Genome-wide search of Schizosaccharomyces pombe genes causing overexpression-mediated cell cycle defects

Victor A. Tallada¹, Rafael R. Daga¹,²,³, Cristina Palomeque², Andrés Garzón¹ and Juan Jimenez¹,²*

¹ Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, Sevilla, Spain
² Departamento de Genética, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain
³ Centro de Investigación del Cancer, Universidad de Salamanca, CSIC, Salamanca, Spain

*Correspondence to: Juan Jimenez, Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, Carretera de Utrera, Km 1 41013 Sevilla, Spain.
E-mail: jimmar@dex.upo.es

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Abstract

Genetic studies in yeasts enable an in vivo analysis of gene functions required for the cell division cycle (cdc genes) in eukaryotes. In order to characterize new functions involved in cell cycle regulation, we searched for genes causing cell division defects by overexpression in the fission yeast Schizosaccharomyces pombe. By using this dominant genetic strategy, 26 independent clones were isolated from a Sz. pombe cDNA library. The cloned cDNAs were partially sequenced and identified by computer analysis. The 26 clones isolated corresponded to 21 different genes. Among them, six were genes previously characterized in Sz. pombe, 11 were homologues to genes identified and characterized in other organisms, and four represented genes with unknown functions. In addition to known cell cycle regulators encoding inhibitory protein kinases (wee1, pka1) and DNA checkpoint proteins (Pena, rad24), we have identified genes that are involved in a number of cellular processes. This includes protein synthesis (ribosomal proteins L7, L10, L29, L41, S6, S11, S17 and the PolyA-Binding Protein PABP), protein degradation (UBI3), nucleolar rRNA expression (fib, imp1, dbp2), cell cytoskeleton (act1) and glycolysis (pfk1). The interference caused in the cell cycle by overexpression of these genes may elucidate novel mechanisms coupling different cellular processes with the control of the cell division. The effect caused by some of them is described in more detail. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: cell cycle control; fission yeast; dominant genetics; overexpression

Introduction

The isolation and characterization of genes involved in cell division control (cdc genes) in yeasts have strongly contributed to the understanding of molecular mechanisms governing the cell cycle in all eukaryotes (Nurse, 1990). A classic genetic strategy to identify cdc genes in yeasts is to screen for recessive — loss of function — mutants causing cell cycle defects after mutagenic treatments. cdc functions are usually essential, therefore mutational inactivation of these genes often requires the use of a conditional procedure (Nurse et al., 1976; Toda et al., 1983; Jimenez and Oballe, 1994). Different genetic screenings may reveal complementary sets of cell division control genes. Strategies based on intergenic suppression (Huynh et al., 1986), protein–protein interactions (Niethammer and Sheng, 1998), differential gene expression (Chu et al., 1998) or differential cellular localization (Sawin and Nurse, 1996), have also been extremely helpful to identify new cell cycle regulators and in building up a network of molecular functions operating at each discrete step of the cell cycle.

Dominant genetics has also been useful for the identification of yeast genes whose overproduction causes special phenotypes (Espinet et al., 1995; Javerzat et al., 1996). In this study, we
searched for \textit{Schizosaccharomyces pombe} genes causing division defects upon overexpression in order to identify new functions involved in cell cycle regulation. This strategy has proved to be useful in budding yeast (Stevenson \textit{et al.}, 2001). For this purpose, a \textit{Sz. pombe} cDNA library under transcriptional control by the \textit{nmt1} promoter (thiamine-repressible) was used. The \textit{nmt1} promoter is not cell cycle-regulated and undergoes a very high level of expression under derepression conditions (Maundrell, 1993). Thus, new genes involved in cell cycle control which require cell cycle-regulated transcription, low or moderate levels of expression or stoichiometric production of the encoded protein (with regard to other partners) are good candidates to be identified by this dominant genetic strategy.

In fission yeast, phenotypes diagnostic of division defects are reliably identified, either as very elongated cells that do not progress throughout the cell cycle (\textit{cdc}− phenotype) (Nurse \textit{et al.}, 1976) or as cells that enter into mitosis prematurely at a reduced size (\textit{wee} phenotype) (Fantes 1981; Russell and Nurse, 1987). Here we report the isolation and partial characterization of a large number of clones that cause division defects (\textit{cdc}− or \textit{wee}-like phenotypes) when overexpressed. Some of them establish unexpected pathways connecting different cellular processes with the control of the cell cycle.

