Yeast
Yeast 2002; **19**: 43–54.
DOI: 10.1002/yea.803

Research Article

SRC1: an intron-containing yeast gene involved in sister chromatid segregation

Susana Rodríguez-Navarro†, J. Carlos Igual and José E. Pérez-Ortín*
Departamento de Bioquímica y Biología Molecular, Universitat de València, C/ Dr. Moliner 50, E-46100 Burjassot, Spain

**Correspondence to:*
J. E. Pérez-Ortín, Departamento de Bioquímica y Biología Molecular, Universitat de València, C/ Dr. Moliner 50, E-46100 Burjassot, Spain.
E-mail: Jose.e.perez@uv.es

†Current address:
Biochemie-Zentrum Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany.

Received: 18 June 2001
Accepted: 18 August 2001

Abstract

Analysis of a three-member gene family in the yeast *Saccharomyces cerevisiae* has allowed the discovery of a new gene that comprises two contiguous open reading frames previously annotated as YML034w and YML033w. The gene contains a small intron with two alternative 5′ splicing sites. It is specifically transcribed during G_2/M in the cell cycle and after several hours of meiosis induction. Splicing of the mRNA is partially dependent on *NAM8* but does not vary during meiosis or the cell cycle. Deletion of the gene induces a shortening of the anaphase and aggravates the phenotype of *scc1* and *esp1* conditional mutants, which suggests a direct role of the protein in sister chromatid separation.

Keywords: ***Saccharomyces cerevisiae*; splicing; sister chromatid; cell cycle; mitosis; meiosis**

Introduction

According to the MIPS database (7 June 2001), the yeast *Saccharomyces cerevisiae* genome contains about 6400 open reading frames (ORFs), of which only 3864 are functionally characterized. Many of the yeast genes have paralogues in the same genome, which means that gene families based on sequence or functional similarities can be established (Bianchi *et al*., 1999; Blandin *et al*., 2000). One of these families is constituted by the YML033w, YML034w and YDR458c ORFs. The first two genes are contiguous in the genome and show low, but significant, similarity to YDR458c (29% amino acid identity). The possible functions for such genes have not been identified.

Whole-genome studies using two-hybrid screens (Ito *et al*., 2000; Uetz *et al*., 2000) or Rosetta stone (Marcotte *et al*., 1999) gave no results for any of the three putative genes. DNA-chip analysis has, however, shown that both YML033w and YML034w have a clear expression peak at the G_2/M transition, being enclosed in the denominated *CLB2* group (Spellman *et al*., 1998) and are strongly induced 7 h after the initiation of meiosis (Chu *et al*., 1998). None of the comprehensive analyses carried out under different growth conditions (DeRisi *et al*., 1997; Jelinsky *et al*., 1999; Gasch *et al*., 2000) or mutant strains (Marton *et al*., 1998; Lelivelt and Culbertson, 1999) or even the whole-genome expression profiles obtained after deleting either of the two ORFs (Hughes *et al*., 2000; http://www.rii.com/publications/cell_hughes.htm), were able to give clues about their function.

The genome positions of YML033w and YML034w suggested that they could be part of the same ORF. We found that both ORFs in fact constitute a single gene (*SRC1*) containing an alternatively spliced intron. We have also demonstrated that *SRC1* splicing is Nam8p-dependent. Our transcriptional analysis shows that *SRC1* is cell cycle regulated with a peak in G_2/M and that it is induced during meiosis. No links between splicing and cell cycle or sporulation were found in this study. Phenotypical analysis of *src1* mutants strains revealed a faster sister chromatid segregation than

in the isogenic wild-type counterparts and synthetic interaction with genes related to the metaphase–anaphase transition, which strongly suggests that Src1p is involved in mitosis.

Materials and methods

Strains, media, plasmids and constructions

Yeast strains used in this work are shown in Table 1. All the mutant strains were constructed by replacing the entire ORF, from the ATG of the gene to the stop codon, following the short flanking homology method (Wach *et al*., 1994). Gene deletions were carried out in the isogenic strains BY4741, BY4742 or in the strain W303-1A. The disruption cassettes for PCR-mediated deletion were amplified from the pRS400 series (Brachmann *et al*., 1998).

The *S. cerevisiae* strains were routinely grown on YPD (1% yeast extract, 2% peptone and 2% glucose) or minimal SD medium (0.67% yeast nitrogen base, 2% glucose) supplemented with the auxotrophic requirements. Kanamycin-resistant strains were grown on YPD plates containing 200 mg/l geneticin (Gibco BRL). Meiosis induction was carried out by growing cells in YPD to saturation, then cells were grown in YPA (1% yeast extract, 2% peptone and 2% acetate) to 2×10^7 cells/ml. After washing cells twice with water they were resuspended in sporulation medium (0.5% potassium acetate).

For phenotypic analysis, growth of haploid disruptants was checked on YPD containing 1.2 M NaCl, 0.8 M KCl, 1.8 M sorbitol, 10 mM caffeine, 10 mM benomyl, 10 mM staurosporin, 10 mM sodium ortovanadate, 5 μg/ml nocodazole or 200 mM hydroxyurea. Yeasts were grown at 15°C, 28°C, 33°C, 35°C and 37°C for 2–3 days, or longer when necessary.

E. coli strain DH5α was employed in cloning procedures. DNA manipulations including plasmid preparation, transformation and subcloning in *E. coli* were carried out following standard protocols (Sambrook *et al*., 1989).

The *SRC1* ORF, from ATG to stop codon, was amplified from genomic DNA by high-fidelity PCR and cloned into pGEM-easy vector. Overexpressions of *SRC1* under the control of tetracycline promoter were obtained by subcloning an *Eco*RI fragment from the pGEM-easy::*SRC1* clone in pCM259 and pCM262 vectors (Bellí *et al*., 1998). Inhibition of tetracycline promoter was carried out by addition of doxycycline at 2.5 μg/ml in YPD

