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A cytokinesis checkpoint requiring the yeast homologue of an APC-binding protein

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Abstract

Checkpoint controls ensure that events of the cell-division cycle are completed with fidelity and in the correct order. In budding yeast with a mutation in the motor protein dynein, the mitotic spindle is often misaligned and therefore slow to enter the neck between mother cell and budding daughter cell. When this occurs, cytokinesis (division of the cytoplasm into two) is delayed until the spindle is properly positioned¹. Here we describe mutations that abolish this delay, indicating the existence of a new checkpoint mechanism. One mutation lies in the gene encoding the yeast homologue of EB1, a human protein that binds the adenomatous polyposis coli (APC) protein, a tumour suppressor. EB1 is located on microtubules of the mitotic spindle and is important in spindle assembly. EB1 may therefore, by associating with microtubules, contribute to the sensor mechanism that activates the checkpoint. Another mutation affects *Stt4*, a phosphatidylinositol-4-OH kinase. Cold temperature is an environmental stimulus that causes misalignment of the mitotic spindle in yeast and appears to activate this checkpoint mechanism.

Yeast lacking dynein show a delay in the cell cycle before cytokinesis; this delay allows the cell to correct defects in mitotic spindle orientation¹. The degree of the defect in mitotic spindle orientation and the delay in the cell cycle necessary for correction vary. About two-thirds of the cells show mild defects with short delays of ~5 min, and about one-third show more severe defects with longer delays of ~30 min. The existence of this delay suggested that a new cell-cycle checkpoint may exist. To investigate the existence of such a checkpoint, we did a genetic screen in *Saccharomyces cerevisiae*, searching for mutations that might abolish the cell-cycle delay.

We screened for mutations that are lethal in combination with the *act5* mutation. Act5 is necessary for dynein function². Two mutants, *stt4-7* and *yeb1-29*, caused a loss of the cell-cycle delay (see below). Using tetrad analysis we found that *stt4* and *yeb1* were lethal in combination with other mutations affecting dynein function, *dhc1* (ref. 1) and *jnm1* (ref. 3). We cloned *STT4* and *YEB1* by complementation. Linkage analysis showed that the mutations resided in the cloned genes. *Stt4* is a phosphatidylinositol-4-OH kinase⁴. *YEB1* encodes the yeast homologue of a human protein, EB1 (ref. 5). Human EB1 binds the carboxy-terminal region of the tumour suppressor APC (for adenomatous polyposis coli) protein⁵; this may be essential for APC's tumour-suppressing function⁶.

To determine whether the cell-cycle delay induced by loss of Act5 was caused by a checkpoint control mechanism, we examined how the different mutations affected this delay (Fig. 1a). A temperature-sensitive *act5* allele induced the defect in spindle orientation and nuclear migration. The level of penetrance of this phenotype observed here is similar to that observed in the dynein-null mutant *dhc1* (ref. 1). We also constructed a complete disruption of the

YEB1 gene. A *yeb1Δ* haploid was viable and similar in phenotype to the original mutant, *yeb1-29*, as described below.

First, we compared *YEB1* wild-type and *yeb1Δ* mutant cells that had temperature-sensitive mutations in *act5* in time-lapse movies. These movies revealed the cell-cycle delay induced by misalignment of the mitotic spindle directly (Fig. 1b). When the mitotic spindle was orientated correctly, the time from spindle elongation to cell separation was ~50 min, with no delay. When the mitotic spindle was misaligned, some cells in both wild-type and mutant strains corrected their misaligned spindle and completed cytokinesis normally. *YEB1* wild-type cells exhibited delays before cytokinesis. Moreover, *YEB1* wild-type cells that did not realign their spindles halted in the cell cycle and did not proceed to cytokinesis.

In contrast, *yeb1Δ* mutant cells proceeded promptly with cell division even when the bud did not contain a nucleus, generating a binucleate mother and an anucleate daughter. Therefore, *YEB1* wild-type cells delay cell division to correct defects in spindle orientation, whereas *yeb1Δ* mutants proceed to cytokinesis even if the daughter does not contain a nucleus.

Second, we synchronized cells at the same point in the cell cycle by arresting the cell cycle using hydroxyurea. These cells were shifted to the temperature that is restrictive for growth of the *act5* temperature-sensitive mutant and released from the cell-cycle arrest. Delay before cell division caused accumulation of cells with large buds with both nuclei in the mother, and loss of delay caused appearance of binucleate unbudded cells (Fig. 1a). In temperature-sensitive-*act5* *YEB1* cells, large-budded cells with both nuclei in the mother accumulated at 20–25% of mitoses (Fig. 2a and Table 1). The cell-cycle delay was also reflected in the increase in cell number, which was low in temperature-sensitive-*act5* cells relative to *ACT5* cells (Fig. 2c) because of a lack of cytokinesis.