\section*{Materials and methods}

\textbf{Yeast strains and general methods}

The standard media and genetic procedures used in this work have been described previously (Moreno \textit{et al.}, 1991). The \textit{Sz. pombe} 972 \textit{(h−} leu1-32 haploid strain and the isogenic \textit{wee1::ura4+ leu1-32} (deleted for \textit{wee1}) and \textit{cdc2-3w leu1-32} mutant strains (Nurse \textit{et al.}, 1976; MacNeill and Nurse, 1993; Russell and Nurse, 1987) were used. Yeast transformation was achieved by electroporation (Prentice, 1992) and basic molecular procedures were carried out as described in Sambrook \textit{et al.} (1989).

For Western blot analysis, a C-terminal HA-tagged version of \textit{Pfk1} was integrated in its locus and a C-terminal HA-tagged version of the truncated gene product of \textit{Pfk1} (\textit{Pfk1-d2p}) was integrated in the \textit{Leu1} locus under the \textit{nmt1} promoter. Both integrations were made using the PJK148 integrative vector (Keeney and Boeke, 1994).

\section*{Screening procedure}

The \textit{Sz. pombe} cDNA library used in this study is cloned in the pREP3X multicopy plasmid under control by the \textit{nmt1} promoter (Maundrell, 1993). Cells of the \textit{Sz. pombe leu1-32} or \textit{cdc2-3w leu1-32} strains were transformed with the cDNA library and \textit{leu+} colonies (the pREP3X plasmid contains the \textit{leu1+} gene), selected in EMM agar plates supplemented with thiamine. These colonies were replica-plated into batches of EMM media with and without thiamine, and incubated at 25°C. Colonies from the EMM media without thiamine were replica-plated once more after 48 h of incubation to a new batch of thiamine-free media containing 2.5 mg/l of phloxin B (Moreno \textit{et al.}, 1991). Cells of non-growing pink colonies identified in these phloxin B plates (after 3 days at 25°C) were further examined under the microscope. All colonies containing either elongated or \textit{wee}-like cells were selected, and plasmids rescued by transformation into \textit{E. coli} by standard procedures (Jimenez \textit{et al.}, 1990). The phenotype caused by the isolated plasmids was verified by re-transformation in yeast cells. Both ends of all positive clones were sequenced and identified by comparison with current DNA databases.

\section*{Fluorescent microscopy and flow cytometry analysis}

Cells exponentially growing in 50 ml EMM media with thiamine were collected by centrifugation, washed and resuspended in 100 ml thiamine-free EMM media. 10 ml samples were taken periodically, and cells fixed and stained with DAPI and Calcofluor white for fluorescent microscopy analysis (Alfa \textit{et al.}, 1992), or with propidium iodine for flow cytometry analysis (Sazer and Sherrwood, 1990) (a Becton Dickinson FACsort system was used).

\section*{Expression analysis}

In Northern blot analysis, total RNA was prepared as described (Moreno \textit{et al.}, 1991) and probed with a 1.8 Kb \textit{wee1} carboxy-terminal PCR fragment, amplified from genomic DNA using the oligonucleotides DWS3: 5′-AGAATTCCATATTTATGATGAAAAG-3′ and DWA3: 5′-TTGGATCCATTCCTATTTCCAT-3′. Filters were analysed using a PhosphorImager system (Fujifilm).
Western blot procedures were performed loading equivalent amounts of protein in SDS–polyacrylamide gels, and the blots carried out using GeneScreen Plus membranes according to the manufacturer’s (NEN) instructions. Tagged proteins were detected using anti-Ha monoclonal antibody (1/40 000 dilution) (Sigma). The blots were developed with an enhanced chemiluminescence method (ECL, Amersham).

