Liz1p, a Novel Fission Yeast Membrane Protein, Is Required for Normal Cell Division When Ribonucleotide Reductase Is Inhibited

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Ribonucleotide reductase activity is required for generating deoxyribonucleotides for DNA replication. *Schizosaccharomyces pombe* cells lacking ribonucleotide reductase activity arrest during S phase of the cell cycle. In a screen for hydroxyurea-sensitive mutants in *S. pombe*, we have identified a gene, $liz1^+$, which when mutated reveals an additional, previously undescribed role for ribonucleotide reductase activity during mitosis. Inactivation of ribonucleotide reductase, by either hydroxyurea or a *cdc22-M45* mutation, causes $liz1^-$ cells in G2 to undergo an aberrant mitosis, resulting in chromosome missegregation and late mitotic arrest. $liz1^+$ encodes a 514-amino acid protein with strong similarity to a family of transmembrane transporters, and localizes to the plasma membrane of the cell. These results reveal an unexpected G2/M function of ribonucleotide reductase and establish that defects in a transmembrane protein can affect cell cycle progression.

INTRODUCTION

Checkpoints are control mechanisms that maintain the order of the cell cycle by ensuring that initiation of certain cell cycle events is dependent on completion of the preceding events (Hartwell and Weinert, 1989). Checkpoints maintain the fidelity of cell proliferation by ensuring that the cell does not replicate or segregate damaged DNA, that the cell does not overreplicate DNA, and that mitosis does not continue in the presence of a damaged spindle (reviewed in Elledge, 1996). The checkpoint that governs entry into mitosis, the G2/M checkpoint, ensures that entry into mitosis is delayed in response to incomplete DNA replication or unrepaired DNA damage. The fission yeast *Schizosaccharomyces pombe* has proved to be a useful genetic system in which to investigate this checkpoint (reviewed in Stewart and Enoch, 1996). In fission yeast, G2/M checkpoint integrity can be assessed by determining whether entry into mitosis occurs either in the presence of hydroxyurea, which blocks DNA replication, or after UV irradiation, which causes DNA damage. Components of the checkpoint mechanism have been identified by finding mutants that show inappropriate entry into mitosis in the presence of one or both of these signals.

Cdc2p is the cyclin-dependent kinase that governs entry into mitosis in *S. pombe* and mediates the checkpoint response (Enoch and Nurse, 1990; reviewed in Sheldrick and Carr, 1993). Cdc2p activity requires binding to a cyclin partner (Cdc13p) and is regulated by phosphorylation. One site of phosphorylation, tyrosine 15, can inhibit Cdc2p activity and is controlled by a set of inactivating kinases (Wee1p and Mik1p) and activating phosphatases (Cdc25p and Pyp3p). Altering the level of this phosphorylation can cause checkpoint defects. For example, overproducing Cdc25p causes DNA replication checkpoint defects (Enoch and Nurse, 1990). A *wee1-50 mik1Δ* double mutant is also checkpoint defective, but cells mutated in $wee1^+$ or $mik1^+$ alone retain their checkpoint function (Sheldrick and Carr, 1993). Mutations in the $cdc2^+$ gene itself, such as in *cdc2-3w,* can cause checkpoint defects, indicating the importance of this kinase in checkpoint regulation (Enoch and Nurse, 1990; Basi and Enoch, 1996).

Analysis of the fission yeast G2/M checkpoint genes has revealed certain differences in the checkpoint sig-

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nals generated by incomplete DNA replication and DNA damage. Although many of the checkpoint mutants are defective in their response to both signals, there are certain mutants that are selectively defective in response to one or the other. Most alleles of the *hus/rad* checkpoint genes, which include *hus1+*, *rad1+*, *rad3+*, *rad9+*, *rad17+*, and *rad26+*, cause cells to be defective in both their checkpoint responses (reviewed in Stewart and Enoch, 1996). In hydroxyurea, these mutants show the "cut" phenotype, in which cells proceed into mitosis in the absence of complete DNA replication, resulting in anucleate cells, or cells with a <1C DNA content. In addition, when exposed to UV irradiation, these mutants fail to arrest their cell cycle, proceeding into mitosis despite DNA damage. In contrast, *cdc2-3w* strains appear to be fully checkpoint defective in response to the DNA replication inhibitor hydroxyurea but show a wild-type checkpoint response to DNA damage (Enoch and Nurse, 1990; Sheldrick and Carr, 1993). Similarly, *cds1−* cells also show hydroxyurea sensitivity but a normal response to UV irradiation. However, *cds1−* mutants may be defective in the S-phase checkpoint, rather than G2/M checkpoint control (Murakami and Okayama, 1995; Lindsay *et al.*, 1998). Conversely, mutants of *chk1+* have a strong checkpoint defect in response to DNA damage and a much less defective checkpoint response to hydroxyurea (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994; Francesconi *et al.*, 1997). Mutants of *rad24+* show a defective DNA damage checkpoint but a normal hydroxyurea response (Ford *et al.*, 1994). Thus, the responses to the two G2/M checkpoint signals are genetically separable, despite the fact that they are transduced by many of the same proteins. This indicates that there is a process by which the cell can discriminate between the two types of defects sensed by the G2/M checkpoint, DNA damage and incomplete DNA replication.

To understand the difference between the responses to the two checkpoint signals, we conducted a screen to identify more mutants like *cdc2-3w* that are defective solely in the incomplete DNA replication checkpoint. The studies in this paper investigate one gene, *liz1+*, identified as a result of this screen, and the role of Liz1p in the regulation of the cell cycle in *S. pombe*. Although cells lacking Liz1p show cuts in hydroxyurea, we show that in fact they are not G2/M checkpoint defective but, rather, have novel cell cycle progression defects in hydroxyurea. Further physiological analysis has revealed that in cells lacking Liz1p, inactivation of ribonucleotide reductase interferes with progression through mitosis as well as S phase. Based on sequence analysis and the localization of Liz1p, *liz1+* is predicted to encode a membrane transport protein. Thus, the *liz1−* phenotypes suggest that ribonucleotide reductase activity may have a role in mitotic processes, and, surprisingly, loss of function

Table 1. *S. pombe* yeast strains and plasmids

Strain	Genotype
TE119	*hus1-14 ura4-294 h+*
TE271	*972 h−*
TE366	*ura4-D18 h−*
TE397	*ade6-M216/ade6-M210 ura4-D18/ura4-D18 leu1-32/ leu1-32 h−/h+*
TE785	*liz1-B44 ade6-M210 leu1-32 ura4-D18*
TE876	*cdc20-M10 leu1-32 ura4-D18 h+*
TE878	*cdc22-M45 leu1-32 ura4-D18 h−*
TE879	*liz1Δ::LEU2 leu1-32 ura4-D18 h−*
TE911	*liz1-B102 ade6-M210 leu1-32 ura4-D18*
TE912	*liz1Δ::LEU2 cdc22-M45 leu1-32 ura4-D18*
TE913	*liz1Δ::LEU2 cdc20-M10 leu1-32 ura4-D18*
TE914	*liz1Δ::LEU2 wee1-50 leu1-32 ura4-D18*
TE915	*liz1Δ::LEU2 leu1-32*
TE958	*liz1Δ::LEU2 cdc25-22 leu1-32 ura4-D18*
TE970	*wee1-50 ura4-D18 h−*

Plasmid		Construct
pTE52	pliz1–4	*liz1+*
pTE665	pliz1Δ	*liz1Δ::LEU2*
pTE666	pREP42-GFPliz1C	*GFP-liz1* (C terminus)
pTE667	pREP42-GFPliz1	*GFP-liz1+*

of a membrane transporter can affect the events of the cell cycle.

MATERIALS AND METHODS

Strains and Media

Standard media and growth conditions were used, and *S. pombe* genetic techniques were performed as previously described (Moreno *et al.*, 1991). The strains and plasmids used in this study are listed in Table 1. The *liz1−* strains used in this study are all marked with *ura4-D18*, with the exception of TE915 (Table 1), because *liz1−* strains accumulate extragenic suppressors when grown with a *ura4+* background. Uracil auxotrophy experiments were performed by streaking strains TE271, TE366, and TE915 on YE5S plates and Edinburgh minimal medium (EMM) plates supplemented with 225 mg/l adenine, histidine, and leucine.