In contrast, temperature-sensitive-*act5 yeb1Δ* cells showed no delay before cell division, as is shown by the increased number (~40%) of binucleate unbudded cells (Fig. 2a and Table 1). The *yeb1-29* (Table 1) and *stt4-7* mutants behaved similarly. Loss of cell-cycle delay in *yeb1* mutants was also reflected in the increase in cell number, which returned to normal (Fig. 2c). Synchronization with α -factor instead of hydroxyurea gave similar results, albeit with less synchrony (Fig. 2d).

When a cell-cycle delay is abolished by a loss-of-function mutation, a checkpoint control mechanism is the likely cause⁷ of the delay. Complete disruption of *YEB1* caused a loss of the delay before cytokinesis in *act5* mutants; therefore, this delay is probably due to a checkpoint mechanism.

In the synchronized cell experiment, temperature-sensitive-*act5 yeb1* mutants experienced a loss of viability of 25–30% (Fig. 2b). The percentage of binucleate unbudded plus anucleate cells was similar (25–30%; Fig. 2a, d and Table 1), indicating that these cells may be inviable. *yeb1* mutants also have a mild mitotic defect (see below), which may contribute to this inviability.

We determined the relationship between this checkpoint and other checkpoint mechanisms. We used hydroxyurea and ultraviolet irradiation to induce the DNA-replication and -damage checkpoints and used nocodazole to induce the spindle-assembly checkpoint^{8,9}. As positive controls, we used a *mad1Δ* strain for the spindle-assembly checkpoint and *mec1* or *rad9* strains for the DNA checkpoints. During growth on solid media, hydroxyurea treatment and release from treatment or ultraviolet irradiation had no effect on wild-type and *yeb1* strains under conditions in which *mec1* mutants did not grow. Nocodazole treatment and release from treatment had no effect on wild-type and *yeb1* strains under conditions in which *mad1Δ* mutants did not grow. Treatment of synchronized cells with hydroxyurea, ultraviolet light or nocodazole

caused wild-type and *yeb1* cells to arrest with undivided nuclei (<5% cells with divided nuclei), 90 min after release from treatment with α -factor. No arrest occurred in a nocodazole-treated *mad1* strain (62% cells with divided nuclei), an ultraviolet-irradiated *rad9* strain (73% cells with divided nuclei), and a hydroxyurea-treated *mec1* strain (63% cells with divided nuclei). Therefore, the *yeb1* mutant has intact spindle-assembly and DNA-replication and -damage checkpoints.

We investigated how EB1 functions *in vivo*. APC binds to microtubules *in vitro* and *in vivo*^{10,11}. EB1 localizes to microtubules *in vivo*, in both budding and fission yeast^{12,13}. EB1 functions in the microtubule cytoskeleton of budding and fission yeast, judging from phenotypes resulting from loss of function and overexpression of EB1^{12,13}. An asynchronous population of *yeb1* Δ cells showed an increased number of large-budded cells with monoastral spindles. To investigate further, we examined the kinetics of spindle assembly and elongation. Formation of the short pre-anaphase bipolar spindle was delayed in *yeb1* Δ cells by ~50 min (Fig. 3a). The overall doubling time also differed by 50 min, with 210 min for *yeb1* Δ cells versus 160 min for wild-type cells. Elongation of the spindle from short to long was normal in the *yeb1* Δ mutant (Fig. 3b). The fine structure of the spindle and spindle pole bodies of *yeb1* Δ cells was unremarkable by thin-section electron microscopy (data not shown). Thus the *yeb1* Δ mutant has a mild defect in its rate of progression through the early phase of spindle assembly.

Cold temperature often aggravates mitosis and nuclear-migration defects^{2,14,15}, presumably because microtubules are unstable in the cold. *yeb1* Δ mutants grew poorly at 14 °C, so we proposed that cold temperature induced the EB1 checkpoint through abnormal spindle orientation due to reduced microtubule stability. Among wild-type cells grown at 14 °C for 36 h, ~10% were large-budded with both nuclei in the mother (Fig. 4); this defect is similar to that seen in dynein mutants at 30 °C. Binucleate unbudded cells were rarely seen. *yeb1* Δ cells grown at 14 °C for 36 h showed binucleate and multinucleate unbudded cells, in addition to large-budded binucleate cells. In *yeb1* Δ cells grown at 14 °C for 80 h, numbers of multinucleate cells increased dramatically. The nuclear-migration defect in wild-type cells at 14 °C and the multinucleate cells seen in *yeb1* Δ cells indicate that the EB1 checkpoint may be activated by the cold. Yeast in the wild experience cold temperatures, which may be the physiological activator of the EB1 checkpoint.