Table 1. List of strains used and constructed in this study

Strain	Genotype	Origin
W303	MAT**a**/α, *ura3-1/ura3-1, leu2−3,112/leu2−3,112, trp1-1/trp1-1, his3-11,15/his3-11,15, ade2-1/ade2-1*	Rothstein, R.
W303-1a	MAT**a**, *ura3-1, leu2−3,112, trp1-1, his3-11,15, ade2-1, can1-100*	Rothstein, R
BY4741	MAT**a**, *leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0*	Brachmann *et al*., 1998
BY4742	MATα, *leu2-Δ0, his3-Δ1, lys2-Δ0, ura3-Δ0*	Brachmann *et al*., 1998
BY4743	MAT**a**/α, *leu2-Δ0/leu2-Δ0, his3-Δ1/his3-Δ1, lys2-Δ0/+, met15-Δ0/+, ura3-Δ0/ura3-Δ0*	Brachmann *et al*., 1998
BQS1026	(BY4741) Δ*src1::LEU2*	This study
BQS1027	(BY4742) Δ*src1::LEU2*	This study
BQS1041	BQS1026 **x** BQS1027	This study
BQS1042	(BY4741) Δ*src1::LEU2*, Δ*ydr458w::MET15*	This study
BQS1039	(BY4741) Δ*ydr458w::MET15*	This study
BQS1074	(BY4742) Δ*ydr458w::LEU2*	This study
BQS1152	MAT**a**, *ura3-52, trp1-289, arg4, ade2, nam8::URA3, leu2-3,112*	Puig, O.
BQS1086	MAT**a**, *ura3-52, trp1-289, arg4, ade2, prp2::URA3, leu2-3,112*	Puig, O.
2788	MAT**a**, *esp1-1^{ts}, ura3-1, leu2-Δ1, can1-100, lys2-Δ0*	Ulhmann, F.
5832	MAT**a**, *scc1-73^{ts}, ade2-1, can1-100, leu2-3,112, his3-11*	Ulhmann, F.
8029	MAT**a**, *cdc20-3^{ts}, ura3-1*	Ulhmann, F.
BQS1123	(BY4741) *SRC1::GFP*	This study
BQS1132	(5832) Δ*yml034w*::KanMX4	This study
BQS1135	(2788) Δ*yml034w*::KanMX4	This study
BQS1133	(8029) Δ*yml034w*::KanMX4	This study
BQS1141	(W303-1a) Δ*yml034w*::KanMX4	This study

liquid medium. A GFP-tagged version of the gene was constructed by inserting a PCR-amplified cassette containing the GFP(S65T) ORF, from pFA6a–GFP(S65T)–KanMX6 (Longtine *et al.*, 1998), into the last codon of the YML033w ORF.

RT–PCR

Total RNA was obtained from cells growing under different conditions using low pH phenol and glass beads, according to Sherman *et al.* (1986). The mRNA was retro-transcribed following the recommendation of the supplier (Gibco, BRL). The oligonucleotides used to detect *SRC1* mRNA were SEC2 (reverse): GCC ATA TTG GCC TTT GAA and SEC3 (forward): TGC ACA AAT TTC ATG CGG, which amplify a fragment of 511 bp from the unspliced copy and a 381–385 bp fragment from the spliced mRNA. For *ACT1* mRNA analysis we used the oligonucleotides ACT1-D2 (forward): GAT TTT TCA CGC TTA CTG C and ACT1-R (reverse): TTG GTC TAC CGA CGA TAG ATG, which amplify a 171 bp fragment from the spliced mRNA. The lack of the 480 bp fragment, corresponding to the unspliced sequence, was taken as a control of the absence of genomic DNA contamination. For *CLB2* mRNA analysis, we used the oligonucleotides CLB2-D (forward): TAA AGA AGG AGG GAA GT and CLB2-R (reverse): GTT ATG GAC TAC CTC AGT, which amplify a fragment of 301 bp. The PCR reaction consisted of 94°C for 2 min, and 20 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, with a final extension of 3 min at 72°C. Several different amounts of template cDNA were tested to find conditions in which PCR serves to quantify the amount of each mRNA species.

Synchronous cultures

An exponential phase culture (2×10^6 cells/ml) was treated with 3 μg/ml α-factor (SIGMA or Diver Drugs) at 28°C. After 2 h treatment the cells were lightly sonicated and the percentage of unbudded cells was determined. When the level of unbudded cells reached more than 95%, the cells were extensively washed with YPD, resuspended in YPD at 2×10^6 cells/ml and incubated at 28°C. Samples for total RNA extraction, FACS analysis, budding index and DAPI staining were taken every 10 or 15 min.

Fluorescence microscopy

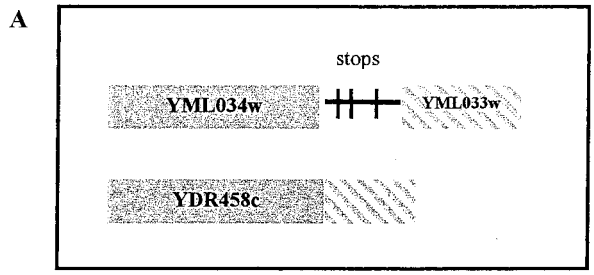
Asynchronous cultures were grown to 2×10^6 cells/m and then the cells were fixed with 70% ethanol. Nuclei were stained by resuspending cells in 4′,6-diamidino-2-phenylindole (DAPI), and cells were examined with an Axioscop (Zeiss Inc.) fluorescent microscope. More than 3000 cells, from at least seven independent cultures, were counted for each strain and cells were classified in three categories: (a) mononucleated cells, including those that had not entered in mitosis and those that had just begun the mitotic process but had not yet started nuclear division; (b) cells with an elongated nucleus expanding along the neck; and (c) binucleated cells, consisting of cells that had undergone sister chromatid separation but not cell division.

Results

SRC1 contains an intron with two non-canonical 5′ splice sites

The search for gene families made by B. Dujon's group found a family comprising three orphan genes, YDR458c, YML033w and YML034w, which are only present in other ascomycetes (Llorente *et al.*, 2000). This family is a special case because YML034w is homologous to the N-terminal part of YDR458c, whereas YML033w, consecutive in the genome to YML034w, is homologous to its C-terminal part (Figure 1A). This arrangement would suggest that either there are sequence errors in the region in between both ORFs or the organization corresponds to a pseudogene. However, the fact that a consensus branching sequence for an intron (UACUAAC) exists in the intergenic region suggested a third possibility: YML034w and YML033w correspond to a single gene containing an intron. We searched for 5′ and 3′ splicing consensus sequences. Two non-canonical 5′ splicing sequences were found, located before the predicted stop codon for YML034w at positions +1916 (GCAAGU) and +1920 (GUGAGU) from the ATG (Figure 1B).