Physiological Methods

All physiological experiments were performed in YE5S. To arrest with hydroxyurea, cultures were grown to an OD_{595} of 0.1–0.2 at 29°C. Hydroxyurea (Sigma, St. Louis, MO) was added from a 200 mM stock to a final concentration of 10 mM. For experiments involving *liz1Δ wee1-50* (TE914), *liz1Δ cdc20-M10* (TE913), and *liz1Δ cdc22-M45* (TE912), cultures were grown at 25°C to an OD_{595} of 0.1–0.2, at which point the cells were shifted to 36°C. For the synchronized cell experiments with *liz1Δcdc25-22* (TE958), cultures were grown at 25°C to an OD_{595} of 0.1, at which point the cultures were shifted to 36°C. After 3 h at 36°C, hydroxyurea was added to the cultures to a final concentration of 10 mM. The cultures were incubated for 1 additional hour at 36°C and then shifted to 25°C to release the arrest.

Cells were prepared for microscopy by heat fixation (J. Creanor, personal communication); 1 ml of cells was spun down, washed once with distilled H_2O, and resuspended in 50 μl of distilled H_2O. Three microliters of the cell suspension were pipetted onto a slide.

The water was allowed to evaporate, and cells were fixed by holding the slide briefly over an open flame. The slide was cooled to room temperature before cell staining. Cell staining with DAPI was performed as described previously (Moreno *et al.*, 1989). Fluorescence microscopy was performed using a Zeiss (Thornwood, NY) Axiophot microscope. To measure cell number, cells were fixed by adding 90-μl aliquots of cells to 10 μl of 37% formaldehyde solution. Two aliquots of each sample of fixed cells were counted using a hemacytometer. To measure radiation sensitivity, 10-μl aliquots of cells were diluted into 10 ml of distilled H_2O. One hundred microliters of the dilution were plated onto YE5S plates. When dry, the plates were irradiated with the indicated UV dose using a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Colonies formed were counted after 3 d. Samples for FACS analysis was prepared as described (Sazer and Sherwood, 1990). FACS analysis was performed with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) and CELLQuest version 3.1f software (Becton Dickinson). For wild-type (TE271) samples, 15,000 events were counted, and for *liz1Δ* (TE879) samples, 25,000 events were counted. FACS data were gated in CELLQuest on a contour plot with a threshold of 0.4%.

Isolation, Sequence Analysis, and Deletion of the *liz1$^+$* Gene

The *liz1$^+$* gene was cloned by transforming TE911 with *S. pombe* genomic DNA libraries pURSP1 and pURSP2 (Barbet *et al.*, 1992). Transformants were screened by replica plating to YE5S-hydroxyurea plates (YE5S plus 10 mM hydroxyurea and 5 μg/ml phloxine B). After 1 d, the plates were rereplica plated to YE5S-hydroxyurea plates, and hydroxyurea-resistant colonies were selected. Plasmid DNA was purified and rescued in *Escherichia coli*. One complementing plasmid, designated p*TE52*, contained a 3.7-kb genomic insert. Integration of the plasmid, Southern blotting, and genetic analysis confirm that this plasmid contains the *liz1$^+$* gene. Deletion analysis reduced the complementing region to a 1.7-kb fragment of genomic DNA. This fragment was sequenced and shown to contain an open reading frame of 1542 bp with no introns. This sequence has been submitted to the GenBank database, accession number AF052688.

p*TE665,* the *liz1Δ* construct, was constructed by subcloning the 3.7-kb *Pst*I–*Kpn*I fragment of p*TE52* into a *Pst*I- and *Kpn*I-digested pUC19 vector in which the *Hin*dIII site had been previously blunted with Klenow. The internal *Eco*RV site of *liz1$^+$* in the resulting plasmid was replaced with a *Hin*dIII site by digestion with *Eco*RV and ligation of a *Hin*dIII linker: 5′-CGAAGCTTCG-3′ and 3′-GCT-TCGAAGC-5′. This construct was digested with *Hin*dIII, and the 1.5-kb *Hin*dIII fragment was replaced with the 2.2-kb *Hin*dIII fragment from p*REP1* (Maundrell, 1990), containing the *S. cerevisiae* LEU2 gene. In the resulting plasmid, p*TE665*, all but 66 bp of the *liz1$^+$* open reading frame have been replaced with the *S. cerevisiae* LEU2 gene fragment, with 1.2 kb of genomic DNA upstream of *liz1$^+$* and 1.0 kb of genomic sequence downstream of *liz1$^+$* remaining. Restriction analysis showed that the LEU2 open reading frame was in the opposite orientation from the original *liz1$^+$* open reading frame.

A *liz1Δ::LEU2* strain was then constructed by one-step gene replacement. The 4.4-kb *liz1Δ::LEU2* fragment was isolated from p*TE665* digested with *Pst*I and *Bam*HI and transformed into TE397. Stable Ade$^+$ Leu$^+$ diploids were isolated and then sporulated. Tetrad dissection revealed that the hydroxyurea-sensitive and Leu$^+$ phenotypes cosegregated and segregated 2:2. A haploid that was hydroxyurea sensitive and Leu$^+$ was selected, and Southern blot analysis confirmed that this strain was deleted for the *liz1$^+$* gene. This strain was back-crossed against wild-type strains three times to produce TE879 and TE915. This strain was also back-crossed against TE911 to confirm that *liz1Δ::LEU2* and *liz1-B102* were allelic.

Construction and Analysis of GFP-liz1

To construct the GFP-Liz1p fusion, we created restriction sites at the 5′ and 3′ ends of the *liz1$^+$* open reading frame. An *Nde*I site was created at the translation initiation site of *liz1$^+$* using PCR mutagenesis, and a *Bam*HI site was created downstream of the open reading frame by cutting at an *Eco*RV site and ligating a *Bam*HI linker. Because *liz1$^+$* also contains an internal *Nde*I site, the *liz1$^+$* gene was cloned into the GFP vector in two steps. First, the plasmid p*TE666* was created by subcloning the 550-kb fragment of *liz1$^+$* (from the *Bam*HI site to the internal *Nde*I site) into vector pREP42-GFPhus1 (a gift from A. Carr, University of Sussex, Brighton, United Kingdom), cut with *Nde*I and *Bam*HI to remove the *hus1$^+$* gene. Then, the 1.1-kb *Nde*I fragment of *liz1$^+$* (from the 5′ *Nde*I site to the internal *Nde*I site) was subcloned into p*TE666* cut with *Nde*I. The resulting plasmids were screened by restriction analysis for clones with the *Nde*I fragment of *liz1$^+$* in the orientation that preserves the *liz1$^+$* open reading frame. The resulting plasmid, p*TE667*, contains the GFP protein fused to the entire 514-amino acid *liz1$^+$* open reading frame, under the control of a moderate strength, thiamine-repressible *nmt1* promoter (pREP42 *nmt1* promoter is described by Basi *et al.*, 1993).

TE879 was transformed with p*TE667*, and Ura$^+$ transformants were selected on EMM plates with 2 μM thiamine. The transformants were replica plated to EMM plates with 10 mM hydroxyurea, both with and without 2 μM thiamine. The transformants showed complementation of the hydroxyurea sensitivity phenotype, both in the presence and absence of thiamine. To assess GFP-Liz1p localization, the transformants were streaked onto EMM plates with 2 μM thiamine and grown for 3 d. These plates were replica plated to EMM plates without thiamine. After 48 h, colonies were picked and individually diluted into 100 μl of distilled H_2O. Three microliters of each dilution were placed on microscope slides for analysis. Fluorescence microscopy was performed using a Zeiss Axiophot microscope.

