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Mutations in genes of *Saccharomyces cerevisiae* encoding pre-mRNA splicing factors cause cell cycle arrest through activation of the spindle checkpoint

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ABSTRACT

Previous work has identified a group of genes whose products play important roles in two seemingly unrelated processes: cell cycle progression and splicing. The products of these genes show a network of physical and genetic interactions suggestive of the existence of a protein complex, the cell cycle and splicing complex (CSC). Here we analyze the genetic interactions between *ISY1*, *SYF2* and *NTC20*, three non-essential components of the CSC. We show that mutations in *ISY1* cause lethality in the absence of Ntc20p, and that the double mutant *isy1*Δ *syf2*Δ shows a temperature-dependent cell cycle arrest. This arrest is due to lower levels of α-tubulin, a protein encoded by *TUB1* and *TUB3*, two intron-containing genes. We show that the low levels of α-tubulin in *isy1*Δ *syf2*Δ trigger activation of the spindle checkpoint, causing cell cycle arrest. Thus, our results have uncovered an unexpected role for pre-mRNA splicing in the maintenance of the fidelity of chromosome transmission during cell division.

INTRODUCTION

The eukaryotic cell cycle is a collection of ordered events in which the initiation of late processes depends upon the completion of early ones. Coordination of the timing and order of these events is critical for the high-fidelity transmission of genetic information. Work in several laboratories has demonstrated that this coordination is genetically controlled by surveillance mechanisms, termed checkpoints. Checkpoints are pathways that affect cell cycle progression in response to genotoxic agents or alteration of particular structures such as the spindle (reviewed in 1,2).

Removal of introns from pre-mRNA is an important component of the regulation of gene expression in eukaryotes. Pre-mRNA splicing requires a large number of *trans*-acting factors, which together constitute the spliceosome (3,4). Biochemical studies have demonstrated that the spliceosome consists of a large number of specific proteins associated with functional small nuclear ribonucleoprotein particles (snRNPs) composed of small nuclear RNA molecules (U1, U2, U4, U5 and U6 snRNA). In addition, a large number of non-snRNP proteins are either transiently associated with, or constitute an integral part of, the spliceosome (3). Genetic screens have identified many proteins involved in splicing in the yeast *Saccharomyces cerevisiae* (5).

The *CDC40/PRP17* gene encodes a protein that plays a role during the second step of the splicing reaction. Mutations in this gene result in accumulation of splicing intermediates (6). This gene was also independently found to function in the mitotic and meiotic cell cycles. At the restrictive temperature, *cdc40/prp17* strains arrest as large-budded cells with one undivided nucleus containing 2N content of DNA (7,8). Thus, the *CDC40/PRP17* gene is involved in two seemingly unrelated processes: pre-mRNA splicing and progression through the G_2/M phases of the cell cycle. For simplicity, we will refer to the gene as *CDC40*.

Previously, we have implemented a screen to identify mutants that are synthetically lethal in the absence of Cdc40p (9,10). This screen identified several genes (*SYF*, synthetic lethal with forty). When some of the *SYF* genes were used as baits in two-hybrid screens, we uncovered a complex network of interactions between a group of splicing factors (including Prp8, Slu7, Prp22, Slt11, Syf1, Syf2, Syf3/Clf1, Cef1, Isy1 and Ntc20). Most of these splicing factors are involved in cell cycle progression and, when mutated, cells arrest at the G_2/M phase of the cell cycle (9,10). Based on these results, we have suggested the existence of a protein complex [cell cycle and splicing complex (CSC)] that plays a role in both splicing and S.cerevisiae and *Schizosaccharomyces pombe* has confirmed the existence of protein complexes containing several of the CSC proteins (11,12).

In this paper, we investigate the genetic interactions between three non-essential components of the CSC, and we report the characterization of the double mutant *isy1*Δ *syf2*Δ. Our results demonstrate that the double mutant is temperature sensitive and arrests in the cell cycle. Moreover, we show that the G_2/M arrest phenotype observed in this mutant is due to the low levels of α-tubulin protein encoded by *TUB1* and *TUB3*, two intron-containing genes. The low levels of α-tubulin activate the spindle checkpoint, leading to cell cycle arrest. Our results point at an unexpected role played by pre-mRNA splicing in guarding the stability of the genome during cell division.

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MATERIALS AND METHODS

Strains, media, growth conditions and general procedures

The *S.cerevisiae* strains used in the present work are as follows. YR4, *MAT***a** *ade2-1 his3-RS21 leu2Δ1 lys2-801 trp1Δ1 ura3-52 can1-100.* YOD84, *MAT***a**/*MATα CDC40/cdc40Δ::URA3 SYF2/syf2Δ::TRP1 ade2-1/ade2-101 his3-RS21/his3-RS21 leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-52.* YOD19, *MAT***a**/*MATα CDC40/cdc40Δ::LEU2 ISY1/isy1Δ::HIS3 ade2-1/ade2-1 his3-RS21/his3Δ200 leu2Δ1/leu2-3,112 LYS2/lys2-801 trp1Δ1/trp1Δ1 ura3-52/ura3-1 CAN1/can1-100.* YOD7, *MAT***a**/*MATα ISY1/isy1::HIS3 SYF2/syf2::TRP1 ADE2/ade2-1 his3Δ200/his3-RS21 leu2-3,112/leu2Δ1 LYS2/lys2-801 trp1Δ1/trp1Δ1 ura3-1/ura3-52 CAN1/can1-100.* YOD15, *MATα isy1::HIS3 ade2-1 his3 leu2 lys2-801 trp1Δ1 ura3.* YOD18, *MAT***a** *syf2::TRP1 ade2-1 his3 leu2 lys2-801 trp1Δ1 ura3.* YOD13, *MAT***a** *isy1::HIS3 syf2::TRP1 ADE2 his3 leu2 lys2-801 trp1Δ1 ura3.* YOD43, *cdc40::URA3 ntc20::KanMX6 ade2-101 his3-RS21 leu2Δ1 lys2-801 trp1Δ1 ura3-52 can1-100.* YOD44, *MAT***a** *syf2::TRP1 ntc20::KanMX6 ade2-1 his3 leu2 lys2-801 trp1Δ1 ura3.* YOD78, *MAT***a**/*MATα SYF2/syf2::TRP1 ISY1/isy1::HIS3 NTC20/ntc20::kanMX6 ade2-1/ade2-1 his3/his3 leu2/leu2 lys2-801/lys2-801 trp1Δ1/trp1Δ1 ura3/ura3.* YOD96, *MAT***a** *isy1::HIS3 syf2::TRP1 mad1::KanMX6 bub2::KanMX6 his3 leu2 ura3.* YOD97, *MAT***a** *mad1::KanMX6 bub2::KanMX6 his3 leu2 ura3*. The disruption of the coding sequence of *ISY1* with *HIS3*, of *CDC40* with either *URA3* or *LEU2* and the replacement of *SYF2* with *TRP1* have been described (9,10,13). Disruption of the entire coding sequence of *NTC20* with KanMX6 was carried out using *NTC20*-specific primers according to the method described (14).