In order to test the existence of a spliced intron a RT–PCR analysis was performed. Previously, several controls were made to ensure that RT–PCR analyses were reproducible and quantitative (see Figure 2). Total RNA, obtained from several yeast strains, was retrotranscribed by employing two primers external to the presumptive intron. In



B

5'-
CGAAGTGGTT GAAAAATCTT TAGAGTTTCT GAGAGCCAAA AATGCACAAA TTTCATGCGG

TGACGGAAAA GACGACATCG AGAGTGGTAT GACGGAAGAT GCCCTCTATC AAATATTTAA

TGAGGCTAGA GCTCCTTGGA TTCGCGACGA TGAATTTGAG GACCTGTGGA TCCAAGTTAT

CAAAGATTTA ACTGAAGAAC CAGAAATATT ATGGAGGCAA GTGAGTACAA TTTATTATCT

TACAAAAATT ATTAAACCTG TTATTATATG ATAACAGTTT TAATTTTTTT TGAATTTTTT

TTTGTTTTTG ATTTATTTAC TAACATCTTT TCCTAATTTT CTCTAGTTAT CACCCACTGA

CAACAACATT GGCGGAAATT CCAACAACAT CATTAAAACC AATGACGTTC CGAGACAGAA

GAGACATCTT CCAGAAAAAT TCATCTCCAA GACGAGGAAT TTTCGGTCGA CGTCCAAGAA

ATACATTGGA ATGAAGTGTC GATTTGAAAG AGAAATCTAC CAAACGTATA AAAAATTTCA

AAGGCCAATA TGGCTAATGT TTTTACTGAT AGTCATTAGT AAAGTAATCG -3'

Figure 1. (A) Schematic view of the YML034w, YML033w and YDR458c ORFs. The homologous regions are represented by identical backgrounds and the stop triplets in the YML034w reading frame by vertical lines. (B) Sequence of the intercoding region of YML034w and YML033w. Locations of the alternative 5′ splice sites (GCAAGTGAGT), branchpoint (TACTAAC) and 3′ splice site (TAG) in the intergenic region are boxed. The sequences corresponding to the two primers used for the RT–PCR analysis of *SRC1* transcript (SEC2: forward, SEC3: reverse) are underlined

addition to the 511 bp fragment predicted from the genomic sequence, an intense 385 bp band was produced in all the strains. Figure 2 shows the result for S288c background but identical result was found for W303 background and for the T73 wine yeast strain (not shown). The 430 bp product was purified from the gel and sequenced. The sequence from the 5′ flank was identical to the one stored in the database up to the presumptive 5′ splice sites. From this point the PCR product was found to contain a mix of two sequences. The main signal corresponds to the elimination of an intron from the +1920 5′ splice site, whereas the second signal reflects the intron elimination at the +1916 5′ splice site (Figure 1B). This PCR fragment was cloned and the sequence of six out of six clones analysed correspond to the main signal seen in the previous experiment. In conclusion, YML034w-YML033w contains an intron with two non-canonical 5′ splice sites, with the +1920 5′ splice site as the one preferentially used. Recently, Davis *et al.* (2000) have also proposed an alternative splicing event. Based on transcriptional (see below) and splicing data we annotated in SGD YML034w-YML033w as *SRC1* for spliced mRNA and cell cycle-regulated gene.

SRC1 splicing is *NAM8*-dependent

The *SRC1* intron has special features: it is located very far from the Cap site (1916/1920 nt) and the two 5′ splice sites are non-consensus sequences. These characteristics suggest a possible involvement of Nam8 protein in *SRC1* splicing, which is known

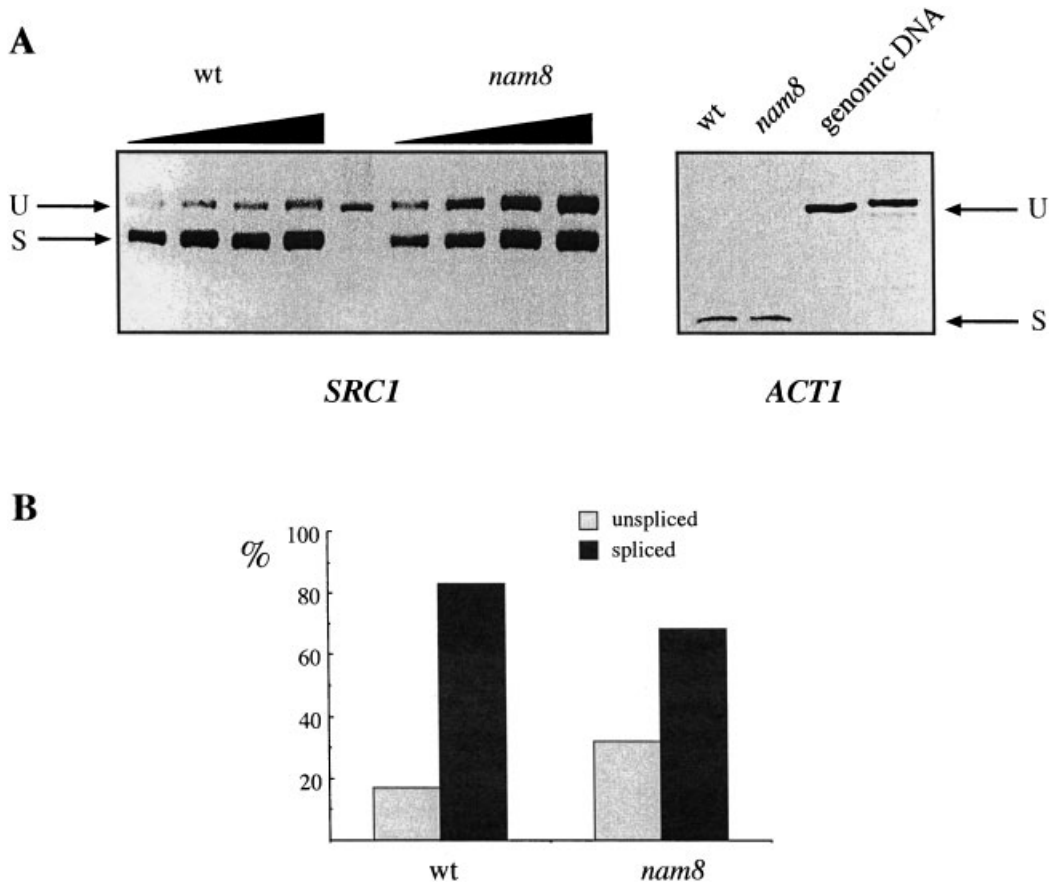


Figure 2. Analysis of *SRC1* splicing. RNA was extracted from exponentially growing cultures of the wild-type (BY4741) and *nam8* (BQS1152) strains and *SRC1* transcript was analysed by RT–PCR using SEC2 and SEC3 oligonucleotides. Two bands, corresponding to the spliced (S) and unspliced (U) transcripts, were detected (panel A). Four different amounts of cDNA were used in each case to find the non-plateau zone of the PCR reaction. Percentages of spliced and unspliced mRNAs were calculated from the leftmost sample and represented in panel B. *ACT1* mRNA which is totally processed in either the presence (wt) or the absence (*nam8*) of Nam8p has been used as a control. We used oligonucleotides ACT1-R and ACT1-D2, which amplify a fragment of 171 bp from the spliced mRNA and a 480 bp fragment from the unspliced one. Genomic DNA was used as a control for the longer fragment

to be required to process some non-canonical introns in yeast (Puig *et al.*, 1999). To test this hypothesis, a RT–PCR analysis was performed in cultures of wild-type and *nam8* strains grown to exponential phase. As shown in Figure 2, the relative amount of the non-processed mRNA compared to that of the spliced mRNA is higher in the *nam8* mutant than in the wild-type strain. A control intron, the one present in *ACT1* that is not dependent on Nam8p, was completely processed in both strains. Similar results were obtained using primer extension analysis (O. Puig, personal communication). These results indicate that Nam8p is involved in *SRC1* splicing, although additional factor(s) are responsible for the elimination of the intron in the absence of Nam8p.