**Results and discussion**

**Isolation of overexpression sensitive clones**

Interference in the cell cycle caused by overexpression is a useful genetic procedure to identify key mitotic regulators. In the fission yeast, this strategy has been achieved by using cDNA libraries subjected to the expression of the thiamine-repressible *nmt1* promoter (Moreno and Nurse, 1994; Javerzat *et al*., 1996). However, the low amount of thiamine required for the repression of the *nmt1* promoter, together with the lag required for its maximal expression after derepression (more than four generation times) (Maundrell, 1993), make difficult the efficient identification of overexpression-sensitive clones. In a reconstruction experiment, replica-plated colonies (from EMM with thiamine to thiamine-free EMM media) containing the *rum1* cDNA under *nmt1* expression in the pREP3X plasmid (the pREP3X *nmt:rum1* construction) caused a severe cell cycle arrest (not shown), easily recognised at the colony level (this CDK inhibitor was isolated by means of a similar overexpression screening procedure; Moreno and Nurse, 1994). However, colonies bearing a pREP3X *nmt:wee1* construction (*wee1* encodes a mitotic inhibitor which causes a lethal cell cycle arrest when overexpressed) (Russell and Nurse, 1987) were not distinguishable by visual inspection from control colonies bearing the empty pREP3X vector. Efficient discrimination of colonies with the *nmt:wee1* construction required a second replica-plating step to fresh media without thiamine and containing the phloxin B dye (not shown). Therefore, this two-step replica-plating procedure (see methods) was applied to search for new genes that may cause cell cycle defects by overexpression.

*S. pombe* *leu1-32* cells were transformed with a *S. pombe* cDNA library subjected to the expression of the *nmt1* promoter (Moreno and Nurse, 1994), and about 35 000 *leu*<sup>+</sup> transformants were screened in the absence of thiamine for poor growth and red staining in phloxin B plates. Cells from these colonies were examined under the microscope, and those showing cell cycle defects (either elongated or *wee-like* cells) were selected, rescued in *E. coli* and retransformed in *S. pombe* to confirm the plasmid-mediated defect. In this way, 23 plasmids were identified. Colonies showing *wee-like* phenotypes were not found.

Both ends of each cDNA from these plasmids were sequenced (more that 300 bp were obtained for each reaction), and the obtained sequences analysed in the available databases. The isolated clones corresponded to 19 different genes represented from one to three times. Six of those genes corresponded to previously characterized *S. pombe* genes. Another 11 were highly homologous (>50% identity at the protein level) to genes of known functions in other organisms. Finally, two genes had been identified in the *S. pombe* sequencing project (Wood *et al*., 2002) but showed less than 50% identity with any other gene of known function. These genes were named *oca2* and *oca3* (for overexpression-mediated cell cycle arrest).

Proteins encoded by genes with known or predictable function included protein kinases (Wee1p and Pka1p), proteins implicated in DNA synthesis and repair (Rad24p and Pcn1p), a large number of ribosomal proteins (L7, L27B, L28B, L42, S6, S9B and S17), a key polypeptide of the protein destruction machinery (Ubi3p), up to three nucleolar proteins involved in rRNA expression (fibrillarin, Imp1p and Dbp2p), a protein involved in the cell cytoskeleton (Act1p), and a glycolytic enzyme (Pfk1p).

The isolation of established negative regulators of the mitotic cycle, such as *rad24*, *pcn1* (PCNA) and *wee1*, indicates that many of the potential inhibitors of the cell cycle should have been identified in our screening. However, many genes were represented by only one clone, and other known inhibitors, such as *rum1* (Moreno and Nurse, 1994) or *spd1* (Woollard *et al*., 1996), were not found, indicating that the screening is not saturated and other genes could yet be identified using this method.
Identification of overexpression-sensitive clones in cdc2-3w mutant cells

Overexpression of rate-limiting cell cycle activators should produce cells undergoing premature events on the cell cycle. For instance, overproduction of the Cdc25p cell cycle phosphatase causes premature entry into mitosis, yielding viable wee cells (Russell and Nurse, 1986). In the screening described above, cells showing wee phenotype were not observed, possibly because overproduction of mitotic regulators shortening G2 length produce viable and healthy wee cells which can not be identified when searching for lethality. However, wee phenotypes are additive when originated from two different pathways yielding synthetic lethality, e.g. overproduction of Cdc25p is not lethal in wild-type cells but it becomes lethal in wee1-deficient mutants (Russell and Nurse, 1987). Thus, to isolate genes producing lethal mitosis, we carried out a new overexpression-sensitive screening in a Sz. pombe cdc2-3w (wee) genetic background. This cdc2-3w mutant is partially insensitive to Cdc25p and produces a lethal mitosis in cells lacking wee1 (MacNeill and Nurse, 1993). Therefore, this strain should allow us to explore mitotic regulators other than the well characterized Cdc25p phosphatase (the search is biased in favour of wee1-inhibitors). From about 70,000 colonies screened, a large number of overexpression-sensitive clones