RESULTS

Isolation of *liz1$^-$* in a Screen for DNA Replication-specific Checkpoint Mutants

We devised a screen (to be described in detail elsewhere) to isolate mutants like *cdc2-3w*, defective solely in their checkpoint response to incomplete DNA replication, and with a wild-type response to DNA damage. We mutagenized wild-type *S. pombe* and then irradiated the cells with 50 J/m^2 UV irradiation. This dose was calculated to eliminate DNA damage checkpoint mutants, while having a minimal effect on wild-type cells. We then screened colonies grown from the surviving cells for mutants that display aberrant mitoses (the cut phenotype) when grown in the presence of the DNA replication inhibitor hydroxyurea but show wild-type resistance to UV irradiation. We screened 55,000 mutagenized colonies and identified eight *liz* (*lives if zapped*) mutants, in seven different genes, with these phenotypes. Among these mutants was an allele of *cdc2$^+$* that shows similar checkpoint-defective phenotypes to the *cdc2-3w* mutant. The isolation of a *cdc2$^-$* allele from the screen establishes that the screen is effective in isolating mutants specifically defective in the response to unreplicated DNA. The other seven *liz* mutants mapped to six different genes, unlinked to other checkpoint genes and to the cell cycle regulators *cdc25$^+$* and *wee1$^+$*. We chose one of these genes, *liz1$^+$*, for further analysis, because it had the strongest phenotypes, and two alleles were isolated in the screen.
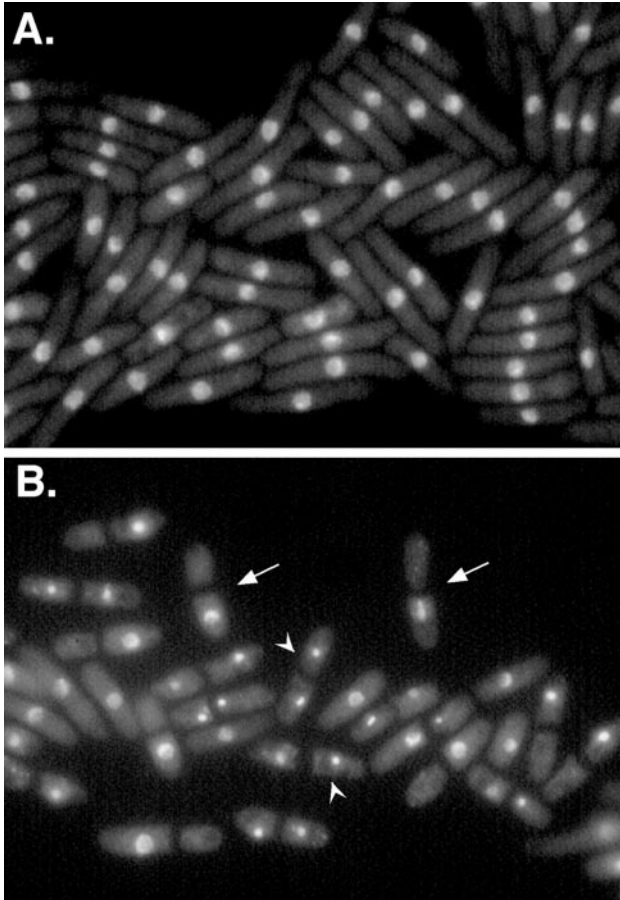
Figure 1. *liz1⁻* mutants show aberrant mitoses in hydroxyurea. Asynchronous cultures of wild-type (TE271) (A) and *liz1Δ* (TE879) (B) cells were incubated in 10 mM hydroxyurea for 6 h, heat fixed, and stained with DAPI. Arrows indicate cells displaying the cut phenotype, and arrowheads indicate cells that have completed nuclear division and arrested with condensed chromosomes and a septum.

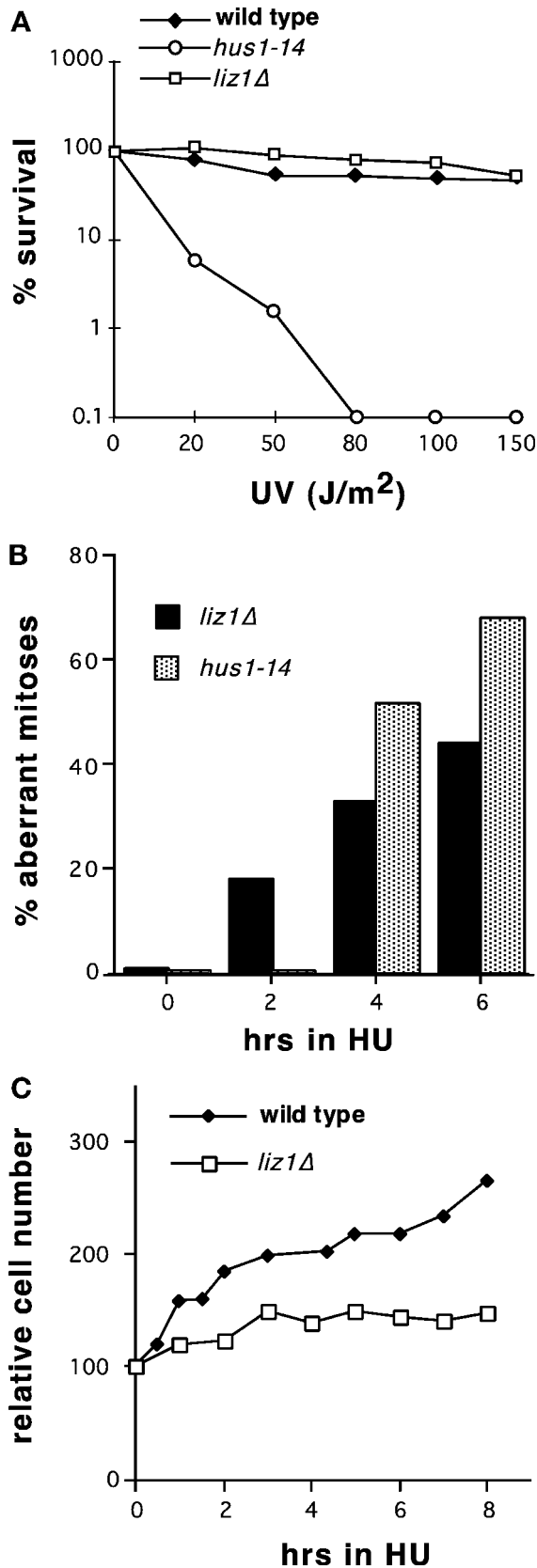### *liz1⁻* Cells Show Defects in Mitotic Progression in Hydroxyurea

Wild-type cells grown in 10 mM hydroxyurea cannot complete DNA replication and arrest in the cell cycle without entering mitosis. Growth continues, however, resulting in highly elongated cells (Figure 1A). In contrast, the addition of 10 mM hydroxyurea to *liz1⁻* cultures results in increased lethality and the cut phenotype, in which a septum forms, dividing the cell, despite the absence of chromosome segregation (Figure 1B, cuts indicated by arrows). These abnormal mitoses result in the formation of anucleate cells, or cells with a partial complement of DNA. This phenotype appears similar to that seen in hydroxyurea-treated G2/M checkpoint mutants, such as *hus1-14* and *cdc2-3w* (Enoch and Nurse, 1990; Enoch *et al.*, 1992).

In addition to displaying the cut phenotype, *liz1⁻* cells appear to undergo another type of aberrant mitotic event. A population of *liz1⁻* cells accumulates, which have completed nuclear division and have formed a septum but continue to have highly condensed chromosomes and a thickened septum and do not appear to undergo cell separation (Figure 1B, indicated by arrowheads). This phenotype is similar to the cell cycle arrest seen in the *S. pombe pim1-d1ᵗˢ* mutant at the restrictive temperature (Sazer and Nurse, 1994), in which the cells are arrested at the point of exit from mitosis.

To assess the nature of the mitotic defects seen in *liz1⁻* cells, the phenotypes of *liz1Δ* cells, in which the *liz1⁺* gene has been deleted (see MATERIALS AND METHODS), were compared with the phenotypes of the checkpoint-defective *hus1-14* mutant (Enoch *et al.*, 1992; Kostrub *et al.*. 1997). *liz1Δ* mutants are phenotypically indistinguishable from the *liz1⁻* alleles isolated in the screen. The *liz1Δ* strain shows wild-type resistance to increasing doses of UV irradiation (Figure 2A), in marked contrast to the high degree of UV sensitivity shown in *hus1-14*. In hydroxyurea, *liz1Δ* cultures form aberrant mitotic cells, which include the cells displaying the cut phenotype and the cells that fail to decondense their chromosomes or undergo cell separation (Figure 2B). Up to 43.9% of *liz1Δ* cells show aberrant mitoses by 6 h in hydroxyurea; 24.3% are cuts, and 19.6% have the condensed chromosome phenotype. For comparison, *hus1-14* cells show up to 68% aberrant mitoses in hydroxyurea, but only cuts are observed.