Yeast cells were grown in YEPD (1% yeast extract, 2% Bacto peptone, 2% dextrose) or SD media (0.67% yeast nitrogen base, 2% dextrose and the appropriate nutrients added). In order to induce expression from the *GAL* promoter, cells were grown on YEPGal plates (1% yeast extract, 2% Bacto peptone, 2% galactose), or SGal media (0.67% yeast nitrogen base, 2% galactose and the appropriate nutrients added). Bacto Agar (1.8%) was added for solid media. Selective media lacking one nutrient are designated SD-nutrient (e.g. SD-Ura). Ura$^-$ colonies were selected on SD complete medium with uracil (50 mg/l) and 5-fluoroorotic acid (5-FOA, 0.8 g/l). Sporulation was carried out in SPO medium (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose).

Nocodazole (Sigma, St Louis, MO) was diluted in YEPD or YEPGal agar at the concentrations indicated from a stock solution of 5 mg/ml in dimethyl sulfoxide. Standard molecular biology procedures such as restriction enzyme and Southern blot analysis were carried out as described (15). Yeast media and molecular biology procedures (transformations, DNA preparations, etc.) were done as described (16).

Cell synchronization

Exponentially growing cells were arrested in G_1 by incubation in the presence of 4 μM α-factor (Sigma, St Louis, MO) for 3 h at 25°C. In order to release cells from G_1 arrest, cells were washed three times and resuspended in fresh YEPD.

Microscopic and flow cytometry analysis

To visualize nuclei, cells were fixed with 70% methanol overnight at 4°C, washed twice with phosphate-buffered saline (PBS), and resuspended in 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) in PBS. To determine nuclear position, the distance from the edge of the nucleus to the bud neck was measured for each budded cell, as well as the diameter of the mother cell. The ratio between these two distances was calculated. A ratio of 0.4 or larger was considered to be indicative of nuclear localization failure. Most wild-type large-budded cells exhibited a ratio of 0.25 or lower. At least 200 cells were analyzed for each strain.

Anti-tubulin antibody staining was performed as described (16). For α-tubulin staining we used YOL1/34 antibodies (Harlan Sera-lab Ltd). The secondary antibody was FITC-conjugated anti-rat IgG (Jackson Immunoresearch Laboratories, Inc.). Flow cytometry analysis was carried out as described (10). Cells were classified as having long spindles if the stained spindle encompassed more than two-thirds of the combined length of mother and daughter cells.

Screening for temperature sensitivity suppressors

YOD13 cells (*isy1Δ syf2Δ*) were transformed with a cDNA expression library, regulated by the *GAL1* promoter on the *URA3-CEN* vector pRS316 (17). The transformation mixture was plated on SGal-Ura plates at 25°C. After 12 h, cells were shifted to 35°C for 3–4 days until the appearance of colonies. Colonies were then streaked on YEPGal plates and replicated on YEPGal and 5-FOA plates at 25 and 35°C. Colonies that were able to grow on YEPGal plates at 25 and 35°C and on 5-FOA plates at 25°C but not at 35°C were further analyzed. Plasmids were extracted, re-checked for their ability to support growth at 35°C and then their cDNA insert was sequenced.

Microcolony formation assays

Exponentially growing cultures were grown at 25°C, transferred to 37°C for 2.5 h, briefly sonicated and streaked on a YEPD plate. Cells were picked using a dissection needle and placed at marked spots on the plates, which were incubated at 37°C. The number of cells in each microcolony was counted at different time points. For assessing growth in the presence of nocodazole, cells were picked and placed on YEPD plates containing 7.5 μg/ml nocodazole.

Western blot analysis

For immunoblot analysis, protein extracts were made as follows: 5×10^8 cells were harvested and the pellets were washed and resuspended in lysis buffer [20% glycerol, 20 mM HEPES (pH 7.9), 20 mM NaCl, 5 mM EDTA (pH 8), 1 mM DTT]. After the addition of protease inhibitors (Complete™; Boehringer Mannheim, Mannheim, Germany) and glass beads, cells were disrupted by vortexing. Lysates were clarified by microcentrifugation. Proteins were fractionated by SDS–PAGE and detected using anti-α-tubulin antibody (YOL1/34) or anti-Vma5p, followed by peroxidase-conjugated anti-rat IgG antibody in the case of α-tubulin and peroxidase-conjugated anti-guinea pig IgG antibody in the case of Vma5p. Signals were detected using ECL (NEN Life

Science Products, Inc.) according to the manufacturer's instructions, and visualized by film exposure.

RT–PCR analysis

RNA was extracted from 5×10^7 cells using the RNeasy kit (Qiagen, Inc.) according to the manufacturer's instructions. Prior to the RT–PCR step, genomic DNA was degraded by RQ1 RNase-free DNase (Promega, Inc.). Complete removal of contaminating DNA was verified by negative control PCRs with a specific set of primers. One microgram of total RNA was used as a template for cDNA synthesized using the Expand™ Reverse Transcriptase Kit (Boehringer Mannheim) and 500 ng of oligo $(dT)_{15}$ as a primer. One quarter of each cDNA preparation was used as a template in a PCR using specific primers spanning the intron of either *TUB1*, *TUB3* or *RPL25*.

RESULTS

Genetic interactions between *ISY1*, *SYF2*, *NTC20* and *CDC40*

The *SYF2* gene was previously identified in our screen for mutants unable to grow in the absence of Cdc40p. It encodes a small protein with no obvious structural motifs (9,10). *ISY1* and *NTC20* also encode two small proteins that lack any clear structural features. Neither gene was identified in our screen for mutations that were synthetically lethal with *cdc40*Δ, but both were found as two-hybrid interactors with Syf1p and Syf3/Clf1p (9,10,13). Syf2p, Isy1p and Ntc20p form part of a large protein complex, the CSC (9,10). Although mutations in most of the genes encoding the CSC proteins cause cell cycle arrest at the G_2/M transition, single mutations in *SYF2*, *ISY1* and *NTC20* have no apparent viability problems or cell cycle phenotype. In order to study the relationship between these three genes and *CDC40*, we sought to create double mutant strains.

Null alleles of *ISY1*, *SYF2* and *NTC20*, in which the ORFs were replaced with DNA fragments containing *HIS3*, *TRP1* or *KanMX*, respectively, were created. These strains were individually crossed to *cdc40*Δ*::LEU2* or *cdc40*Δ*::URA3* strains. The double heterozygous strains were subjected to meiosis and tetrad analysis. As expected from our previous results (9,10), we were unable to obtain any *syf2*Δ *cdc40*Δ double mutant spores among 40 tetrads dissected, indicating a synthetic lethality between mutations in these genes. Similarly, no *isy1*Δ *cdc40*Δ spores were obtained in 38 tetrads analyzed. In contrast, double mutants *ntc20*Δ *cdc40*Δ were obtained in the numbers expected and grew well at 25°C, showing no synthetic phenotypes. Thus, mutations in *SYF2* and *ISY1*, but not *NTC20*, are lethal in the absence of Cdc40p (Fig. 1).