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Biological function</th>
<th>Det.</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wee1</td>
<td>Protein kinase</td>
<td>Mitotic inhibition by phosphorylation of Cdc2p</td>
<td>E</td>
<td>SPCC1885.03</td>
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<tr>
<td>pka1</td>
<td>Protein kinase (cAMP-dependent)</td>
<td>Meiotic control, signal transduction and protein degradation</td>
<td>E</td>
<td>SPBC106.10</td>
</tr>
<tr>
<td>pcn1</td>
<td>Nuclear antigen (PCNA). Interacts with d-cyclins and p21</td>
<td>DNA synthesis and repair</td>
<td>E</td>
<td>SPBC16D10.09</td>
</tr>
<tr>
<td>rad24</td>
<td>14–3–3 Homologue</td>
<td>DNA damage checkpoint</td>
<td>E</td>
<td>SPAC8E11.02c</td>
</tr>
<tr>
<td>rps6-1, rpi7-b/7-c, rpl27-2, rps9-2, rpl42, rpl1-2</td>
<td>Ribosomal proteins S6, L7, L28B, L27B, S9B, L42, S17</td>
<td>Protein synthesis</td>
<td>S</td>
<td>SPAC13G6.07c, SPBC18H10.12c, SPCC5E4.07, SPCC74.05, SPBC29A3.12, SPAC15E1.03, SPCC24810.09</td>
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<tr>
<td>ubi3</td>
<td>Ribosomal protein S3–ubiquitin fusion protein</td>
<td>Protein degradation</td>
<td>S</td>
<td>SPAC6G10.11c</td>
</tr>
<tr>
<td>dbp2</td>
<td>p68 RNA helicase</td>
<td>rRNA processing</td>
<td>S</td>
<td>SPBP887.16c</td>
</tr>
<tr>
<td>fdr</td>
<td>Fibonlin</td>
<td>Nucleolar protein required for pre-rRNA processing</td>
<td>S</td>
<td>SPBC2D10.10c</td>
</tr>
<tr>
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<td>Actin</td>
<td>Cell cytoskeleton and polarity</td>
<td>E</td>
<td>SPACTOKYO453.25c</td>
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<tr>
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<td>Phosphofructo-kinase I</td>
<td>Glycolysis</td>
<td>E</td>
<td>SPBC16H5.02</td>
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<td>popb*</td>
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<td>mRNA stability and modulation of translation initiation</td>
<td>S</td>
<td>SPAC57A7.04c</td>
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<tr>
<td>imp1</td>
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<td>Nucleolar structure, RNA</td>
<td>S</td>
<td>SPBC1604.08c</td>
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<tr>
<td>acc2</td>
<td>Putative ser–thr kinase</td>
<td>Regulation of transport of low molecular weight molecules</td>
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<td>Putative cytochrome B5</td>
<td>Electron transport</td>
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<td>SPCC16A11.10c</td>
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*Identified in the cdc2-3w genetic background screening.
Det., Determination of function.
were also identified in this genetic background, but only three colonies yielded cells suffering a lethal mitosis. Sequence analysis of the cDNA in plasmids rescued from these colonies indicated that lethality in two of them was due to the same gene, here named oca8, coding for a cytochrome B5 homologue. The third plasmid contained a cDNA corresponding to Sz. pombe polyA-binding protein (PABP), a protein involved in RNA stability and translation initiation (Le et al., 1997; Craig et al., 1998).

All genes identified in the overall screening (either in wild-type or in cdc2-3w genetic backgrounds) are listed in Table 1. The cell cycle phenotypes caused by these genes, and a preliminary characterization of the effects caused by some of them (representing diverse cellular processes), are described and discussed below.