The Abnormal Mitosis of liz1Δ Cells in Hydroxyurea Occurs with a 2C DNA Content

Detailed analysis of the kinetics of mitosis in hydroxyurea-treated *liz1Δ* mutants reveals that aberrant mitoses appear sooner in *liz1Δ* cultures than in cultures of checkpoint-defective cells. As shown in Figure 2B, after 2 h in hydroxyurea 17.9% of the *liz1Δ* cells display aberrant mitoses, whereas there are none in the *hus1-14* culture. There is a delay in the appearance of aberrant mitoses in *hus1-14* cells, because most cells in an asynchronous culture of *S. pombe* are in the G2 stage of the cell cycle. Thus, *hus1-14* cells undergo a normal first mitosis after the addition of hydroxyurea, because the cells have already completed DNA replication. DNA replication in the following S phase is arrested by hydroxyurea, but because *hus1-14* cells lack a checkpoint, they continue into a second mitosis. In this mitosis, the cell attempts to segregate the unreplicated DNA, resulting in the formation of cuts. Because these cuts do not occur until the second mitosis in hydroxyurea, they are not observed for approximately a generation and a half, or 4 h (Figure 2B). In contrast, because aberrant mitoses are observed in

Figure 2. Phenotypic analysis of the *liz1Δ* mutant. (A) *liz1Δ* cells show wild-type resistance to UV irradiation. The viability of wild-type (TE271), *hus1-14* (TE119), and *liz1Δ* (TE879) was assayed by colony formation after UV irradiation. % Survival is the viability of cells after irradiation expressed relative to the viability of unirradiated cells. (B) *liz1Δ* cells form aberrant mitoses in hydroxyurea. *hus1-14* (TE119) and *liz1Δ* (TE879) cells were fixed and stained with DAPI at times after the addition of 10 mM hydroxyurea. Aberrant mitoses include both cuts and cells with separated, condensed chromosomes. (C) *liz1Δ* cells do not double in cell number in hydroxyurea. Wild-type (TE271) and *liz1Δ* (TE879) cells were fixed and counted at times after addition of 10 mM hydroxyurea. Relative cell number is cell number at each time point expressed relative to cell number at the point when hydroxyurea is added.
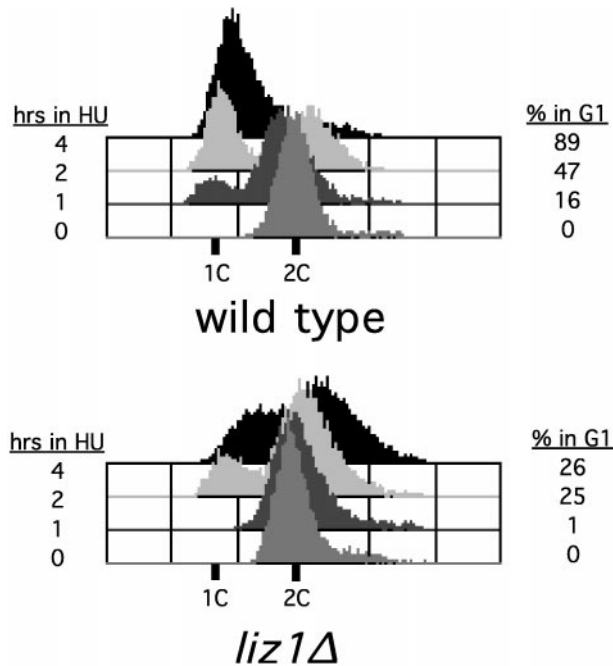
the *liz1Δ* cultures only 2 h after the addition of hydroxyurea, we wondered whether hydroxyurea might interfere with the first, rather than the second, mitosis in *liz1Δ* cells.

To investigate this possibility, we examined the increase in cell number of an asynchronous *liz1Δ* culture in hydroxyurea. Asynchronous wild-type cells in hydroxyurea double in cell number as they proceed through the first mitosis and then arrest in the subsequent S phase (Figure 2C). In contrast, when hydroxyurea is added to a *liz1Δ* culture, the cell number does not double; there is only an increase of ~40%. This indicates that the majority of *liz1Δ* cells are not completing cell division before arresting in hydroxyurea. (In the cell number assay, *liz1Δ* cells showing aberrant mitoses are counted as one cell, because they do not undergo cell separation.) This failure to complete cell division is not due to a growth arrest in hydroxyurea, because the cell mass of the *liz1Δ* culture continues to increase (our unpublished results). The failure of *liz1Δ* cultures to double in cell number in hydroxyurea, together with the early appearance of aberrant mitoses, suggests that in *liz1Δ* cultures hydroxyurea interferes with the first rather than the second mitosis.

If hydroxyurea-treated *liz1Δ* cells are undergoing an abnormal first mitosis, with fully replicated DNA, the culture should consist of cells with a 2C, rather than a 1C, DNA content when aberrant mitoses appear. To determine whether this is the case, FACS was performed on wild-type and *liz1Δ* cells (Figure 3). Like wild-type cells, untreated asynchronous *liz1Δ* cells (0 h) predominantly have a 2C DNA content and are therefore in the G2 phase of the cell cycle. However, after 4 h in hydroxyurea, 89% of wild-type cells have accumulated in G1 with a 1C DNA content, whereas only 26% of *liz1Δ* cells have done so (Figure 3). Therefore, incubation in hydroxyurea causes a large population of *liz1Δ* cells to arrest with a 2C DNA content, confirming that the majority of these cells are arresting in hydroxyurea at the first mitosis. The cells with aberrant mitoses are predicted to be in the population

Figure 3. *liz1*Δ cells maintain a 2C DNA content in hydroxyurea. Wild-type (TE271) and *liz1*Δ (TE879) cells were fixed and stained with propidium iodide, for analysis of DNA content by FACS, at times after the addition of 10 mM hydroxyurea. It should be noted that the apparent upward drift of the 1C and 2C peaks over time is due to a common FACS artifact of both wild-type and mutant *S. pombe* cells. As the cell length increases, the apparent DNA content also appears to slightly increase.

of 2C cells, because they have undergone nuclear division but not cell separation.

Figure 4 schematically compares the hydroxyurea response of *liz1*⁻ cells with that of checkpoint-defective and wild-type cells. Both wild-type and checkpoint-defective cultures proceed through a normal first mitosis when hydroxyurea is added, because most of the cells have already completed DNA replication. In the subsequent S phase, the cells either arrest (wild type) or proceed through an aberrant second mitosis with a 1C DNA content (checkpoint mutants). Unlike both wild-type and checkpoint-defective cells, a percentage of *liz1*Δ cells are unable to progress through the first mitosis in hydroxyurea. Therefore, many *liz1*Δ cells never reach the normal arrest point in DNA replication. From these studies we conclude that a proportion of G2 *liz1*Δ cells treated with hydroxyurea are unable to complete mitosis or anaphase normally. This is unexpected, because hydroxyurea has not been previously shown to affect mitotic events.

liz1⁻ Cells Presynchronized in G2 Show Aberrant Mitoses in Hydroxyurea

The previous studies suggest that hydroxyurea interferes with mitotic processes in *liz1*⁻ cells in G2, with

replicated DNA. However, it is also possible that the abnormal mitoses occur in cells that are still undergoing late DNA replication. Such cells might have an apparent 2C DNA content, because FACS analysis is not sensitive enough to detect small amounts of unreplicated DNA.

To investigate this possibility, we examined the hydroxyurea response of *liz1*⁻ cells presynchronized in G2 using the temperature-sensitive *cdc25-22* mutant. *cdc25-22* harbors a mutation in a gene encoding an essential activator of *cdc2*⁺, which arrests at the G2/M boundary with fully replicated DNA, at the nonpermissive temperature (Nurse *et al.*, 1976; Russell and Nurse, 1986). We constructed a *liz1*Δ*cdc25-22* strain and incubated it at 36°C, the nonpermissive temperature, for 3 h. Hydroxyurea was then added to the culture, and the cells were incubated at 36°C for another hour. At this point, cells were shifted to the permissive temperature without removing hydroxyurea. Cell plate index and mitosis were monitored by fluorescence microscopy in samples taken every 15 min for the next 5 h.

Because of the prolonged G2/M arrest, the synchronized cells should have fully replicated DNA at the time of release. If the reason some *liz1*⁻ cells formed cuts in the asynchronous culture was because of ongoing late DNA replication, in the synchronous culture aberrant mitoses should not be observed in the mitosis after release from the arrest. On the other hand, if hydroxyurea interferes with mitotic processes in *liz1*⁻ cells, we would expect to observe a significant number of aberrant mitoses immediately after release.