To investigate the genetic interactions between the three non-essential genes of the CSC, we carried out crosses in all possible combinations between the genes *ISY1*, *SYF2* and *NTC20*. Tetrad analysis showed that *isy1*Δ is synthetically lethal in combination with *ntc20*Δ: no viable double mutant spores were observed among 25 tetrads dissected. In contrast, *isy1*Δ *syf2*Δ and *syf2*Δ *ntc20*Δ double mutants were obtained at the expected numbers. Whereas the latter strain is viable at all temperatures and shows no obvious synthetic phenotypes,

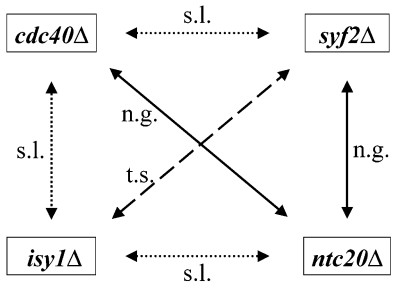

Figure 1. Genetic interactions between *CDC40*, *ISY1*, *SYF2* and *NTC20*. The following interactions were observed between full deletion mutants: s.l., synthetic lethality; t.s., temperature sensitivity; n.g., normal growth.

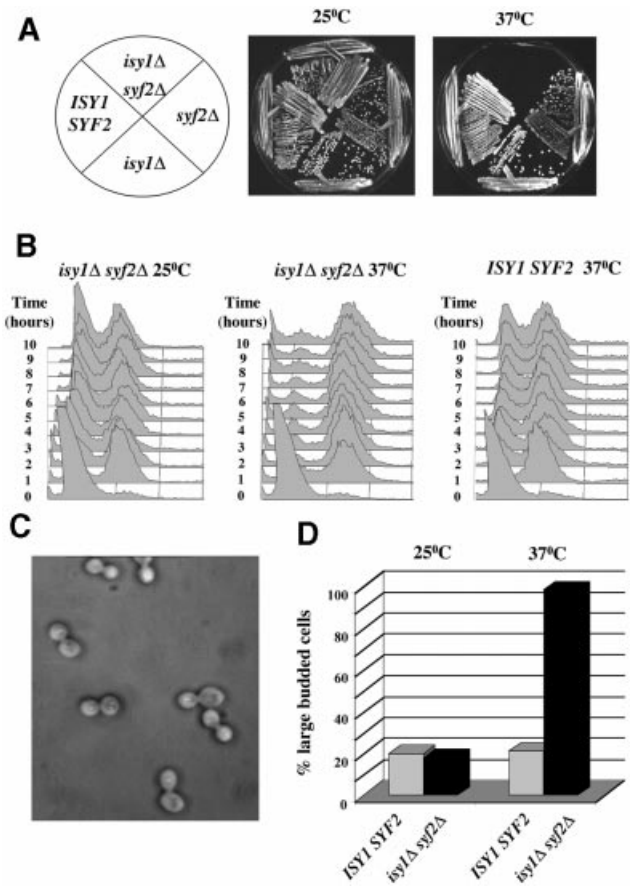

Figure 2. Temperature sensitivity of the *isy1*Δ *syf2*Δ mutant and accumulation of large-budded cells at the restrictive temperature. (**A**) Deletion of both *ISY1* and *SYF2* confers a temperature-sensitive phenotype. Growth of isogenic wild-type, *isy1*Δ, *syf2*Δ and *isy1*Δ *syf2*Δ strains at 25 and 37°C is shown. (**B**) Altered cell cycle distribution in the *isy1*Δ *syf2*Δ strain. Double mutant or wild-type cells were grown at 25°C, synchronized at the G_1 stage with α-factor and then released at either 25 or 37°C. After the release, samples were analyzed by flow cytometry. (**C**) The *isy1*Δ *syf2*Δ strain accumulates as large-budded cells at the restrictive temperature. (**D**) The percentages of large-budded cells in wild-type and *isy1*Δ *syf2*Δ strains incubated in either 25 or 37°C are shown. Large-budded cells were scored if the bud was greater than two-thirds of the size of the mother cell.

*isy1*Δ *syf2*Δ is temperature sensitive, being unable to grow at 37°C (Figs 1 and 2A). Below we describe a detailed analysis of the phenotypes of the *isy1*Δ *syf2*Δ strain.

Table 1. cDNAs able to suppress the cell cycle arrest of an *isy1*Δ *syf2*Δ strain at 35°C

Gene/ORF name	Function	Number of hits	Intron
ISY1	Splicing	15	No
SYF2	Splicing	10	No
TUB1	α-Tubulin subunit	4	Yes
TUB3	α-Tubulin subunit	4	Yes
MOT2	Transcription regulation	1	No
RPN5	Proteasome subunit	1	No
YOL162w	Unknown	1	No

The *isy1*Δ *syf2*Δ double mutant exhibits cell cycle arrest at the non-permissive temperature

To study if the temperature sensitivity of the *isy1*Δ *syf2*Δ strain is related to defects in the cell cycle, we examined whether the cells exhibit the typical phenotype of *cdc* mutants at restrictive temperatures. To this end, *isy1*Δ *syf2*Δ cells were synchronized at the G_1 phase of the cell cycle with α-factor and then released at either permissive (25°C) or restrictive (37°C) temperatures. At different time points, samples were analyzed by flow cytometry. Figure 2B shows that *isy1*Δ *syf2*Δ cells released from G_1 at the restrictive temperature replicated their DNA and subsequently accumulated with a 2N DNA content. Light microscopy of samples taken 3 h after release to 37°C showed that almost all the population was composed of large-budded cells (Fig. 2C and D). Staining the cells with DAPI and α-tubulin antibodies demonstrated that the arrested cells had undivided nuclei and short spindles (see below). We thus conclude that *isy1*Δ *syf2*Δ cells are unable to proceed through the G_2/M stage at the restrictive temperature.