SRC1 expression, but not mRNA splicing, is induced during sporulation

Expression profiles of the whole genome suggest an induction of *SRC1* during sporulation (Chu *et al.*, 1998). To confirm this data, samples were taken for RNA extraction at 0, 5, 10 and 15 h after shift to sporulation medium from a wild-type diploid strain. RT–PCR analysis showed a five-fold induction of *SRC1* during sporulation (Figure 3). Interestingly, the relative amounts of spliced and unspliced cDNA

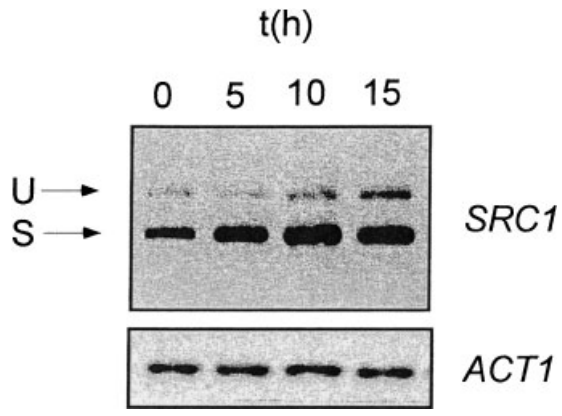


Figure 3. Analysis of *SRC1* expression and splicing during sporulation. RNA was extracted from wild-type (BY4743) diploid cells at 0, 5, 10 and 15 h after the shift to sporulation medium and *SRC1* transcripts were analysed by RT–PCR, as in Figure 1. As a loading control, a *ACT1* 171 bp fragment was amplified from the same samples

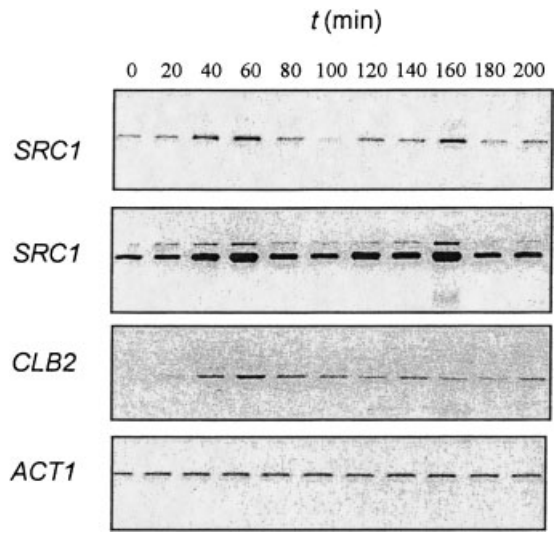


Figure 4. Analysis of *SRC1* expression and splicing through the cell cycle. RNA was extracted from W303-1a cells at the times indicated after the release from an α-factor arrest. *SRC1*, *CLB2* and *ACT1* (as loading control) transcripts were analysed by RT–PCR by using oligonucleotides SEC2 and SEC3, CLB2-D and CLB2-R, and ACT1-D2 and ACT1-R, respectively. Two different exposure times of the *SRC1* samples are shown to better observe the periodic expression of both spliced and unspliced transcripts

did not vary during the sporulation process. In conclusion, these results indicate that, although *SRC1* expression is strongly induced, the splicing of the transcript is not regulated during sporulation. The observed pattern of expression suggests a role for Src1p in sporulation. However, no significant differences were observed in the sporulation rate of a wild-type and the isogenic *src1*/*src1* diploid strains, or in the viability of wild-type and *src1* spore clones (data not shown).

SRC1 expression, but not mRNA splicing, is regulated during the cell cycle

A genomic wide survey of cell cycle-regulated gene expression suggests that the *SRC1* gene is periodically expressed peaking in early mitosis (Spellmann *et al.*, 1998). In order to confirm the cell cycle-regulated expression of *SRC1* gene a RT–PCR analysis was carried out. Cells were synchronised by α-factor and mRNA was extracted at different times after release from the arrest. As seen in Figure 4, there was an increase in *SRC1* expression at 40–60 min after the release. This peak of maximum expression coincided with the activation of *CLB2* gene in G_2/M. Further confirmation of the cell cycle regulation of *SRC1* gene was obtained by Northern analysis of a α-factor synchronized culture of another non-related strain (data not shown). In conclusion, the *SRC1* gene is periodically expressed through the cell cycle, showing a pattern of expression similar to the *CLB2* gene with a peak in G_2/M.

A link has been proposed between cell cycle control and splicing (Boger-Nadjar *et al.*, 1998; Ben-Yehuda *et al.*, 1998, 2000a,b). We wondered whether the splicing of the *SRC1* transcript could be cell cycle-dependent. As occurred for spliced *SRC1* mRNA, the levels of the unspliced transcript oscillated through the cell cycle, peaking in G_2/M (Figure 4). It is worth pointing out that no significant differences were detected in the proportion of spliced and unspliced transcripts. Thus, the extent of *SRC1* transcript splicing is constant throughout the cell cycle.

SRC1 is involved in mitosis

The regulation of *SRC1* gene expression suggests that it may be involved in cell cycle progression in mitosis. However, *SRC1* inactivation does not result in an obvious growth defect. *src1* cultures in different growth conditions have the same doubling time as isogenic wild-type cultures (data not shown). Moreover, budding index and FACS

analysis of asynchronous cultures do not indicate significant differences between the *src1* and the wild-type strains, suggesting that Src1p is not involved in budding or DNA replication (data not shown). Src1p does not seem to play a role in checkpoints either, as the *src1* strain is as sensitive as the isogenic wild-type to EMS, UV radiation, nocodazole, benomyl and hydroxyurea (data not shown). By contrast, when segregated nuclei were analysed by fluorescence microscopy, a clear difference between *src1* and wild-type strains was observed. The frequency of a single undivided nucleus (interphase + metaphase cells), an elongated dividing nucleus along the neck (anaphase cells) and two segregated nuclei one in the mother and the other in the future daughter (telophase cells), were determined. In order to obtain statistically significant results, an exhaustive analysis was carried out, involving more than 3000 cells from at least seven independent cultures for each strain. Both wild-type and *src1* mutant present the same proportion of interphase + metaphase cells. However, in wild-type cells there is the same percentage of cells in anaphase as in telophase, while the anaphase : telophase distribution is 1 : 2 in the *src1* mutant (Figure 5A). This result indicates that the *src1* mutant exhibits faster sister chromatid segregation than the parental strain. The same results were obtained in the S288C background (data not shown). Taking into account that the duplication time is 100 min for both strains, from the percentage in Figure 5A one can deduce a shortening of the anaphase in the *src1* strain of approximately 6 min (35% of the total anaphase) compared to wild-type cells.