Inhibitory protein kinases (weel and pka1)

Protein kinases are key enzymes involved in intracellular signalling pathways and cell cycle control. Two of the identified clones coded for weel and pka1, respectively, two protein kinases which are well known inhibitors of the Sz. pombe cell cycle (Russell and Nurse, 1986; Maeda et al., 1994; Yu et al., 1994). Restriction analysis indicated that the isolated weel clone did not contain a full-length cDNA. This observation was confirmed by DNA sequence and Northern blot (Figure 1c). The derived sequence indicated that translation of the putative Wee1 protein encoded by this cDNA is initiated at the Met314 residue, thereby yielding a Wee1 mutant protein which lacks the first 313 amino acids (see scheme in Figure 1a). However, the truncated weel version (here named weel-d313) efficiently inhibited the cell cycle, causing very elongated cells (Figure 1b), indicating that this NH2-terminal domain of the protein is not essential for its biochemical activity.

Inhibitory phosphorylation of Wee1p occurs in Sz. pombe cells throughout the activity of the Nim1p kinase (Feilotter et al., 1991). This weel-inhibitor acts on the carboxy-terminal end, and therefore the truncated Wee1-d313p protein should retain nim1-dependent regulation. It has been shown that the Sz. pombe Wee1p protein is inhibited by phosphorylation at the NH2-terminus in Xenopus mitotic extracts by an unidentified kinase different from Nim1p (Tang et al., 1993). Interestingly, our truncated Wee1-d313p protein loses 15 of the 22 putative phosphorylation sites of the amino-terminal domain (Tang et al., 1993), indicating that phosphorylation of most of these NH2-terminal sites can be lost without abolishing activity (see Figure 1). In Sz. pombe, the Cds1p kinase is thought to activate Wee1p by phosphorylating its NH2-terminal domain in response to DNA damage (Boddy et al., 1998). Thus, the amino-terminus of Wee1p is not required for its activity (Figure 1), but it could play a regulatory role in coupling mitosis to the DNA checkpoint machinery (Boddy et al., 1998).

The multi-copy expression of the genomic pka1 gene bypasses meiotic differentiation and provokes

![Figure 1](image-url)
a G2 delay in fission yeasts (Maeda et al., 1994, Yu et al., 1994). The higher level of overexpression attained in the nmt:pka1 construction isolated in our screening produced a different phenotype. To better characterize the effect of this gene in the cell cycle, a DNA fragment containing the corresponding nmt:pka1 construction was subcloned into the pJK148 Sz. pombe integrative plasmid (Keeney and Boeke, 1994) and stable leu+ integrant selected for each gene (integration is targeted to the leu1 gene in a leu1-32 strain). As determined by flow cytometry analysis and microscopic observation, the overexpression of this protein kinase produces a very efficient cell cycle arrest at the G2 phase after 18 h in the absence of thiamine (Figure 2a,b). Further incubation in the absence of this vitamin produced cells that became extremely swollen in the middle (see in Figure 2c). Sz. pombe regulates intracellular cAMP levels, and therefore cAMP-dependent protein kinase A (PKA) activity, in response to changes in nutrient conditions (Jin et al., 1995). The effect of pka1 in the G2–M transition suggests that the activity of this kinase could influence cell cycle progression in response to nutritional conditions.

DNA checkpoint genes (rad24 and pcn1)

During the cell cycle, DNA is replicated and segregated into two daughter cells. rad24 is involved in a DNA checkpoint system ensuring that damaged DNA is repaired before mitotic segregation is attempted (Ford et al., 1994). Like other 14–3–3 homologues, Rad24p binds to phosphorylated substrates and promotes their export from the nucleus. The mitotic activator Cdc25p is included among these substrates and, after DNA damage, Rad24p blocks entry into mitosis by sequestering Cdc25p in the cytoplasm until DNA is repaired (Zeng and Piwnica-Worms, 1999). Thus, the overexpression-mediated cell cycle arrest found in this study agrees with its established role in the control of the cell cycle.

The Sz. pombe proliferating cell nuclear antigen (PCNA) is a multifunctional protein. It is able to enhance DNA synthesis and is essential for DNA repair (Waseen et al., 1992; Arroyo et al., 1996). In agreement with our results, a cell cycle block caused by the overexpression of the pcn1 Sz. pombe gene (coding for PCNA) has been previously reported. This cell cycle arrest establishes a direct link between cell cycle regulation and the DNA damage checkpoint pathway (Tournier et al., 1996).