Our results suggest that hydroxyurea interferes with mitotic processes in *liz1*⁻ cells. Upon release all the cells in the culture entered mitosis synchronously, and by 210 min 97% of the cells were either postmitotic (71%) (our unpublished results) or showed the typical *liz1*⁻ phenotype (27%). To determine whether the cuts and condensed chromosomes we observed were due to mitotic events, we compared the timing of the appearance of abnormal mitoses with the kinetics of cell plate formation in the cells that were dividing normally. (The cell plate is a structure that forms transiently just before cytokinesis.) As shown in Figure 5, the abnormal mitoses reach a maximum at 100 min, at the same time that the cell plate index of normally dividing cells peaks (Figure 5, compare filled and open squares). These kinetics were identical to the kinetics of cell plate formation in a control *cdc25-22* culture (our unpublished results). We conclude that cuts and condensed chromosomes observed in hydroxyurea-treated *liz1*⁻ cultures are the result of abnormal mitotic events in cells with fully replicated chromosomes.

Hydroxyurea apparently only interferes with mitosis in some of the cells, because only 27% of the cells undergo an abnormal mitosis. We do not know why
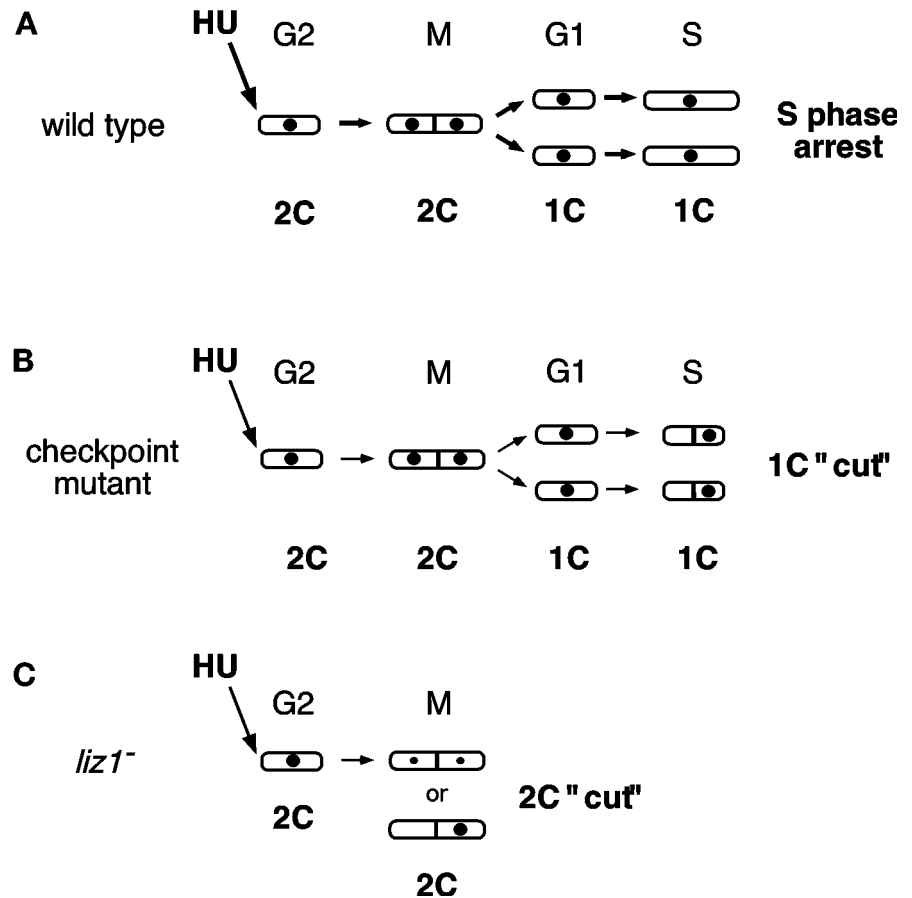
Figure 4. *liz1Δ* cells show a novel arrest point in hydroxyurea. Schematic representation of the differences in cell cycle progression after hydroxyurea is added to wild-type cells, a checkpoint mutant, and *liz1Δ* mutant. (A) Asynchronous wild-type cells have a 2C DNA content and after addition of hydroxyurea proceed through a normal mitosis before arresting with a 1C DNA content. (B) Checkpoint mutants also proceed through a normal mitosis after the addition of hydroxyurea, but in the subsequent cell cycle, they undergo an aberrant mitosis with a 1C DNA content. (C) Although asynchronous *liz1Δ* cells also have a 2C DNA content, addition of hydroxyurea causes aberrant progression through the first mitosis, indicating that hydroxyurea can affect mitotic processes as well as DNA replication in a *liz1Δ* mutant.

the phenotype is only partially penetrant under these conditions. The remaining cells undergo a normal mitosis and then arrest in S phase, because there is no further increase in the number of abnormal mitoses. This normal arrest argues that *liz1⁻* cells have an intact G2/M checkpoint, because we would expect checkpoint-defective cells to continue to accumulate aberrant mitoses during the 5-h incubation in hydroxyurea. To confirm this point, *liz1⁻* cells in G2 were collected by centrifugal elutriation and then allowed to proceed into G1 (60 min) before treatment with hydroxyurea. Under these conditions, all of the cells stopped dividing and arrested with a 1C DNA content, and no aberrant mitoses were observed (our unpublished results), arguing that *liz1⁻* does not alter the checkpoint response to unreplicated DNA.

liz1⁺ Shows Similarity to a Family of Transmembrane Transporters

The *liz1⁺* gene was cloned from a genomic library by functional complementation of the hydroxyurea-sensitive phenotype of *liz1⁻* cells (see MATERIALS AND METHODS). *liz1⁺* was sequenced and found to encode a 514-amino acid protein, which surprisingly shows similarity to a family of putative and known transmembrane transporters from yeast and bacteria (Figure 6). The most similar sequences to Liz1p are the open reading frames of *YCR8*, *SEO1*, and *YG28* from *S. cerevisiae* (accession numbers P25621, P39709, and P53241). All three open reading frames encode proteins of unknown functions but are predicted to be transmembrane transporters from sequence similarity. *YCR8* appears to be a *liz1⁺* homologue in *S. cerevisiae*. It has a high degree of both identity (37.8%) and similarity (61.2%) with *liz1⁺* and is very similar in length. However, a deletion of *YCR8* (a gift from Giovanna Lucchini, Universita degli Studi di Milano, Milano, Italy) does not show hydroxyurea sensitivity (our unpublished results).

In addition, several transmembrane transporters with known functions, from yeast and bacteria, also show similarity to Liz1p. The three that show the greatest amount of similarity are TtuBp, a tartrate transporter from *Agrobacterium vitis* (Salomone *et al.*, 1996), Pht1p, a phthalate transporter from *Pseudomonas putida* (Nomura *et al.*, 1992), and Dal5p, the allantoate transporter from *S. cerevisiae* (Rai *et al.*, 1988) (Figure 6). The substrates of these transporters, and of
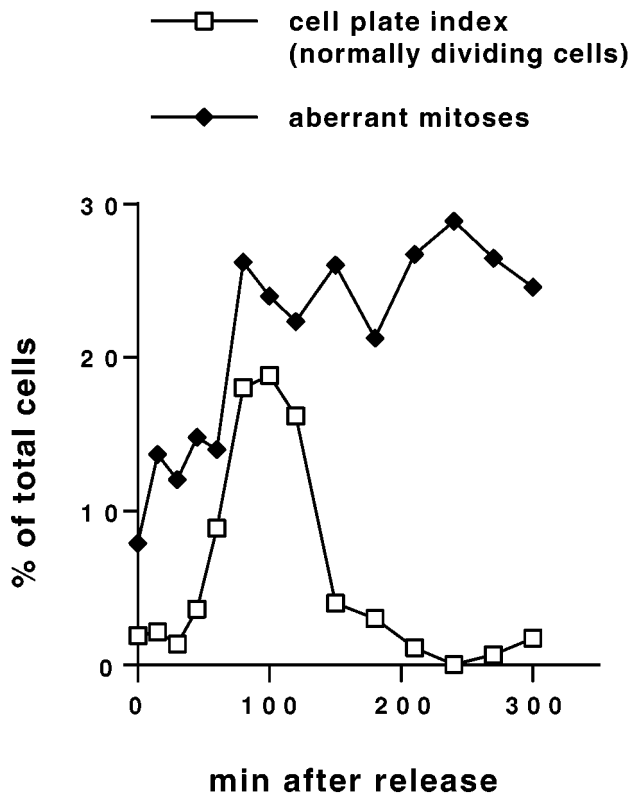
Figure 5. *liz1Δcdc25-22* cells show aberrant mitoses in hydroxyurea when presynchronized at G2/M. *liz1Δcdc25–22* (TE958) cells were arrested at G2/M at 36°C for 3 h. Hydroxyurea was added to 10 mM, and the cells were incubated at 36°C for another hour and then released at 25°C. Cells were fixed and stained with DAPI at the indicated times after release. Aberrant mitoses and cell plate index were quantified at each time point. The cell plate index includes only normal cells that have completed nuclear division and display a septum. Aberrant mitoses include cells with missegregated chromosomes, and with separated, condensed chromosomes. Ninety-seven percent of the cells in the culture completed mitosis by 210 min (our unpublished results).

other transporters with less similarity to Liz1p, all are small acidic metabolites with one or more carboxylic acid groups. The sequence similarity of Liz1p to these proteins suggests that Liz1p may share a function with this family of transmembrane transporters.