To further characterize the abnormal chromatid segregation associated with the inactivation of Src1p, cultures of wild-type and *src1* mutant were synchronized by α-factor and the appearance of binucleated cells was measured after release from α-factor arrest. As shown in Figure 5B, the *src1* mutant accumulated binucleated cells approximately 4 min earlier than the isogenic wild-type. It is interesting to point out that exit from mitosis, detected as a reduction in the percentage of binucleated cells, occurs at approximately the same time in both wild-type and *src1*. The faster appearance of binucleated cells was not detected in the second cycle, probably as a consequence of synchrony loss. After 200 min, once synchrony is lost, *src1* mutant showed a higher proportion of binucleated cells than wild-type. These results are in concordance with the result obtained in asynchronous cultures and support the idea that Src1p is involved in chromatid segregation.

We wondered whether the sister chromatid segregation function characterized for Src1p is shown by the other member of the family, *YDR458c*. Analysis of deletion mutants by fluorescence microscopy showed that in the single *ydr458w* strain nuclear division is identical to that of wild-type strain and that in the double mutant, *src1 ydr458w*, it is identical to the single *src1* mutant (not shown). In addition, *YDR458c* gene is neither differentially expressed along the cell cycle (Spellman *et al.*, 1998) nor induced during sporulation (Chu *et al.*, 1998). These observations suggest that *YDR458c* is not functionally related to *SRC1*.

The metaphase–anaphase transition is a key step in cell cycle control (reviewed in Zachariae and Nasmyth, 1998; Morgan, 1999; Nasmyth *et al.*, 2000). Segregation of chromatids is triggered by the activation of APC–Cdc20p complex, which ubiquitinize and target for degradation the securin Pds1p. Destruction of Pds1p liberates separin Esp1p, which then cleaves the cohesin Scc1p. Scc1p, together with other cohesin proteins, constitutes the 'glue' that keeps sister chromatid joined and its destruction initiates sister segregation. In order to gain insight into the function of Src1p in chromatid segregation, *SRC1* gene was disrupted in yeast strains mutated in *CDC20*, *ESP1* or *SCC1*, and the phenotype of the double mutant was analysed. No genetic interaction between *cdc20* and *src1* mutations could be observed, neither did overexpression of *SRC1* suppress the thermosensitive growth defect of the *cdc20*, *esp1* and *scc1* strains. However, a synthetic interaction between *src1* mutation and *esp1* or *scc1* mutations was detected: both *esp1src1* and *scc1src1* double mutants, in contrast to single mutants, failed to grow at 33°C (Figure 6). The synthetic interaction between mutations in the *SRC1* gene, and in genes such as *SCC1* and *ESP1*, known to be involved in the onset of anaphase, confirms the role of Src1p in chromatid segregation.

Scr1p is a nuclear protein

The intracellular location of Src1p was studied by constructing a GFP-tagged version of the protein. GFP coding region was fused to the C-terminus of the YML033w ORF at its genomic locus. The Src1p–GFP fusion was functional as no alterations in chromatid segregation was detected in the strain