Protein synthesis and degradation (ribosomal proteins and ubiquitin precursor)

Many of the genes identified in our screen are integral components of the protein synthesis machinery. As shown in Figure 3, overexpression of ribosomal protein S6 (an integrated nmt:S6 construction was used) caused a delay in the cell cycle, yielding arrested cells with about twice the wild-type cell size, a single interphase nucleus and 2C DNA content. About 10% of these arrested cells were septated and their nuclear position located off-centre.
New cell cycle regulators in Sz. pombe

Ribosomal protein S6 has been demonstrated to be rapidly phosphorylated as cells are stimulated to grow or divide, suggesting that S6 phosphorylation may be involved in upregulating translation (Jefferies and Thomas, 1996). The overproduction of S6 could arrest the cell cycle by unbalancing the phosphorylation state of this protein in Sz. pombe cells. However, many other ribosomal proteins which are not regulated by phosphorylation caused identical G2 arrests to that produced by S6 (Table 1), suggesting that a common mechanism different to posttranslational phosphorylation is responsible for the cell cycle block caused by overexpression of the ribosomal components.

Ribosomes are large ribonucleoprotein complexes which require precise equimolar amounts of their components for function (Meyujas et al., 1996). The equimolar accumulation of ribosomal proteins is maintained by coordinated regulation at various levels of gene expression (Meyujas et al., 1996). Thus, ectopic overproduction of individual ribosomal proteins could unbalance the stoichiometry of ribosomal components, resulting in a dominant negative effect on the translational machinery. Despite the molecular reason, the large number of ribosomal proteins causing uniform G2-arrested cells upon overexpression suggests the existence of a mechanism coupling the translational machinery to the cell cycle control operating at the G2–M transition. This is in strong agreement with the previously described results coupling translation initiation with entry into mitosis (Daga and Jimenez, 1999).

Degradation by the 26S proteasome of specific proteins that have been targeted by the ubiquitin pathway is the major intracellular non-lysosomal proteolytic mechanism and is strongly implicated in the destruction of mitotic cyclins and other regulatory proteins, such as the Cdc25p cell cycle phosphatase (Nefsky and Beach, 1996; Spataro et al., 1998). Ubiquitination mechanisms use as ubiquitin donors hybrid proteins comprised by ubiquitin peptides fused to a ribosomal protein (Ozkaynak et al., 1987). The identification of a hybrid ubiquitin donor protein in this screening (Ubi3p) suggests that the regulated expression of this peptide could have a significant role on the programmed destruction of cell cycle regulators. Alternatively, the cell cycle defect observed may be due to the unbalance of ribosomal stoichiometry caused by the overexpression of the ribosomal moiety, as discussed above.

Nucleolar functions (p68 RNA helicase, fibrillarin and imp1)

We identified three genes related to nucleolar function/structure and rRNA expression yielding elongated cells upon overproduction (Figure 4). The p68 RNA helicase was first identified in humans because of its immunological cross-reaction with a viral RNA helicase, simian virus 40 large T antigen. Genes related to p68 protein were identified
by low-stringency screening of yeast libraries. The cDNA isolated in our screening corresponds to this p68-related *S. pombe* gene (*dbp2*) previously identified (Iggo *et al*., 1991). It has been shown that p68 undergoes dramatic changes in nuclear location during the cell cycle, lying in the nucleoplasm during interphase and transiently entering the nucleoli during telophase. Despite the pleiotropic phenotype of cells overexpressing p68 (Figure 4), it is remarkable that about 25% of the cells were septated and their nucleus positioned off-centre, a similar phenotype to that observed in 10% of the cells overproducing the ribosomal S6 protein (see Figures 3a, 4). RNA helicases (p68 belong to the DEAD box family of RNA helicases) are involved in many aspects of rRNA metabolism, including splicing, translation and ribosome assembly (Iggo *et al*., 1991). Thus, among other pleiotropic defects, the nmt1-expression of p68 could produce a cell cycle defect by interfering with protein synthesis. A similar situation could explain the cell cycle effect caused by overexpression of the nucleolar protein fibrillarin (encoded by the NOP1 gene in budding yeast), a protein which is required for many post-transcriptional steps in yeast ribosome synthesis (Tollervey *et al*., 1991).