The cell cycle arrest phenotype of *isy1*Δ *syf2*Δ cells can be suppressed by *TUB1* or *TUB3* cDNA

Since splicing constitutes a key cellular mechanism, genes encoding splicing factors are often essential. Many temperature-sensitive splicing mutants have been isolated; most of them arrest at the restrictive temperature without any defined cell cycle morphology (10). We reasoned that the cell cycle-specific arrest of the *isy1*Δ *syf2*Δ double mutant strain might be caused by inefficient processing of a small number of intron-containing genes particularly dependent on Isy1p and Syf2p for their pre-mRNA splicing. If this were the case, we expected that introduction of the relevant target gene(s) in the form of cDNA should be sufficient to bypass the need for Isy1p and Syf2p and therefore should allow growth at the restrictive temperature. To test this hypothesis, we conducted a screen to isolate cDNAs able to suppress the temperature sensitivity of *isy1*Δ *syf2*Δ cells. Strain YOD13 (*isy1*Δ *syf2*Δ) was transformed with a cDNA library and screened for cell growth at 35°C. Table 1 shows that in addition to the *ISY1* and *SYF2* genes, two intron-containing genes, *TUB1* and *TUB3*, were isolated. *TUB1* is the major gene encoding the α-tubulin subunit in *S.cerevisiae. TUB3* is a second gene encoding the α-tubulin subunit. It is expressed at lower levels than *TUB1*. The protein products of *TUB1* and *TUB3* are very similar, showing 90% sequence identity (18,19). *TUB1* and *TUB3* cDNA showed better suppression than three other cDNAs isolated, supporting growth of *isy1*Δ *syf2*Δ cells at temperatures up to 35°C (although not at 37°C). The three additional cDNA clones (encoding *MOT2*, *RPN5* and *YOL162w*) were isolated only once each and allowed only weak growth at 35°C. They encode, respectively, a general transcription factor, a proteasome subunit and an unknown ORF. Since their effect seemed relatively weak, these cDNAs were not further analyzed.

The double mutant *isy1*Δ *syf2*Δ exhibits sensitivity to the microtubule-disrupting agent nocodazole

Increased sensitivity of cells to anti-tubulin drugs is a common phenotype of tubulin mutants (18,19). To establish whether the phenotypes of the *isy1*Δ *syf2*Δ mutant were related to tubulin, we checked the ability of the double mutant cells to grow in the presence of increasing nocodazole concentrations. As shown in Figure 3, at a semi-restrictive temperature (30°C), the *isy1*Δ *syf2*Δ double mutant displays increased sensitivity to nocodazole relative to single mutants or to the wild-type control. Such sensitivity could not be seen at the permissive temperature. Figure 3B also shows that nocodazole sensitivity was completely suppressed by *TUB1* or *TUB3* cDNA expression. The fact that both the temperature sensitivity and nocodazole sensitivity of *isy1*Δ *syf2*Δ cells can be suppressed by *TUB1* or *TUB3* cDNA suggests a common cause for these phenotypes.

Isy1p and Syf2p are required for the maintenance of the mitotic spindle

In *S.cerevisiae*, spindle pole body duplication and spindle assembly take place concomitantly with initiation of DNA replication (20). Thus, during the S, G_2 and early M phases, a short spindle is observed, whereas spindle elongation occurs during anaphase. Since *TUB1* and *TUB3* encode the α-tubulin subunit, which is a major component of the mitotic spindle, we considered the possibility that lower expression of *TUB1* and *TUB3* in the *isy1*Δ *syf2*Δ double mutant strain incubated at the restrictive temperature may disrupt the mitotic spindle and therefore cause cell cycle arrest. To test this hypothesis, we carried out a careful analysis of the spindle length of *isy1*Δ *syf2*Δ cells at the permissive and non-permissive temperatures. The cells were grown at 25°C and then shifted to the restrictive temperature (37°C). At different time points samples were taken for tubulin staining by indirect immunofluorescence with anti-α-tubulin antibodies. At the permissive temperature, the *isy1*Δ *syf2*Δ cells contained spindles of all sizes, as did the wild-type control. However, after 2.5 h of incubation at the non-permissive temperature, mutant cells exhibited either a short spindle or a dot of α-tubulin material. Following a longer incubation at 37°C, the mitotic spindle eventually disappeared, and after 5 h incubation at the non-permissive temperature almost no staining was detected (Fig. 4). These results indicate that Isy1p and Syf2p are required for maintenance of the mitotic spindle.

Nuclear migration phenotypes of *isy1*Δ *syf2*Δ mutant cells

In wild-type cells, nuclear migration takes place early in the cell cycle. Therefore, in large-budded cells the nucleus is almost always seen near, or extended through, the bud neck. This process of nuclear migration to the bud neck requires

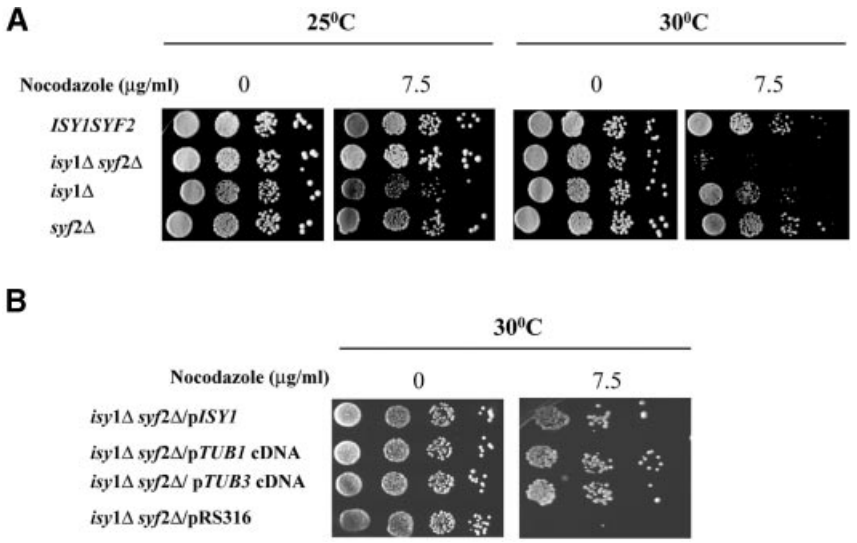

Figure 3. The *isy1*Δ *syf2*Δ strain is sensitive to nocodazole. (**A**) Nocodazole sensitivity of wild-type, *isy1*Δ, *syf2*Δ and *isy1*Δ *syf2*Δ strains. Serial dilutions of cells were spotted onto YEPD plates containing 0 or 7.5 μg/ml nocodazole and incubated at the permissive (25°C) and semi-permissive temperature (30°C). (**B**) Suppression of the nocodazole sensitivity by expression of *TUB1* and *TUB3* cDNA. *isy1*Δ *syf2*Δ cells carrying *ISY1*, *TUB1* or *TUB3* cDNA under the inducible *GAL1* promoter, or the vector alone (pRS316) were spotted onto YEPGal plates containing 0 or 7.5 μg/ml nocodazole at the semi-permissive temperature (30°C).

extranuclear microtubules (21). In tubulin mutants, the nucleus does not divide; moreover, some tubulin mutants also exhibit defects in nuclear migration (22). To test the effect of Isy1 and Syf2 on these tubulin-dependent events, we followed nuclear migration and nuclear division in *isy1*Δ *syf2*Δ mutant and in wild-type cells. As we have shown (Fig. 2C and D), the *isy1*Δ *syf2*Δ strain accumulated large-budded cells when incubated at the non-permissive temperature. DAPI staining showed that more than 95% of these cells contained one undivided nucleus. Under these conditions, a significant nuclear migration defect is observed: in 50% of the arrested cells the nucleus is located away from the bud neck (Fig. 5). This number may be an underestimate, as a very conservative classification limit was used: only cells in which the distance between the edge of the nucleus to the neck was at least 40% of the diameter of the mother cells were classified as having a nucleus away from the neck. Similar defects, although less profound, can be seen in *isy1*Δ *syf2*Δ cells incubated at the permissive temperature. This is in stark contrast to the wild-type control cells, in which more than 95% of the large-budded cells had the nucleus localized to the bud neck (Fig. 5B). We therefore conclude that *isy1*Δ *syf2*Δ cells are defective in nuclear migration.