Liz1p Localizes to the Plasma Membrane of S. pombe

If Liz1p does encode a transporter, we would expect Liz1p to be localized to a cellular membrane, such as the plasma membrane or the nuclear membrane. GFP fusion proteins have previously been used in fission yeast to examine the localization of proteins (Sawin and Nurse, 1996). We created a construct in which GFP was fused to the N terminus of the Liz1p protein (see MATERIALS AND METHODS). This fusion protein was expressed from a plasmid under the control of the *S. pombe nmt1* promoter, which is largely repressed in the presence of thiamine (Maundrell, 1990). The GFP-Liz1p fusion protein is functional as it complements the hydroxyurea sensitivity of *liz1⁻* mutants both in the presence and absence of thiamine (our unpublished results), in contrast to a plasmid expressing the GFP protein alone.

Fluorescence microscopy reveals the GFP-Liz1p fusion localizes in the plasma membrane of the cells and may also be in the cytoplasm but is clearly not in the nucleus or nuclear membrane (Figure 7). When the *nmt1* promoter is derepressed, the GFP-Liz1p protein first appears at the cell ends, as has been observed with other *S. pombe* plasma membrane proteins (Dibrov *et al.*, 1997). The GFP-Liz1p protein also appears to localize to the septa of dividing cells. The localization of Liz1p is consistent with the predicted role of Liz1p as a transport protein.
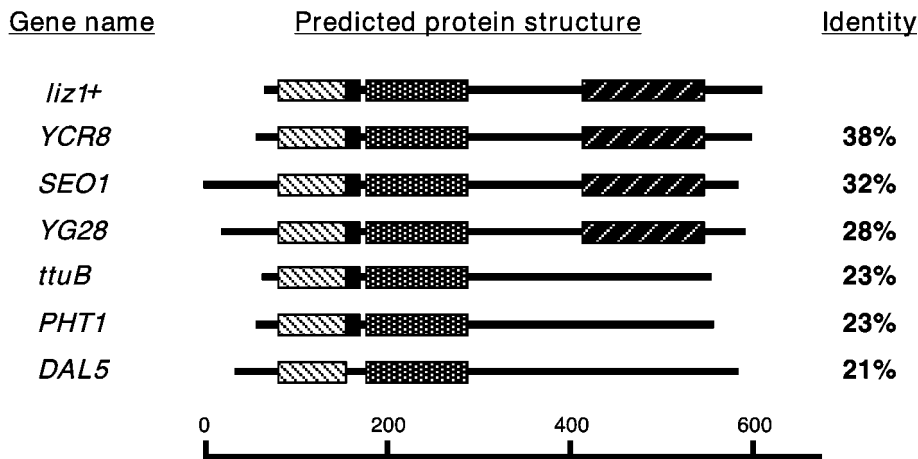

Figure 6. Liz1p shows similarity to a family of transmembrane transporters. Schematic representation of Liz1p and related proteins; the name of the gene is shown on the left, and the percent of amino acids identical to Liz1p is shown on the right. The scale below indicates length in amino acids. The length of the predicted protein sequence is shown by the black line. Shaded boxes indicate regions of high similarity. The proteins were arranged by aligning regions of highest similarity, using MACAW software (National Center for Biotechnology Information, Bethesda, MD). The GenBank accession number for *liz1⁺* is AF052688.
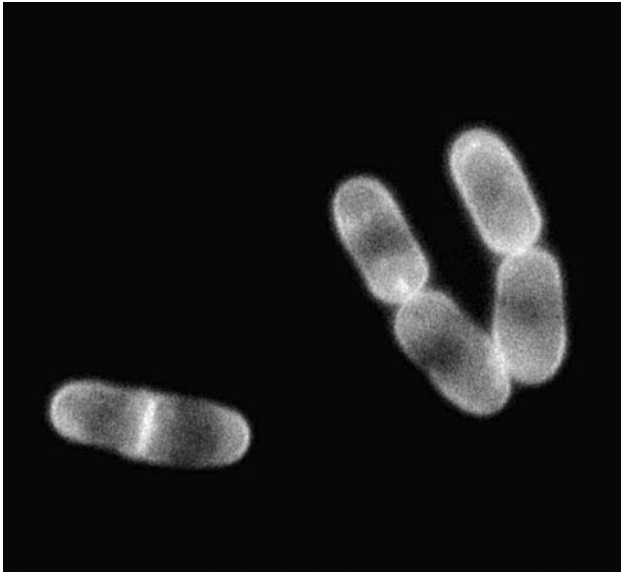
Figure 7. GFP-Liz1p localizes predominantly to the plasma membrane of *S. pombe* and is excluded from the nucleus. *liz1Δ* (TE879) cells containing p*TE667* were examined microscopically after 48 h on EMM media lacking thiamine. Bright staining is seen in the plasma membrane and septum, with some cytoplasmic staining. GFP-Liz1p appears to be excluded from the nucleus.

liz1Δ Mitotic Defects Are Due to Inhibition of Ribonucleotide Reductase

The similarity of *liz1+* to known transporters suggested that the *liz1−* phenotype could be due to abnormal transport of hydroxyurea. To exclude this possibility, we examined the phenotype of a *liz1Δ cdc22-M45* double mutant. *cdc22-M45* is a temperature-sensitive mutation in the gene encoding the large subunit of ribonucleotide reductase, which is the target of hydroxyurea (Fernandez Sarabia *et al.*, 1993). At 36°C, *cdc22-M45* cells arrest in S phase because of insufficient nucleotides for DNA replication. Shifting the strain to the restrictive temperature thus provides an alternative means of inactivating ribonucleotide reductase that is not dependent on transport of hydroxyurea.

As shown in Figure 8, *liz1Δ cdc22-M45* cells show aberrant mitoses, similar to the *liz1Δ* phenotype in hydroxyurea, 6 h after the shift to the nonpermissive temperature (Figure 8A). The aberrant mitotic phenotype in *liz1Δ cdc22-M45* cells is less dramatic than in *liz1Δ* cells in hydroxyurea; there are quantitatively fewer aberrant mitoses (Figure 8B), and the chromosomes appear to be less condensed. However, *liz1Δ cdc22-M45* cells show significantly more aberrant mitoses than either *liz1Δ* or *cdc22-M45* cells at 36°C (Figure 8B), indicating that this synthetic phenotype is due to a requirement for ribonucleotide reductase function in *liz1Δ* cells.
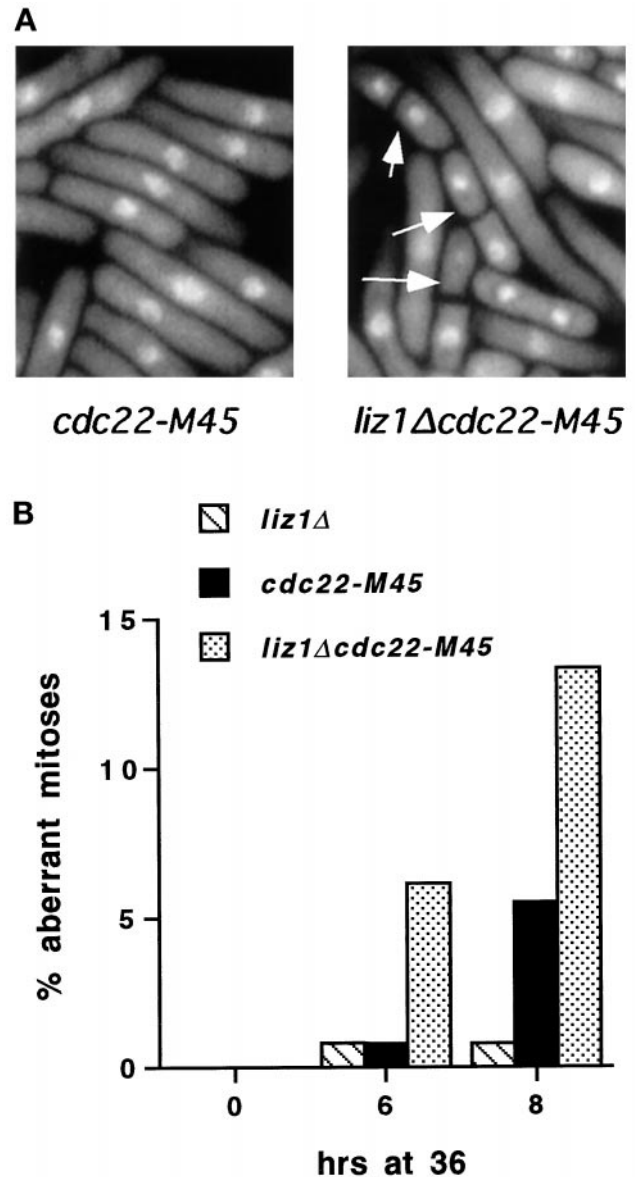