*Imp1* (for importin 1) is homologous to the *Saccharomyces cerevisiae* SRP1 gene. SRP1 was first identified as a suppressor of certain temperature-sensitive mutations in RNA polymerase I in budding yeast (Yano *et al*., 1994), suggesting that, as discussed above, the nmt:imp1 expression could perturb rRNA synthesis. However, this Srp1p protein is homologous to nuclear importins, it is associated with nuclear pores and interacts with multiple components of the cell nucleus that are required for mitosis (Kussel and Frasch, 1995). Thus, overexpression of imp1 could also interfere with the import of mitotic proteins from the cytoplasm into the nucleus.

**Actin**

Actin mediates a variety of essential biological functions in all eukaryotic cells. In addition to providing a structural framework around which cell shape and polarity are defined, its dynamic properties provide the driving force for cells to divide. As we report here, the ectopic expression of this and other cytoskeleton proteins are known to inhibit the mitotic cycle (Hall, 1998).

**A glycolytic enzyme (Pfk1)**

Phosphofructokinase is the limiting enzyme of glycolysis, acting as a key regulator to this metabolic pathway (Lloyd *et al*., 1992; Arvanitidis and Heinisch, 1994; Reuter *et al*., 2000). The conventional cdc*−* phenotype and the abnormal septation pattern observed in cells overexpressing a cDNA coding for Pfk1p (see Figure 5a,b), suggest that this protein may play a dual function in glycolysis and cell cycle regulation that could serve to coordinate this main metabolic pathway with progression throughout the cell cycle. Sequence analysis indicated that the isolated cDNA lacks the 5′-half of the gene (translation of the truncated Pfk1 protein is initiated at residue Met584). This result

**Figure 5. Effects on the cell cycle caused by overexpression of *pfk1*.** (a) Microphotographs of cells (Dapi-stained) containing an integrated nmt:pfk1 construction growing in the presence of thiamine (+T) and after 18 h incubation in the absence of this vitamin (−T). Arrows indicate cells showing abnormal septa. (b) Calcofluor staining of these cells (−T 18H) and of cells incubated for 24 h in the absence of thiamine (−T 24H) was used to show the abnormal pattern of septation induced by overexpression of *pfk1*. (c) Western blot analysis of cells expressing a HA-tagged copy of Pfk1p (1) or a HA-tagged copy of Pfk1-d2p (2)
has been confirmed by Western blot analysis (see Figure 5c). It can be concluded that the effect of Pfk1p on the cell cycle resides on the carboxy-terminal domain of the protein.

Genes with unknown functions (oca2 and oca3)

Two additional cDNAs (oca2 and oca3) causing cell cycle arrest when overexpressed were isolated in this screening.

Sequence comparison identifies Oca2p as a putative ser–thr protein kinase, showing the highest similarity with a family of S. cerevisiae kinases (Hunter and Plowman, 1997) regulating metabolic functions such as amino acid transport (Craene et al., 2001) and RAS/cAMP signalling (Johnston et al., 2001; NPR1), plasma membrane ATPase (Goossens et al., 2000; HRK1) or pheromone biogenesis (Chen et al., 1997; PRR2). As shown in Figure 6, this similarity extends over the C-terminal half of the protein where the catalytic domain is located.

The oca3 gene encodes a small protein composed mainly of two tetratricopeptide repeats. This protein motif is usually involved in protein–protein interactions in multi-protein complexes (Lamb et al., 1995). Some of these complexes might be involved in cell cycle regulation, as is the case of the anaphase-promoting complex (APC) (Lamb et al., 1994). If this is the case for Oca3p, its overexpression can disrupt the stoichiometry of the complex.

Genes identified in a cdc2-3w background (oca8 and PABP)

Only two different cDNAs, one coding for the poly(A) binding protein and another representing...
a novel function (oca8, see Table 1), were identified among genes causing a lethal wee-like phenotype when overexpressed in a cdc2-3w background. As shown in Figure 7a, the integrated nmt:oca8 construction displayed a terminal mitotic defect in the absence of thiamine. Most of the arrested cells had highly condensed DNA, allowing the three Sz. pombe chromosomes to be visualized microscopically. This phenotype indicates that overexpression of oca8 specifically and efficiently impedes progression through mitosis. The mitotic arrest also takes place in a wild-type background (Figure 7a,b), although the lethal effect is slightly exacerbated in cdc2-3w mutant cells. The identified gene did not alter the cell cycle control in a conventional wee-like manner (shortening G2 length), but produced a mitotic arrest which is influenced by the cdc2 cell cycle regulator. Oca8 is one of the two genes encoding for putative cytochrome B in Sz. pombe (Figure 7c). Cytochrome B is involved in electron transport and its role in cell cycle control could be to couple cell division with the energetic state of the cell in a similar way that Pfk1p might coordinate cell cycle with glycolysis. Cells overexpressing Oca8p often showed two or four condensed chromosomes, suggesting that these cells could frequently lose and gain chromosomes. The wide range of DNA content observed by flow cytometry in these cells, as well as the occurrence of cells without DNA in this analysis (see arrows in distributions of DNA content per cell in Figure 7a), is in agreement with the above microscopic observation, supporting the proposition that an excess of Oca8p could contribute to chromosome missegregation during mitosis.