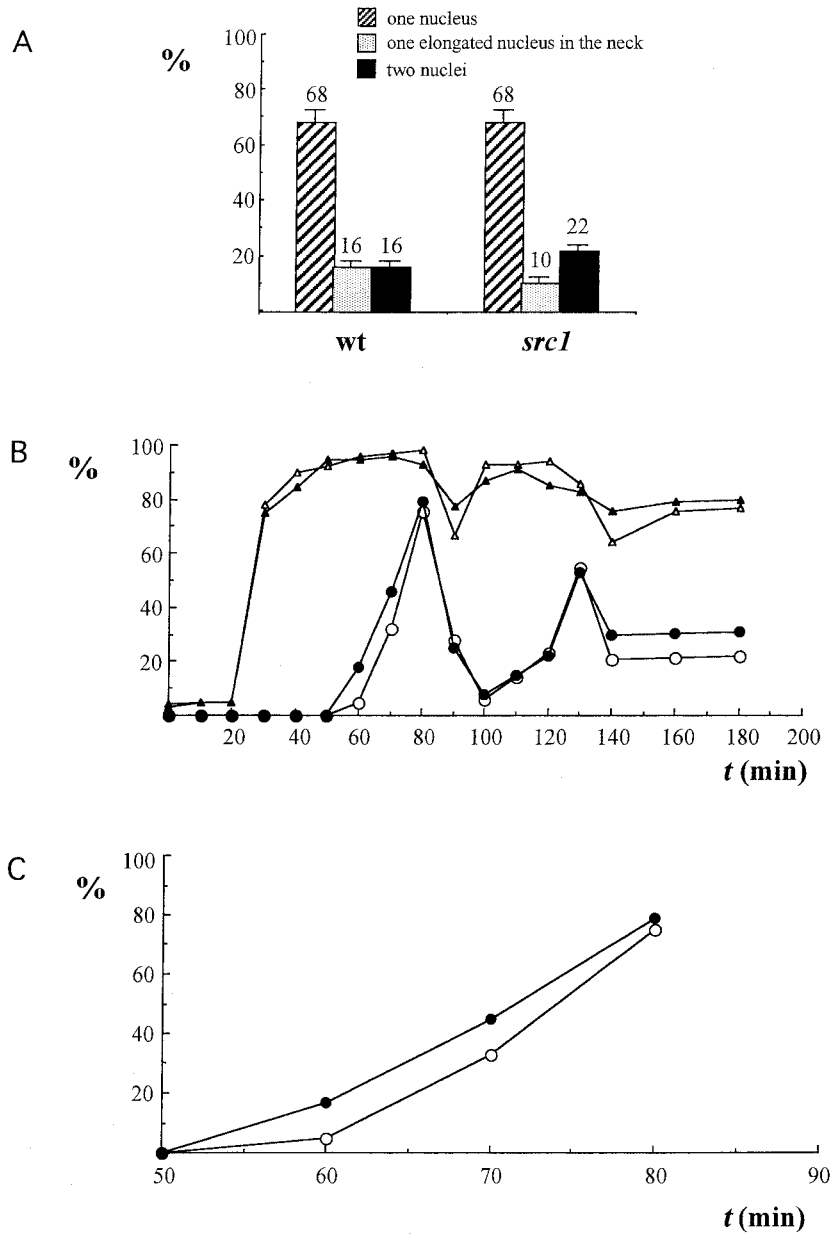


Figure 5. Analysis of nuclear division by fluorescence microscopy. (A) Proportion of cells with one nucleus (interphase plus metaphase cells), one elongated nucleus in the neck (anaphase cells) and two separated nuclei (telophase cells) in wild-type (left) and *src1* (right) exponential cultures. Means and SD were calculated from the results obtained from seven independent cultures in each strain. The total number of cells analysed was ca. 3000. (B) Percentage of binucleated cells in wild-type W303-1a (○) and in the isogenic *src1* (BQS1141) (●) cultures at the indicated time after release from an α-factor arrest. Budding index of both wild-type (△) and *src1* (▲) strains are shown as marker of cell cycle progression. (C) Amplified detail from (B)

carrying the *SRC1 : GFP* gene (data not shown). Cells in exponential culture were stained with DAPI and observed by fluorescence microscopy. As shown in Figure 7, the GFP signal of the Src1p–GFP protein co-localized with the nuclear DAPI signal, indicating that Src1p–GFP is preferentially localized in the nucleus. No changes in the subcellular localization through the cell cycle were

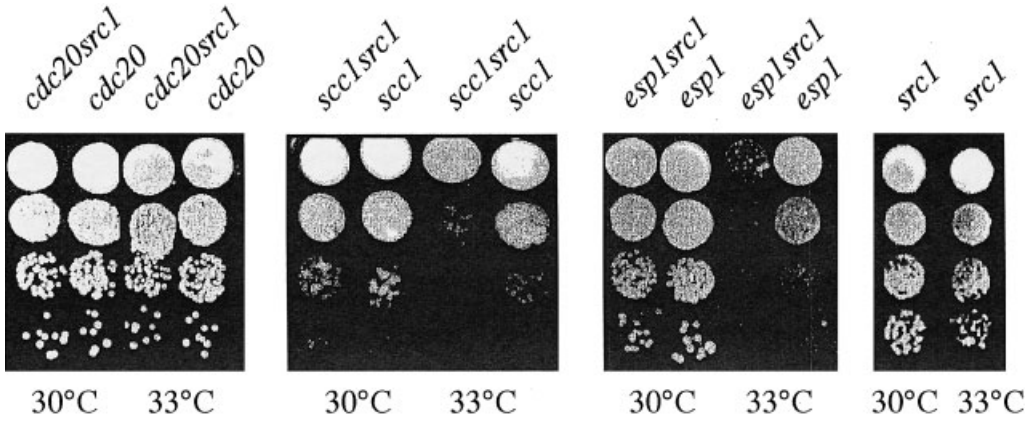


Figure 6. Analysis of synthetic interaction between *src1* and *cdc20*, *scc1* or *esp1* mutations. Ten-fold serial dilutions from an exponential culture of the different strains were spotted on YPD plates and incubated for 2–3 days at the indicated temperatures

detected, as Src1p was observed in the nucleus in cells at different stages of the cell cycle. The nuclear localization of Src1p is consistent with a role for Src1p in chromatid segregation.

Discussion

The study of the physiological roles of a new gene, which has neither homology to another known gene nor any obvious phenotype for its mutation, is a difficult task. Synthetic phenotypes may be look for within genes that belong to a family (Dujon *et al.*, in preparation). However, in many cases, as seems to occur in the *SRC1/YDR458c* family, sequence homology does not necessary reflect a related function. Additional clues to the gene function can be found by examining the results of whole genome analyses and by closely reviewing the sequence features of the unknown gene. In this case we have used both approaches to try to find out the roles of *SRC1*.

Close inspection of the YML033w–YML034w loci showed that the intergenic distance (222 bp) was too small for the standard promoter and terminator lengths in the yeast genome (Dujon,

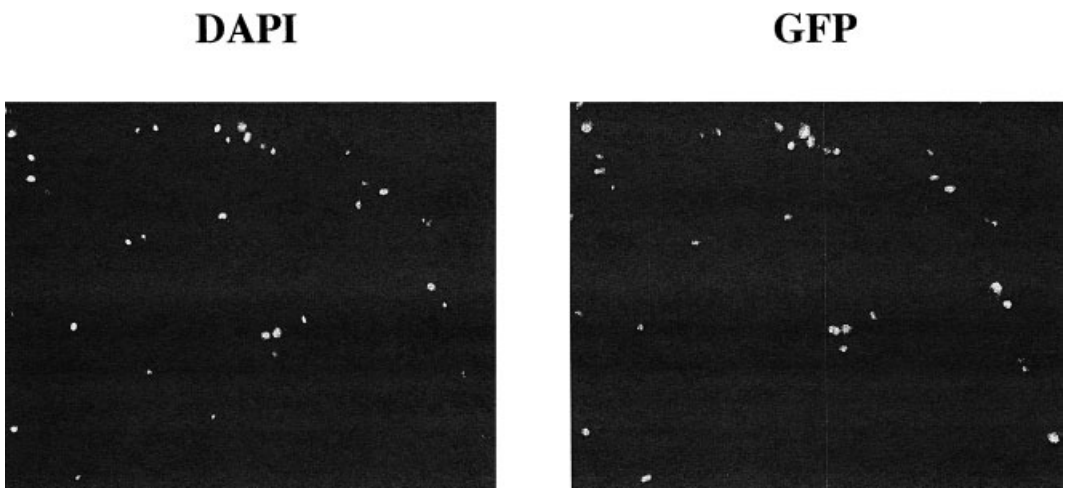


Figure 7. Intracellular location of Src1p–GFP. A wild-type strain containing a GFP-tagged version of the *SRC1* gene was grown to mid-log phase, stained with DAPI and observed by florescence microscopy. DAPI and GFP signals are shown, corresponding to nucleus and Src1p locations, respectively. In a control wild-type strain with untagged *SRC1* gene no GFP signal was detected (not shown)

1996). We conjectured several possibilities for these two ORFs, including the possibility that they could be part of a single gene. We have proved experimentally that a single small intron, whose putative branching sequence is located in between both ORFs, is the reason for the apparently short intergenic distance. This intron is +1916–1920 bp from the ATG, which means that it is the farthest from the Cap site found in yeast to date. Moreover, its 5′ splice sites are not canonical, having two changes in six bases with regard to the consensus site GUAUGU. Probably because of that, it has escaped prediction in all databanks until very recently (Davis *et al.*, 2000).