Figure 8. Temperature sensitivity of ribonucleotide reductase also causes abnormal mitoses in *liz1−* cells. *cdc22-M45* (TE878) and *liz1Δ cdc22-M45* (TE912) cells were incubated at 36°C for 6 hr, heat fixed, and stained with DAPI. (A) *liz1Δcdc22-M45* cells display aberrant mitotic phenotypes. Arrows indicate cells displaying aberrant mitotic phenotypes. (B) The *liz1Δcdc22-M45* double mutant displays more aberrant mitoses than either single mutant. Aberrant mitoses include cells with missegregated chromosomes or separated, condensed chromosomes and a septum.

The quantitative difference between *liz1Δ cdc22-M45* cells and *liz1Δ* cells in hydroxyurea may be due to incomplete or delayed deactivation of the *cdc22-M45* allele at the nonpermissive temperature. Indeed, FACS analysis of a *cdc22-M45* culture indicates that this is a "leaky" allele (Enoch, unpublished results). If

Cdc22p is not rapidly deactivated upon shift to the nonpermissive temperature, the *liz1Δ cdc22-M45* cells may have sufficient ribonucleotide reductase activity to complete a normal mitosis. The cells would then arrest in the following G1 phase, being unable to complete DNA replication. This would account for a smaller percentage of cells showing the mitotic arrest phenotype. The difference in the appearance of the cells (the chromosomes appear to be less condensed) may be due to the difference in the growth temperature (36 vs. 29°C). At 36°C, these cells may be less able to maintain the highly condensed chromosomes. The appearance of aberrant mitoses in *liz1Δ cdc22-M45* mutants establishes that the mitotic defect in *liz1Δ* mutants is a consequence of the inactivation of ribonucleotide reductase. Because *liz1Δ cdc22-M45* cells show aberrant mitoses in the absence of hydroxyurea, this phenotype is not dependent on altered transport of hydroxyurea.

In addition, we examined the phenotype of *liz1Δ* in combination with another mutation that affects DNA replication, *cdc20-M10. cdc20-M10* is a temperature-sensitive mutation in DNA polymerase ϵ (D'Urso and Nurse, 1997). *cdc20-M10* mutants also arrest in S phase because of an inability to fully replicate DNA. *liz1Δ cdc20-M10* double mutants do not show the mitotic defects seen in *liz1Δ cdc22-M45* mutants (our unpublished results). This indicates that the mitotic defects seen in *liz1Δ* mutants are specifically seen only when ribonucleotide reductase is inhibited and do not occur in other conditions in which DNA replication is arrested.

Additional Phenotypes of liz1Δ

To further investigate the role of Liz1p in the fission yeast mitotic cycle, we examined the phenotype of *liz1Δ wee1-50* double mutants. Wee1p is a kinase, which phosphorylates the cyclin-dependent kinase Cdc2p on tyrosine 15, inhibiting its activity and consequently blocking entry into mitosis (Russell and Nurse, 1987). At the nonpermissive temperature, *wee1-50* mutants are viable but have a short G2. They therefore accelerate entry into mitosis and divide at a small size. Although Wee1p is not essential in wild-type cells, it is essential in cells lacking other cell cycle control functions. For example, *cdc2-3w wee1-50*, *chk1::ura4 wee1-50*, and *rum1Δ wee1-50* are all inviable, under restrictive conditions (Russell and Nurse, 1987; Walworth *et al.*, 1993; Moreno and Nurse, 1994). Such double mutants display a characteristic "mitotic catastrophe phenotype" under restrictive conditions. Many of the genes that are lethal with mutations in *wee1+* are known to be lacking negative regulation of cell cycle control, such as checkpoint controls.

As shown in Figure 9A, *liz1Δ* mutants resemble cells lacking negative regulators in this respect. The *liz1Δ*
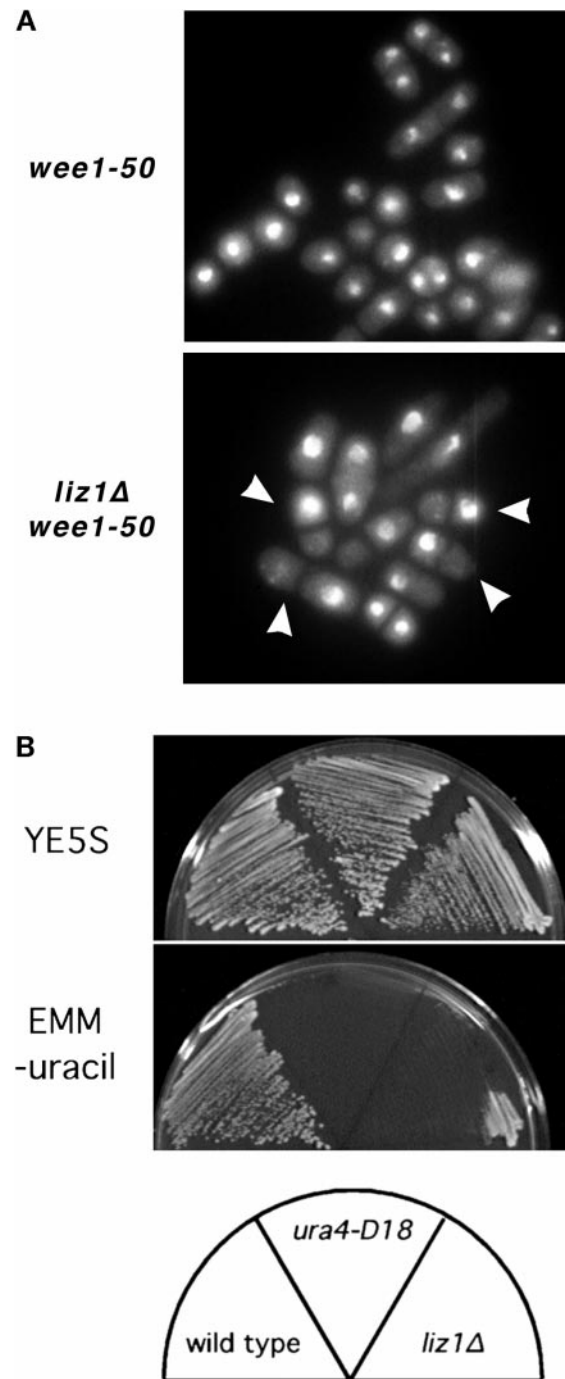

Figure 9. Additional phenotypes of *liz1Δ* cells. (A) *liz1Δ wee1-50* double mutants show aberrant mitotic progression at 36°C. *wee1-50* (TE970) and *liz1Δ wee1-50* (TE914) cells were incubated at 36°C for 6 hr, heat fixed, and stained with DAPI. Arrows indicate cells displaying aberrant mitotic phenotypes. There is a cell not in focus in the lower right corner of the *wee1-50* panel. (B) *liz1Δ* cells show a partial auxotrophy for uracil. Wild-type (TE271), *ura4-D18* (TE366), and *liz1Δ* (TE915) strains were streaked on EMM plates supplemented with 225 mg/l adenine, histidine, and leucine, and YE5S plates. They were incubated for 3 d at 32°C and assessed for growth. *liz1Δ* (TE915) strains eventually form colonies of standard size on EMM at ~7 d.

wee1-50 double mutants are not viable at 36°C, the nonpermissive temperature for *wee1-50* (our unpublished results). Microscopic examination of the double mutants at the nonpermissive temperature shows that the double mutants display aberrant mitotic phenotypes, including the cut phenotype (Figure 9A). This is similar to the phenotype typical of checkpoint mutants and reveals a requirement for Liz1p function to prevent premature mitosis when the cells are accelerated in the cell cycle by the *wee1-50* mutation. No cuts are observed in *wee1-50* cells under the same conditions.