The effect of the nmt:pabp construction in the cell cycle was similar to that of oca8, its lethal effect being greatly enhanced in a cdc2-3w mutant background. Although its precise function remains to be elucidated, the highly conserved polyA-binding protein mediates polyA-dependent events in translation initiation and mRNA stability (Le et al., 1997; Craig et al., 1998, Pestova et al., 2001). Overexpression of this protein could interfere the translatability of specific cellular mRNAs involved in cell cycle regulation.

Final remarks

Overall, the genes identified in this study causing overexpression-mediated cell cycle defects may highlight new pathways regulating the cell division cycle and mechanisms that are required to coordinate the mitotic cycle with the different activities of the cell (Mitchinson, 1971). In particular, the

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**Figure 7.** (a) Microphotographs of cells (Dapi-stained) containing an integrated nmt:oca8 construction growing in the presence of thiamine (+T) and after 18 h of incubation in the absence of this vitamin (−T) in a cdc2-3w background (left panels) or in a wild-type background (right panels). Arrows indicate cells showing three condensed chromosomes. (b) The distribution of the cell size and the DNA content per cell, determined by flow cytometry analysis, is also shown for each of these strains. Arrows in these distributions indicate peaks corresponding to cells with 1C and 2C, respectively, in wee cells (cdc2-3w background, +T) and that of 2C in wild-type strains (wild-type background, +T). The distribution changes in both cases under overexpression of oca8, and also appears a small fraction of cells without nuclear DNA (see arrows in −T). The cell size only suffers a modest reduction when oca8 is overexpressed (arrow in wild-type background −T). (c) Sequence comparison of the Oca8p protein with Spbc29A10cp; the other Sz. pombe putative cytochrome B5; HsCYB5, the human cytochrome B5; and ScCyb5p, the S. cerevisiae cytochrome B5.
arrest caused by overexpression of ribosomal components, or by nucleolar proteins that could interfere with ribosomal RNA expression, suggests the existence of a mechanism coupling the translational machinery to the cell cycle control. The effect of the cAMP-dependent protein kinase A (pka1) could reveal a mechanism involved in coupling levels of cAMP (a general sensor of the nutritional state of the cell) with entry into mitosis. Similarly, the effect caused by a partial cDNA coding for pfk1 also establishes a novel relationship that could serve to coordinate the energetic flux provided by the glycolytic pathway with progression throughout the cell cycle.

The relevance of this screening is increased by comparison with parallel studies performed in other organisms. A similar overexpression screening has been applied to S. cerevisiae (Stevenson et al., 2001), identifying genes participating in the same cellular pathways as those described in this paper. Similarly to our screening, protein kinase A subunits TPK1 and TPK2, cytoskeletal proteins such as actin (ACT1) or β-tubulin (TUB2), ribosomal proteins, metabolic enzymes such as pyruvate dehydrogenase (PDB1) and acetyl-CoA synthetase (ACS2), RNA helicases and rRNA processing functions (SUB2 and NOP2) and proteins involved in poly-A function (PAPI) are described as possible cell cycle regulators in S. cerevisiae.

The fact that two organisms that diverged as early as one billion years ago (Heckman et al., 2001) retain similar pathways joining cell cycle with cellular processes, strongly suggests that they represent universal mechanisms of cell division control. The idea that main actors in cellular processes can be used to communicate with the cell division machinery, either as a result of some kind of bifunctionality or through the activation of new checkpoint functions, is appealing. These alternative roles have been traditionally difficult to uncover by classical genetic approaches due to the dedicated cellular roles that they perform.

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