The existence of alternative splicing is also very rare in yeast, with only two cases having been described (Davis *et al.*, 2000). A consequence of alternative splicing is the generation of different proteins with an alternative C-terminal part. Up to three different proteins could be obtained from *SRC1* gene if we consider the different splicing possibilities. The use of the main 5′ splicing site eliminates a 126 bp intron, avoiding the predicted stop codon for YML034w and keeping the reading frame for the YML033w ORF, therefore a protein of 834 amino acids and 95.5 kDa is translated. The use of the minor 5′ splice site, by contrast, changes the reading frame and would produce a 687 amino acid protein of 77.8 kDa. Because a significant percentage of the mRNA in the cell is unspliced under any growth condition, a third type of protein of 656 amino acids and 74.2 kDa could be synthesized, corresponding to that originally assigned to the old version of YML034w ORF. Because the Src1–GFP protein was obtained by insertion of the GFP tag into the C terminus of the YML033w ORF, it is clear that the 95.5 kDa Src1p variant is synthesized in the cell and that it is localized in the nucleus. The existence of the other two alternative proteins has yet to be proved.

Several intron-containing genes have been described to contain a non-canonical 5′ splice site: MER2 (GUUCGU), *RPL32* (GUCAGU) (Dabeva and Warner, 1987) and *MER3* (GUA_GU) (Nakagawa and Ogawa, 1999). The processing of these 5′ splice site variant introns is dependent on the *NAM8* gene. In addition, Nam8p has been reported to recognize and bind U/A-rich sequences placed just downstream of the 5′ splice site (Puig *et al.*, 1999). In the case of *SRC1* intron, the 5′ splice site is also non-canonical, resembling that of *MER2*, *RPL32* and *MER3*, and its immediate downstream sequence is very U/A-rich. All these facts suggest that this intron should need the participation of Nam8p to be removed and, in fact, our results show that *NAM8* is involved in splicing the *SRC1* intron. However, in contrast to the genes cited above, *SRC1* splicing is not completely dependent on *NAM8*, which suggests that other factor(s) take(s) part in the removal of its intron.

Because the *SRC1* gene is differentially expressed during the cell cycle and during sporulation, an attractive possibility would be that splicing was regulated during these processes. Different observations support this possibility. Elimination of the introns of the *MER2* and *MER3* genes is restricted to the sporulation process. However, we have not detected that the regulation of *SRC1* pre-mRNA splicing is dependent on sporulation. On the other hand, different factors (among them Cdc40p) have been described to be involved in both cell cycle progression and pre-mRNA splicing (Boger-Nadjar *et al.*, 1998; Ben-Yehuda *et al.*, 2000a). Nevertheless, our results show that there is no variation in *SRC1* pre-mRNA splicing through the cell cycle. Moreover, *SRC1* splicing was not affected in a *cdc40* strain (data not shown). Thus, *SRC1* splicing is not regulated during sporulation or the cell cycle, although the expression of the gene is strongly induced under these conditions.

The search for homologous genes to *SRC1* in other yeasts has shown that the *SRC1* gene has a 21% amino acid identity to two *Schizosaccharomyces pombe* ORFs: SPAC14c4.05c and SPAC18g6.10p. It has also been found that *S. bayanus*, *S. servazzii*, *Zygosaccharomyces rouxii*, *Debranomyces hansenii* (E. Bon and C. Gaillardin, personal communication) and *Candida albicans* have putative genes homologous to *SRC1*, with sequence identities of 20–50%. At least the *C. albicans*, *Z. rouxii* and *D. hansenii* counterparts seem to contain an intron located at a position similar to that of the *SRC1* intron. This situation resembles that of *REC114*, a gene that is only transcribed in meiosis, contains an intron placed very far (1241 nucleotides) from the Cap site, and has two paralogues in *S. pastorianus* and *S. paradoxus* with introns located at the same position as in the *S. cerevisiae* gene (Malone *et al.*, 1997). Thus, these observations suggest that the position of the intron in the Src1 protein family might be more important than the amino acid sequence conservation.

Regarding the cellular function of the *SRC1* gene, our data demonstrate that Src1p is involved in mitosis. Fluorescence microscopy analysis of nuclear division reflects a faster segregation of sister chromatids, i.e. a shortening of the anaphase period of the mitosis, when the *SRC1* gene is deleted. Taking into account that cultures of wild-type and *src1* strains show the same percentage of mononucleated cells, this shortening of the anaphase is not accompanied by a reduction in the duration of the whole mitosis. Instead, the shortening of the anaphase must be compensated by the elongation of the post-anaphase period preceding cell division. This is clearly observed in the experiment shown in Figure 5B, where binucleated cells accumulated earlier in the *src1* mutant but cell division occurred at the same time as in the wild-type strain. Thus, the inactivation of *SRC1* affects the anaphase length but not the timing of cell division at the exit of mitosis.

Genetic interactions between *SRC1* with *SCC1* and *ESP1* genes strongly support a role of Src1p in the anaphase. However, the results are not conclusive regarding the molecular function of Src1p. Segregation of sister chromatids to opposite poles of the cell is a two-step process. First, a cohesion step is carried out by the cohesin complex, which acts as a 'glue' between chromatids. Second, a separation step is carried out by the separin Esp1p, which degrades the Scc1p cohesin and thus liberates chromatids for movement to poles by the pulling force exerted by the spindle. A faster segregation of sisters could be due to a defective cohesion that facilitates the separation of chromatids, or to a more effective separin function. The synthetic interaction between *src1* and *scc1* mutations indicates that Src1p functions in establishing or maintaining an appropriate cohesion between sisters. But, surprisingly, a synthetic interaction between deletion of *SRC1* gene and a mutation in the separin Esp1p was also detected, which suggests that the loss of Src1p also causes a defect in the separin function involved in sister segregation. These results could be interpreted by considering that Src1p plays a dual function, acting both in cohesion and in separation, as has also been suggested for the securin *PDS1* (Nasmyth *et al.*, 2000). For instance, Src1p could be required to establish or maintain cohesion, but at the same time be required to locate the separin to the site of action or to enable separin to adopt an active conformation. In the context of the possible alternative splicing of the *SRC1* transcript discussed above, it is tempting to speculate that specific Src1p variant proteins could carry out the different cohesion or separation functions. An alternative explanation of our results, which can not be ruled out, is the possibility that Src1p plays a role in the mitotic spindle function and that the faster segregation of the chromatids in the *src1* mutant was due to a stronger pulling force from the spindle apparatus. Finally, considering the induction of the *SRC1* gene during meiosis, our results suggest that Src1p is probably also involved in the segregation of homologous chromosomes and/or sister chromatids during the meiotic nuclear divisions. Future work will be needed to answer these questions.

