhibiting cell differentiation and promoting the cell cycle start (Obara-Ishihara and Okayama, 1994; Mondesert *et al.*, 1996). Interestingly, the sterility but not the G_1 arrest inability of Δ*srw1* cells was effectively suppressed by the deletion of Cig2. The lack of the restoration of the G_1 arrest ability was unexpected but may be explained by the presence of Cdc13 mitotic cyclin, which shares the S phase start function with Cig2 (Fisher and Nurse 1996; Mondesert *et al.*, 1996). Thus, Srw1 plays a key role in the coor-

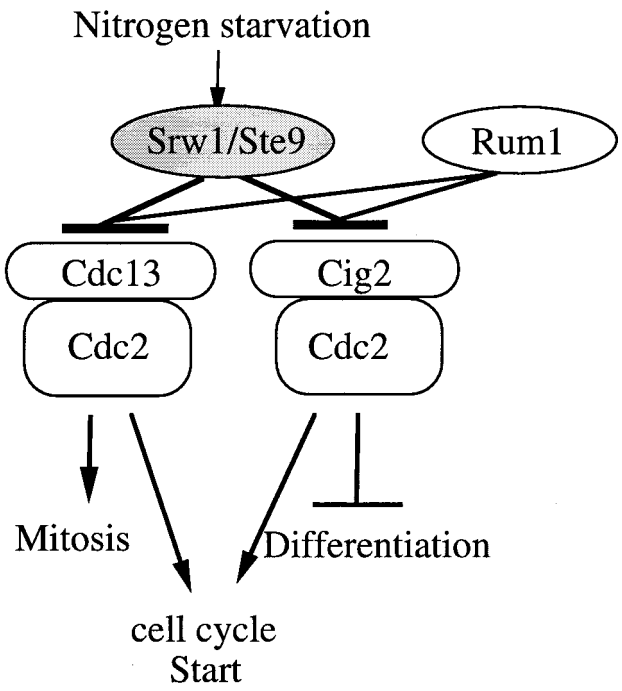

Figure 7. Proposed model for Srw1 function. Srw1 controls differentiation and the onsets of S phase and mitosis by negatively regulating Cdc2/B-type cyclin complexes.

dinated regulation of cell differentiation and proliferation by nutrient starvation.

Our genetic data suggested that the primary target(s) for the Srw1 action for mitotic control were not Tyr15 of Cdc2 but Cdc13 B-type cyclin. This was confirmed by the biochemical data demonstrating that Srw1 negatively regulates the level of Cdc13 upon nitrogen starvation (Figure 6). Taking the structural similarity with *fizzy* into consideration, it is most probable that Srw1 might directly promote degradation of Cdc13. However, contrary to our expectation, Srw1 is unlikely to play a role regulating the level of Cig2. Regardless of the presence or absence of Srw1, Cig2 was degraded upon nitrogen starvation, suggesting that Srw1 might inhibit $p34^{cdc2}$/Cig2 by a different mechanism.

Interestingly, Srw1 shares striking functional similarity with Rum1 despite their structural dissimilarity. Both deletion mutants are sterile, and their sterility is suppressed at least in part by the inactivation of Cig2. In addition, both mutants are defective in G_1 and mitotic controls, and their defects in mitotic control are displayed when the mitotic start regulation by tyrosine phosphorylation is compromised. Both overexpressed *srw1*$^+$ and *rum1*$^+$ block the onset of mitosis and induce endoreplication. The major target for both factors is Cdc2 kinase associated with B-type cyclins. But, the molecular mechanisms by which they inhibit $p34^{cdc2}$/B-type cyclins appear to differ at least partly. Rum1 directly binds $p34^{cdc2}$/Cdc13 and inhibits its activity (Correa-Bordes and Nurse, 1995), whereas the primary action of Srw1 to the mitotic kinase seems to be to regulate the level of Cdc13.

In addition to *rum1*$^+$ deletion mutants, cells deficient in *nuc2*$^+$ are phenotypically similar to Δ*srw1* cells. They are sterile and defective in nitrogen starvation-induced G_1 arrest (Kumada *et al.*, 1995). Despite such similarity, *srw1*$^+$ and *nuc2*$^+$ seem to differ fundamentally at least in some functional aspects and therefore in their targets. *srw1*$^+$ inhibits the onset of mitosis whereas *nuc2*$^+$ promotes the metaphase–anaphase transition while inhibiting formation of septum.

Recently we learned that *srw1*$^+$ is identical to *ste9*$^+$ (Kitamura *et al.*, personal communication). The *ste9* mutant was initially isolated a long time ago but has not well been characterized until recently (Sipiczki, 1988).

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