Efficient splicing of *TUB1* and *TUB3* requires *ISY1* and *SYF2*

To test the possibility that the efficient splicing of *TUB1* and *TUB3* pre-mRNA is dependent on Isy1p and Syf2p, we examined the splicing efficiency of the *TUB1* and *TUB3* pre-mRNA by RT–PCR analysis. Total RNA from *isy1*Δ *syf2*Δ and wild-type strains was isolated before and after the cells were shifted to the non-permissive temperature. RNA was subjected to RT–PCR analysis using primers specific to either *TUB1*, *TUB3* or, as a control, to the intron-containing ribosomal gene, *RPL25*. As shown in Figure 6, when cells deleted for *ISY1* and *SYF2* were incubated at the non-permissive temperature, an accumulation of unspliced *TUB1* and *TUB3* was observed. Such an accumulation was not seen in cells incubated at the permissive temperature, or in wild-type cells at either temperature. No pre-mRNA accumulation was seen using primers specific to the ribosomal gene *RPL25* (data not shown). These results indicate that the splicing of *TUB1* and *TUB3* pre-mRNA is highly dependent on Isy1 and Syf2 proteins.

*isy1*Δ *syf2*Δ cells have less α-tubulin protein at the restrictive temperature

The disappearance of the mitotic spindle observed in double mutant *isy1*Δ *syf2*Δ cells at elevated temperatures might be due to a reduction in α-tubulin protein levels caused by defects in *TUB1* and *TUB3* pre-mRNA splicing. We therefore measured directly the levels of α-tubulin by immunoblotting. As a control we measured the amount of Vma5p, the c subunit of the vacuolar H (+) ATPase, which is also encoded by an intron-containing gene. As shown in Figure 7, α-tubulin protein levels decreased rapidly when *isy1*Δ *syf2*Δ cells were incubated at the non-permissive temperature. In contrast, constant amounts of α-tubulin were detected in *isy1*Δ *syf2*Δ cells incubated at the permissive temperature or in wild-type cells. The reduction in α-tubulin levels is not due to a general decrease in protein stability in *isy1*Δ *syf2*Δ cells at the non-permissive temperature, since the level of other proteins (such as Vma5) remained constant under all conditions (Fig. 7).

The cell cycle arrest of *isy1*Δ *syf2*Δ strains is mediated by the spindle checkpoint

When the mitotic spindles are defective (due to the action of drugs or to mutated spindle components) cells delay the onset of chromosome segregation by activating the spindle checkpoint (reviewed in 2). This mechanism is dependent on the activity of two groups of genes, the *mad* genes (23) and the *bub* genes (24). Cell cycle arrest is achieved by inhibiting the anaphase-promoting complex-dependent degradation of specific proteins. Sister chromatid separation requires proteolysis

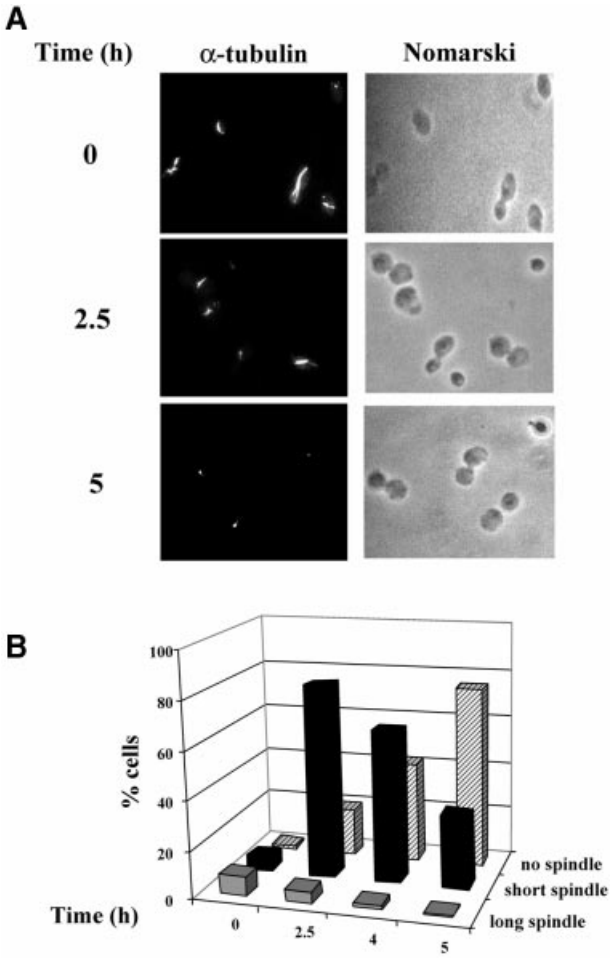

Figure 4. The *isy1*Δ *syf2*Δ mutant cells arrest at G_2/M with unstable microtubules. (**A**) Asynchronous cultures of wild-type or *isy1*Δ *syf2*Δ cells were shifted to the restrictive temperature and stained for microtubules using an anti-α-tubulin antibody followed by a FITC-conjugated secondary antibody. The same samples were also examined by Nomarski microscopy. (**B**) Graphic presentation of spindle morphology in *isy1*Δ *syf2*Δ cells incubated at the restrictive temperature. Percent of cells with long or short spindle, or without anti-tubulin signal, are shown.