Acknowledgements

We would like to thank A. Llopis for technical assistance, M. Pérez-Alonso and E. Queralt for helpful suggestions and discussion, F. Ulhmann for *scc1*, *esp1* and *cdc20* mutant strains, O. Puig for *nam8* mutant strain and his collaboration in the splicing analysis, and C. Gaillardin for sharing non-published sequences of yeast species. This work was supported by Grant No. BIO4-CT97-2294 from the Commission of the European Union within the EUROFAN program, Grants Nos BIO98-1316-CE and PP97-1468-CO-02 from the Spanish Government, and Grant No. GV98-5-86 from the Generalitat Valenciana.

References

Bellí G, Garí E, Piedrafita L, Aldea M, Herrero E. 1998. An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic Acids Res* **26**: 942–947.

Ben-Yehuda S, Dix I, Russell C, Levy S, Beggs JD, Kupiec M. 1998. Identification and functional analysis of hPRP17, the human homologue of the *PRP17/CDC40* yeast gene involved in splicing and cell cycle. *RNA* **4**: 1304–1312.

Ben-Yehuda S, Russell C, Dix I, Levy S, Begg JD, Kupiec M. 2000a. Extensive genetic interactions between *PRP8* and *PRP17/CDC40*, two yeast genes involved in pre-mRNA splicing and cell cycle progression. *Genetics* **154**: 61–71.

Ben-Yehuda S, Dix I, Russell C, *et al.* 2000b. Genetic and physical interactions between factors involved in both cell cycle progression and pre-mRNA splicing in *Saccharomyces cerevisiae*. *Genetics* **156**: 1503–1517.

Blandin G, *et al.* 2000. Genomic exploration of the hemiascomycetous yeasts: 4. The genome of *Saccharomyces cerevisiae* revisited. *FEBS Lett* **487**: 31–36.

Boger-Nadjar E, Vaisman N, Ben-Yehuda S, Kassir Y, Kupiec M. 1998. Efficient initiation of S-phase in yeast requires Cdc40p, a protein involved in pre-mRNA splicing. *Mol Gen Genet* **260**: 232–241.

Brachmann CB, Davies A, Cost GJ, Caputo E, Hieter P, Boecke

JD. 1998. Designer deletion strain derived from *Saccharomyces cerevisiae* S288C: a useful set of strain and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**: 115–132.

Bianchi MM, Sartori G, Vandenbol M, *et al*. 1999. How to bring orphan genes into functional families. *Yeast* **15**: 513–526.

Chu S, De Risi J, Eisen M, *et al*. 1998. The transcriptional program of sporulation in budding yeast. *Science* **282**: 699–705.

Dabeva MD, Warner JR. 1987. The yeast ribosomal protein L32 and its gene. *J Biol Chem* **262**: 16055–16059.

Davis CA, Grate L, Spingola M, Ares M. 2000. Test of intron predictions reveals novel splice sites, alternatively spliced mRNAs and new introns in meiotically regulated genes of yeast. *Nucleic Acids Res* **8**: 1700–1706.

DeRisi JL, Iyer VR, Brown PO. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**: 680–686.

Dujon B. 1996. The yeast genome project: what did we learn? *Trends Genet* **12**: 263–270.

Gasch AP, Spellman PM, Kao CM, *et al*. 2000. Genomic expression programs in the response of yeast to environmental changes. *Mol Biol Cell* **11**: 4241–4257.

Holloway SL, Glotzer M, King RW, Murray AW. 1993. Anaphase is intiated by proteolysis rather than by inactivation of maturation-promoting factor. *Cell* **73**: 1393–1402.

Hughes TR, *et al*. 2000. Functional discovery via a compendium of expression profiles. *Cell* **102**: 109–126.

Ito T, Tashiro K, Muta S, *et al*. 2000. Toward a protein–protein interaction map of yhe budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc Natl Acad Sci U S A* **97**: 1143–1147.

Jelinsky SA, Samson LD. 1999. Global response of *Saccharomyces cerevisiae* to an alkylating agent. *Proc Natl Acad Sci U S A* **96**: 1486–1491.

Lelivelt MJ, Culbertson MR. 1999. Yeast Upf proteins required for RNA surveillance affect global expression of the yeast transcriptome. *Mol Cell Biol* **19**: 6710–6719.

Llorente B, *et al*. 2000. Genomic exploration of the hemiascomycetous yeasts: 20. Evolution of gene redundancy compared to *Saccharomyces cerevisiae*. *FEBS Lett* **487**: 122–133.

Longtine MS, McKenzie A, Demarini DJ, *et al*. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14**: 953–961.

Malone RE, Pittman DL, Nau JJ. 1997. Examination of the intron in the meiosis-specific recombination gene *REC114* in *Saccharomyces*. *Mol Gen Genet* **225**: 410–419.

Marcotte EM, Pellegrini M, Thompson MJ, Yeates TO, Eisenberg D. 1999. A combined algorithm for genome-wide prediction of protein function. *Nature* **402**: 83–86.

Marton MJ, *et al*. 1998. Drug target validation and identification of secondary drug target effects using DNA microarrays. *Nature Med* **4**: 1293–1301.

Morgan D. 1999. Regulation of the APC and the exit from mitosis. *Nature Cell Biol* **1**: E47–53.

Nakagawa T, Ogawa H. 1999. The *Saccharomyces cerevisiae MER3* gene, encoding a novel lelicase-like protein, is required for crossover control in meiosis. *EMBO J* **20**: 5714–5723.

Nasmyth K, Peters JM, Ulhmann F. 2000. Splitting the chromosome: cutting the ties that bind sister chromatids. *Science* **288**: 1379–1384.

Puig O, Gottschalk A, Fabrizzio P, Seraphin B. 1999. Interaction of the U1 snRNP with nonconserved intronic sequences affects 5′ splice site selection. *Genes Dev* **13**: 569–580.

Sambrook J, Fritsch E, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: New York.

Sherman F, Fink GR, Hicks JB. 1986. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press: New York.

Spellman PT, Sherlock G, Zhang MQ, *et al*. 1998. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol Biol Cell* **9**: 3273–3297.

Uetz P, *et al*. 2000. A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature* **403**: 623–627.

Ulhmann F, Lottspeich F, Nasmyth K. 1999. Sister-chromatid separation at anaphase onset promoted by cleavage of the cohesin subunit Scc1p. *Nature* **400**: 37–41.

Wach A, Brachat A, Pöhlmann R, Philippsen P. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793–1808.

Zachariae W, Nasmyth K. 1999. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev* **13**: 2039–2058.