We also noticed fortuitously that *liz1⁻* cells are partial uracil auxotrophs. *liz1⁻* mutants grow much more slowly than wild-type cells on media lacking uracil, but *liz1⁻* and wild-type cells form colonies with similar kinetics on rich media (Figure 9B). No auxotrophy is seen for adenine, histidine, or leucine. Because Liz1p shows sequence similarity to transmembrane transporters, Liz1p may transport a precursor in the uracil biosynthesis pathway.

DISCUSSION

A Role for Ribonucleotide Reductase in Mitosis in *liz1⁻* Cells

Ribonucleotide reductase is an enzyme that reduces ribonucleotides to deoxyribonucleotides (dNTPs). Because dNTPs are required for DNA synthesis, inhibition of ribonucleotide reductase blocks DNA replication, leading to cell cycle arrest in early S phase (reviewed in Elledge *et al.*, 1992). Here we report that ribonucleotide reductase is also required for normal mitosis in the absence of Liz1p function. Treatment of asynchronous cultures of *liz1⁻* cells with hydroxyurea, an inhibitor of ribonucleotide reductase, results in abnormal mitotic events, including missegregation and mitotic arrest with highly condensed chromosomes (Figure 1). Similar mitotic abnormalities are also observed when ribonucleotide reductase is inactivated by mutation in *liz1⁻* cells (Figure 8).

Although the morphology of *liz1⁻* cells in hydroxyurea resembles the morphology of checkpoint-defective cells, the underlying defect is completely different. Checkpoint-defective cells undergo mitosis before DNA is completely replicated. This creates cytological abnormalities because there is not enough genetic material for each of the daughters, and because structures required for chromosome segregation such as kinetochores may not have assembled correctly. In contrast, the mitotic abnormalities in *liz1⁻* cells occur when G2 cells with fully replicated DNA lose ribonucleotide reductase activity. This was established by measuring the DNA content of *liz1⁻* cells arrested in mitosis by hydroxyurea (Figure 3) and by showing that hydroxyurea induces aberrant mitoses

in *liz1⁻* cells that have been presynchronized in G2/M (Figure 5). *liz1⁻* cells apparently have a normal checkpoint response to unreplicated DNA, because cells presynchronized in G1 arrest normally in S phase when treated with hydroxyurea (our unpublished results).

Mutations in a Putative Transmembrane Transporter Disrupt the Cell Division Cycle

We believe that *liz1⁺* is likely to encode a transmembrane transporter because of its high degree of sequence similarity to several known and predicted transmembrane transporters (Figure 6). The predominant localization of the GFP-Liz1p protein to the plasma membrane of cells is consistent with this hypothesis (Figure 7). If Liz1p shows functional as well as structural conservation with the known transporters, Liz1p may function to transport a small acidic metabolite with at least one carboxylic acid group. Discovering a potential transporter with a role in cell cycle progression was unexpected.

Because Liz1p shows significant sequence similarity to transporters, an initial concern was that the defects seen in *liz1⁻* mutants in hydroxyurea were due to changes in hydroxyurea transport. Multiple lines of evidence suggest that this is not the case. Unlike the substrates of transporters similar to Liz1p, hydroxyurea is not acidic and does not have any carboxylic acid groups. *liz1⁻* mutants are not unusually sensitive to hydroxyurea; dose–response curves show that *liz1⁻* cells do not show altered response to lower concentrations of hydroxyurea (our unpublished results). Most importantly, the same defects seen in *liz1⁻* cells in hydroxyurea can also be observed when ribonucleotide reductase is inactivated by a temperature-sensitive mutation, in the absence of hydroxyurea (Figure 8). Interestingly, *liz1⁻* mutants also show cell cycle defects in combination with mutations in the cell cycle regulator *wee1⁺* with no hydroxyurea present (Figure 9A). Therefore, although the *liz1⁻* cell cycle defects may be caused by altered transport, it is unlikely to be hydroxyurea transport.

A schematic overview of our findings is shown in Figure 10. We have found that combining mutations in Liz1p, a putative transporter, with treatments that inhibit ribonucleotide reductase, causes abnormal mitoses. We speculate that Liz1p transports a molecule designated X, which functions in concert with ribonucleotide reductase during mitosis. If one of these activities is absent, such as in a *liz1⁻* mutant or in a *cdc22⁻* mutant, mitotic progression may occur normally because of the other remaining activity. However, in either *liz1⁻* mutants in hydroxyurea or *liz1Δcdc22-M45* double mutants, the loss of both activities simultaneously results in the mitotic defects of missegregation and mitotic arrest.
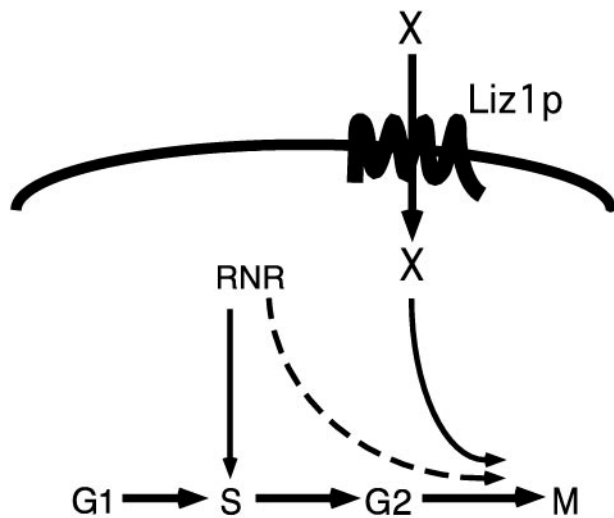
Figure 10. Model for Liz1p function with ribonucleotide reductase in mitosis. Schematic representation of a model for Liz1p function in mitosis. We propose that Liz1p transports a molecule, designated X, with a role in mitotic processes. Although ribonucleotide reductase (RNR) activity has its main role during S phase, during mitosis it may be required in conjunction with X for a normal mitosis to occur. If cells are depleted solely for either Liz1p function or RNR activity, normal mitosis can still occur, but the absence of both functions compromises mitosis.

Could Liz1p Be Transporting a Metabolite Required for Uracil Biosynthesis?

If Liz1p is a transporter, what is its substrate, and how does this affect cell cycle progression? One clue may come from the partial auxotrophy for uracil seen in *liz1⁻* mutants (Figure 10). The slow growth of *liz1⁻* mutants in media lacking uracil may indicate that *liz1⁻* mutants cannot transport a molecule required for efficient uracil biosynthesis. Many of the molecules in the uracil biosynthesis pathway, such as orotic acid and ureidosuccinate (Denis-Duphil, 1989), resemble the substrates of membrane proteins with similarity to Liz1p. Indeed, one of the transporters related to Liz1p is *DAL5*, the allantoate transporter from *S. cerevisiae*, which also has been shown to transport ureidosuccinate (Turoscy and Cooper, 1987). Possibly, Liz1p is involved in the transport of ureidosuccinate or another similar molecule in the uracil biosynthesis pathway. Inefficient transport of such an intermediate could make de novo uracil biosynthesis less efficient in *liz1⁻* mutants, leading to a partial uracil auxotrophy.

Because ribonucleotide reductase is known to be necessary to maintain deoxyribonucleotide pools, and Liz1p may be involved in some aspect of nucleotide metabolism, it is possible that ribonucleotide reductase and Liz1p function together to maintain sufficient nucleotide levels during mitosis. Our results thus hint at an unexpected dependence of mitosis on normal deoxynucleotide pools.

Our results also indicate that mutation of a putative membrane transporter can affect cell cycle progression. Whole genome sequencing and analysis of *S. cerevisiae* has predicted that 186 of the 5885 open reading frames encode transmembrane permeases (Nelissen *et al.*, 1997). Sixty-six of these putative transporters have unidentified functions, and are classified on the basis of similarity to known transporters (Mewes *et al.*, 1998). Our studies predict that these proteins could turn out to play roles in many aspects of cellular metabolism that are not obviously dependent on membrane transport.

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