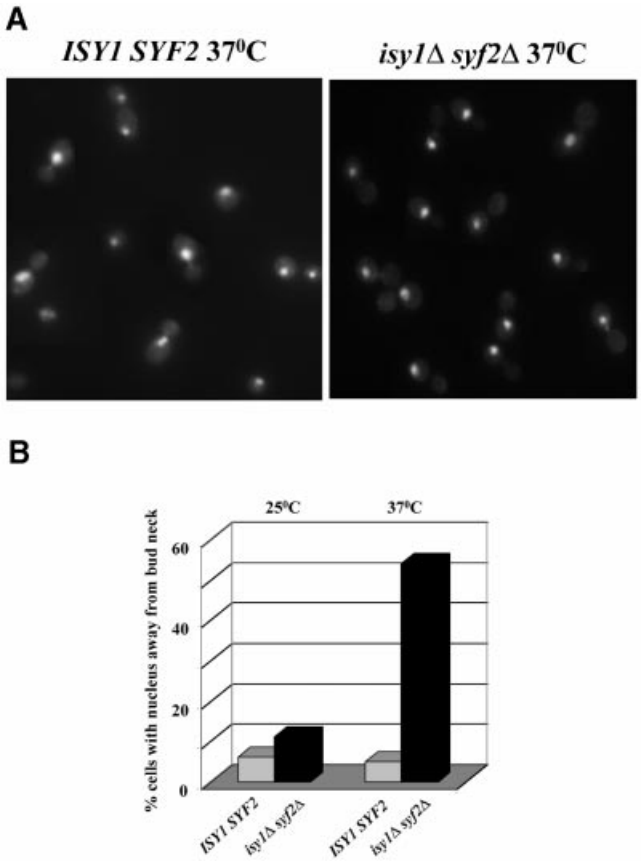

Figure 5. *isy1*Δ *syf2*Δ cells are defective in both nuclear division and nuclear migration. (**A**) The *isy1*Δ *syf2*Δ cells were grown at 37°C, DAPI stained and examined under the microscope by UV light. As a comparison, *ISY1 SYF2* cells grown at 37°C are shown. (**B**) The percentage of large-budded cells with one nucleus away from its position near the bud neck was determined in both *isy1*Δ *syf2*Δ and wild-type cells after 3 h of incubation at 25 or 37°C.

of Pds1p (25), whereas assembly of replication-competent complexes on origins (to ensure DNA replication in the next S-phase) requires degradation of B-type cyclins, such as Clb2 (26). Recent work has shown that prevention of Pds1 degradation depends on the Mad1, Mad2, Mad3 and Bub1 checkpoint proteins, whereas the prevention of Clb2 proteolysis is mediated by Bub2 (27). Since mutations in genes encoding tubulin activate the spindle checkpoint (28), we tested whether the inefficient splicing of *TUB1* and *TUB3* transcripts causes cell cycle arrest by activating this control mechanism. We reasoned that if that were the case, mutations that inactivate the spindle checkpoint would prevent cell cycle arrest and would lead to catastrophic cell divisions and to cell death. We therefore created *isy1*Δ *syf2*Δ strains in which the *MAD1* and *BUB2* genes were deleted.

The *isy1*Δ *syf2*Δ double mutant strain and the quadruple *isy1*Δ *syf2*Δ *mad1*Δ *bub2*Δ mutant strain were grown to mid-logarithmic phase, and were then transferred to the non-permissive temperature. Individual cells were monitored (under the microscope) for their ability to undergo cell cycle arrest. Figure 8 shows that whereas the double mutant arrested as large-budded cells in response to the restrictive temperature, the quadruple mutant continued to divide and created microcolonies that did not continue to develop (Fig. 8). Although the quadruple *isy1*Δ *syf2*Δ *mad1*Δ *bub2*Δ mutant strain was able to undergo several divisions in non-permissive conditions, the cells rapidly lost viability, as can be seen by their reduced ability to recover when returned to permissive conditions (Fig. 8C). Similar results were observed when tubulin was depolarized by nocodazole: whereas the wild-type strain arrested for several hours as large-budded cells, the *mad1*Δ *bub2*Δ mutant strain failed to arrest and produced inviable microcolonies (Fig. 8B).

These results indicate that the absence of the Isy1 and Syf2 proteins leads to reduced tubulin levels, which trigger cell cycle arrest by activating the spindle checkpoint.

DISCUSSION

Cell cycle progression and pre-mRNA splicing are two apparently independent processes in eukaryotic cells. However, a growing body of evidence has recently accumulated indicating that these processes may be connected. The

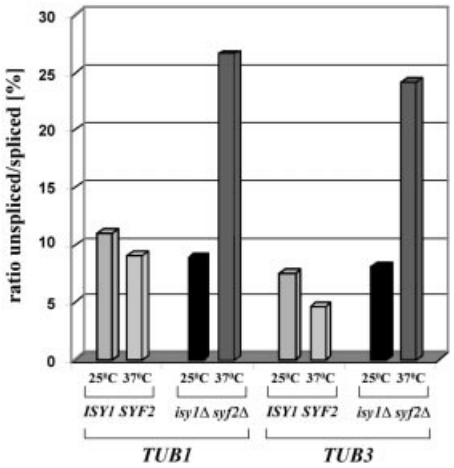

Figure 6. Isy1p and Syf2p are required for efficient splicing of the *TUB1* and *TUB3* introns. RT–PCR analysis of *isy1*Δ *syf2*Δ and wild-type cells using *TUB1*- or *TUB3*-specific oligonucleotides spanning the intron. The level of products for spliced and unspliced transcripts of *TUB1* and *TUB3* was quantified, after gel electrophoresis, using the program NIHimage (v. 1.62). For each strain/temperature, the ratio unspliced/spliced is shown.

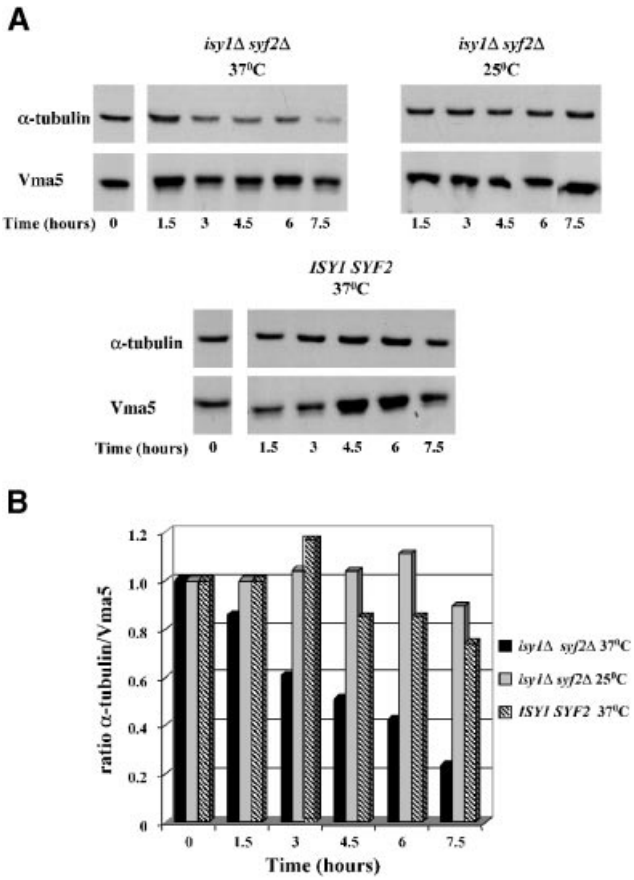

Figure 7. The level of α-tubulin is reduced in *isy1*Δ *syf2*Δ mutant cells incubated at 37°C. (**A**) Asynchronous cultures of wild-type or *isy1*Δ *syf2*Δ strains were incubated at either 25 or 37°C. At different time points total protein was extracted and subjected to western blot analysis using anti-α-tubulin antibody. As a control, anti-Vma5p antibody was used. (**B**) Quantitative analysis of α-tubulin in *isy1*Δ *syf2*Δ cells. The western blots shown in (A) were quantified using the program NIHimage (v. 1.62) and the calculated ratio α-tubulin/Vma5 is shown.

CDC40/PRP17 gene, for example, encodes a protein essential for G_2/M transition (8), which also plays a role in splicing (29–31). Using synthetic lethal and two-hybrid screens we have previously identified a putative protein complex that genetically interacts with Cdc40p (i.e. CSC). The CSC contains proteins involved in both cell cycle progression and in splicing (9,10,13). Most of the genes encoding proteins of this complex are essential; however, the CSC also contains (at least) three proteins encoded by non-essential genes: *ISY1*, *SYF2* and *NTC20*. In this paper we have dissected the genetic relationships between these genes, which exhibit a complex set of interactions. While deletion of *ISY1* is lethal in the absence of *NTC20* and is temperature sensitive in combination with a deletion of *SYF2*, the *syf2*Δ *ntc20*Δ double mutant shows no viability or growth defect (Fig. 1).

The nature of the interactions observed between the CSC genes is still under investigation; currently the results could be interpreted in two alternative ways. (i) The three proteins could be part of one large complex with a role in both cell cycle progression and pre-mRNA splicing. The different gene interactions observed among the non-essential members of this complex could be due to different effects on the stability or function of the complex. For example, it is possible that for the protein complex to be fully stable/active, only the Isy1 protein is needed. In its absence, however, Ntc20p can provide some degree of stability, such that an *isy1*Δ *syf2*Δ strain is only temperature sensitive, and not completely lethal. In contrast, Syf2p alone is insufficient for CSC stability and thus the *isy1*Δ *ntc20*Δ double mutant is inviable. (ii) An alternative view posits that the elaborate interactions observed are due to the existence of several alternative protein complexes with a similar core, each containing different non-essential proteins. These complexes may act redundantly or together as mediators that link splicing to cell cycle progression. For example, functional complexes may exist in the cell, some carrying Isy1p, some carrying Ntc20p. In the absence of both proteins, no active complex can be formed, and the cells die.

The nature of the connection between *CDC40* and the group of interacting proteins is still unclear. We were unable to detect physical or two-hybrid interactions between Cdc40p and any of the CSC proteins. Furthermore, protein complexes similar to the CSC have been recently purified biochemically from *S.cerevisiae* (11,32) and *S.pombe* (12). In neither case was Cdc40p found among the proteins isolated. On the other hand, in recent studies of a large-scale analysis of protein complexes of yeast, Cdc40p was identified in association with some (but not all) of the complexes precipitated with antibodies directed against members of the CSC (33,34). This may indicate that Cdc40p is loosely associated with some of the CSC components. Thus, Cdc40p may act alone, or in conjunction with other proteins, to affect the same cellular activity as the CSC. Alternatively, Cdc40 may associate transiently with the CSC, to facilitate the access or activity of other proteins. This process could also take place in the absence of Cdc40, provided Syf2 or Isy1 are functional.

When shifted to the restrictive temperature, the *isy1*Δ *syf2*Δ double mutant cells arrest as large-budded cells with a short spindle. We isolated *TUB1* and *TUB3* cDNAs as suppressors of the cell cycle arrest of *isy1*Δ *syf2*Δ. RT–PCR analysis

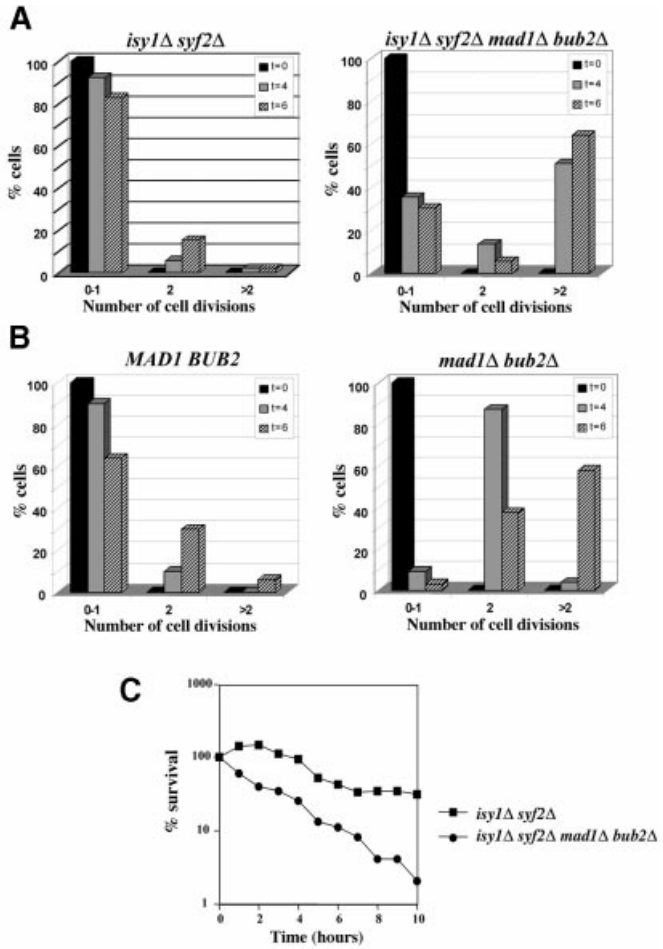

Figure 8. Quantification of cell cycle arrest and survival of *mad1 bub2* deleted strains. (**A**) Double mutant *isy1*Δ *syf2*Δ and quadruple mutant *isy1*Δ *syf2*Δ *mad1*Δ *bub2*Δ cells were grown at 37°C for 2.5 h, spotted on YEPD plates and incubated at 37°C. At different time points the cells were examined by light microscopy and the number of cell divisions in each microcolony was determined. (**B**) Wild-type *MAD1 BUB2* and double mutant *mad1*Δ *bub2*Δ cells were placed on YEPD plates containing 7.5 µg/ml nocodazole, and incubated at 30°C. At different time points the cells were examined by light microscopy and the number of cell divisions in each microcolony was determined. (**C**) Double mutant *isy1*Δ *syf2*Δ and quadruple mutant *isy1*Δ *syf2*Δ *mad1*Δ *bub2*Δ cells were grown at 37°C, at timely intervals samples were taken, briefly sonicated, diluted and spotted onto YEPD plates. Colonies were counted after 3 days of incubation at 25°C.

showed that *isy1*Δ *syf2*Δ cells incubated at the restrictive temperature accumulate *TUB1* and *TUB3* pre-mRNA at a higher level than other pre-mRNAs, indicating that the dependence on Isy1p and Syf2p for the removal of the *TUB1* and *TUB3* introns is specific. The *TUB1* and *TUB3* introns may contain unique features that make their pre-mRNA splicing highly dependent on the CSC. Dix *et al.* (13) have previously shown that Isy1p is required *in vivo* for the splicing of introns carrying suboptimal consensus splicing sequences. The need for specific splicing factors for the efficient splicing of a subset of genes is a well known phenomenon. For example, the splicing factor Slu7 is required for the efficient splicing of introns in which the distance between the 3′-splice site and the branch point is larger than 7 bp (35). The fact that *TUB1* and *TUB3* cDNA support growth of *isy1*Δ *syf2*Δ cells up to 35°C, but not at 37°C, indicates that under these conditions another transcript(s) becomes rate limiting. This putative transcript may contain introns that share some characteristics with those of *TUB1* and *TUB3*.

Microtubules are ubiquitous cytoskeletal structures, consisting of α- and β-tubulin hetrodimers (36). In *S.cerevisiae*, as in all eukaryotic cells, microtubules are necessary for chromosome movement. Unlike most other eukaryotes, however, in yeast the only other functions known to depend on microtubules are the movement of nuclei to the bud neck before mitosis and the fusion of nuclei after mating (21,37). Most *tub1* and *tub3* mutants show several common phenotypes such as supersensitivity to spindle disrupting agents, accumulation of large-budded cells, and defects in nuclear division, nuclear migration or both (18,19,22,38). We have shown that the double mutant *isy1*Δ *syf2*Δ cells exhibit similar phenotypes when incubated at non-permissive or semi-permissive conditions. The sensitivity to nocodazole, as well as the cell cycle arrest, could be fully suppressed by the presence of intronless α-tubulin cDNA, indicating that the phenotypes observed are truly due to lack of α-tubulin protein. Furthermore, the *syf2*Δ *ntc20*Δ strain, which grows well at all temperatures, was not sensitive to nocodazole (data not shown). These results strongly imply that the nocodazole sensitivity phenotype of *isy1*Δ *syf2*Δ cells is connected to the cell cycle arrest phenotype.

The mechanism that links cell cycle progression and splicing exhibits an exquisite sensitivity to the level of pre-mRNA splicing. Our RT–PCR results show a ~3-fold increase in accumulation of *TUB1* and *TUB3* pre-mRNAs in *isy1*Δ *syf2*Δ cells at the restrictive temperature (Fig. 6). Although splicing of the *TUB1* and *TUB3* transcripts is only reduced and not eliminated, a strong decrease in the levels of α-tubulin is seen. It is not yet clear why accumulation of unspliced transcripts has such a strong effect on the level of expression. Unspliced intermediates may exert their negative effect by affecting RNA stability or the efficiency of translation. Likewise, α-tubulin levels, as detected by immunoblot experiments, are strongly reduced; the protein, however, is not totally eliminated. It has been well established that the ratio between α- and β-tubulin is of crucial importance for microtubule stability in yeast. Microtubule function is sensitive to very small changes in tubulin stoichiometry. Several reports have demonstrated that gene dosages of β-tubulin in excess of α-tubulin, or overexpression of β-tubulin, are not tolerated and result in rapid depolymerization of normal microtubules and loss of viability (39,40). Thus, the small effect in splicing of the α-tubulin-encoding genes is amplified, resulting in a strong destabilization of the microtubules.

The spindle checkpoint of *S.cerevisiae* is able to sense a multitude of spindle defects and halt cell cycle progression before the exit from mitosis. The genes in this pathway are necessary for the cell cycle arrest phenotype observed in the presence of spindle-depolarizing drugs: spindle checkpoint mutants fail to arrest in the presence of benomyl or nocodazole (Fig. 8B) (23,24,41). We have shown that the cell cycle arrest of *isy1*Δ *syf2*Δ cells as a consequence of inefficient *TUB1* and *TUB3* splicing is not direct (i.e. the cells do not arrest with a short spindle simply because they are unable to elongate it in order to proceed in the cell cycle). Instead, the defect in

microtubule structure is detected by the spindle checkpoint, which initiates a cascade of events leading to cell cycle arrest. In contrast to the *isy1*Δ *syf2*Δ strain, the quadruple *isy1*Δ *syf2*Δ *mad1*Δ *bub2*Δ mutant (defective in the two branches of the spindle checkpoint) continues to divide at the non-permissive conditions in the presence of damaged spindles resulting in loss of viability (Fig. 8). Our results thus show that pre-mRNA splicing plays an important role in promoting the fidelity of chromosome segregation and cell cycle progression. While this work was in progress, Burns *et al.* (42) reported that *TUB1* pre-mRNA splicing is also defective in temperature-sensitive *cef1* mutants. Similar to results presented here, *TUB1* cDNA could also suppress the cell cycle arrest of these mutants. These results further support our findings, since Cef1 is one of the components of the CSC. It is very likely that defects in *CEF1* lead to cell cycle arrest by activating the spindle checkpoint, as we have shown here for Isy1 and Syf2.

Maintaining genome stability is essential for all eukaryotes. The survival of cells and organisms requires accurate chromosomal segregation. The cell has developed several regulatory networks to maintain genome stability. Recent studies have identified some CSC components as important for sister chromatid separation and segregation (41,43). Thus, one of the possible roles of the CSC could be to ensure proper chromosome segregation by linking pre-mRNA splicing to spindle integrity.

The mechanism unveiled here is probably evolutionarily conserved. Orthologs for most of the CSC proteins exist in other eukaryotes, and mutations in many splicing mutants in *S.pombe* cause cell cycle arrest (44,45). Interestingly, a recent report (45) has shown that mutations in some genes encoding splicing factors in *S.pombe* can lead to a reduction in α-tubulin levels and to defects in microtubule integrity. The splicing defects result in unequal chromosome separation and in polyploidy. These findings suggest that the link between pre-mRNA splicing and spindle integrity in both *S.cerevisiae* and *S.pombe* may represent a common mechanism in eukaryotes.

Are all splicing factors required for maintaining the stability of the genome? As mentioned above, mutants defective in several components of the CSC cause cell cycle arrest by a similar mechanism. Cells lacking the Cdc40 protein also arrest at the G_2/M transition upon the shift to the restrictive temperature. As in the case of *isy1*Δ *syf2*Δ, prolonged incubation at the restrictive conditions induces spindle depolymerization (8). However, unlike the *isy1*Δ *syf2*Δ strain, introduction of *TUB1* or *TUB3* cDNA does not suppress the temperature-sensitive phenotype of *cdc40*Δ cells (data not shown). Furthermore, *TUB1* and *TUB3* pre-mRNA do not accumulate in the *cdc40*Δ strain (data not shown) (46). These results suggest that the CSC and *CDC40* may be differentially required for the efficient splicing of two subsets of transcripts involved in cell cycle progression. Control of cell cycle progression via pre-mRNA splicing appears to be a complex process involving several regulatory pathways.

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