We tested whether the cold sensitivity of *yeb1* mutants was due to enhancement of the mitotic phenotype. The percentage of large-budded cells with a monoastral spindle, which reflects the mitotic phenotype of *yeb1* mutants, was 7% in *yeb1* cells at 30 °C, 4% in *yeb1* cells at 14 °C, and 1% in wild-type cells at either temperature. Therefore, the *yeb1* mitotic phenotype was not enhanced in the cold.

We have provided evidence for the existence of a new cell-cycle checkpoint that occurs before cytokinesis. This checkpoint is important for cells to maintain neutral ploidy under conditions that induce mitotic spindle misorientation, including cold temperature. As EB1 localizes to spindle microtubules and as the loss of EB1 slows spindle assembly, EB1 may be a necessary component of the checkpoint's sensor mechanism. After sensing the misorientated spindle, the EB1 checkpoint may activate a signal to delay the cell cycle. The phosphatidylinositol-4-OH kinase Stt4 may be an element of this signalling pathway. As aneuploidy is often observed in cancer cells, human EB1 may be necessary for APC's tumour-suppressor function through this checkpoint. Yeast do not have obvious sequence homologues of APC, but the function of EB1 in yeasts and humans may be similar because human EB1 rescues the *Schizosaccharomyces pombe* EB1 mutant¹². This checkpoint may also function in development when the orientation of the mitotic spindle is important for determination of cell fates.

Methods

Strains

Strains were derived from YCH125:MATa *ade2 ade3 ura3 leu2 trp1 LYS2* (YJC1428) and YCH128:MATa *ade2 ade3 ura3 leu2 TRP1 lys2* (YJC1429) cells, donated by C. Hardy. Media and α -factor were prepared and used as described². Nocodazole (Sigma, St Louis, MO) and benomyl (DuPont, Wilmington, DE) at 15 mg ml⁻¹ in dimethylsulphoxide were diluted 1,000-fold into media. 2 M hydroxyurea in water was diluted 20-fold into media.

Mutant isolation

YJC1259:MATa *ade2 ade3 ura3 leu2 TRP1 lys2 act5Δ::HIS3* [CEN *ACT5 URA3 ADE3*] and YJC1355:MATa *ade2 ade3 ura3 leu2 trp1 LYS2 act5Δ::HIS3* [CEN *ACT5 URA3 ADE3*] strains were treated with the mutagen ethyl methanesulphonate to 30% viability, and plated at 25 °C for 4–6 days until red colonies appeared. Solid red colonies and red colonies with small white sectors were tested for growth on 5-fluoro-orotic acid. An *ACT5* plasmid shuffle assay tested for specificity. Mutants were defined as recessive or dominant and placed into complementation groups.

Gene cloning and disruption

Mutants were transformed with a genomic library (ATCC 77162) and incubated at 25 °C until sectoring colonies were identified. Polymerase chain reaction (PCR) was used to exclude *ACT5* plasmids. Insert ends were sequenced and identified in the genome. The overlapping regions of different clones identified candidate open-reading frames (ORFs). Subcloning identified one ORF as necessary and sufficient for rescue. Linkage analysis showed that the rescuing DNA fragment was tightly linked to the original mutation.

Sequence analysis, including our analysis here, confirmed the high level of similarity between *S. cerevisiae* and human EB1, and the existence of EB1 homologues in *S. pombe* and mouse^{12,13}. Database searches with an EB1 protein identified only other EB1 proteins as having high sequence similarity.

A PCR-based method² was used to generate an allele with a complete disruption, *yeb1Δ::HIS3*. Haploid mutants derived from several independent diploids were viable.

Double mutants

yeb1Δ was synthetic-lethal with *dhc1-Δ7* (from K. Bloom) on the basis of tetrad dissection of 32 asci. All predicted double-mutant haploids were inviable. Similar results were obtained for *yeb1Δ* and *jnm1-Δ3* (provided by K. Tatchell) with 21 asci, for *stt4-7* and *dhc1-Δ7* with 20 asci, and for *stt4-7* and *jnm1-Δ3* with 19 asci.

Temperature-sensitive *act5*

A temperature-sensitive allele of *act5* was constructed with the temperature-sensitive degtron system¹⁶. Temperature-sensitive *act5*, plasmid-borne or integrated, conferred temperature sensitivity to growth of three different synthetic-lethal mutants from the screen.

Movie assay

Microtubule and nuclear positions were determined with fusions of green fluorescent protein (GFP) to tubulin and to the SV40 nuclear-localization signal (NLS), respectively. GFP-TUB1 was from A. Straight¹⁷. GFP-SV40-NLS was from J. Haseloff. These strains were also used in cell-cycle-delay assays with α -factor-mediated synchronization.

Cells were grown in synthetic media lacking methionine for 2 h to induce GFP–TUB1. During the second hour, the temperature was raised to 37 °C to inactivate temperature-sensitive *act5*. Cells were placed on agarose with non-fluorescent growth medium supplemented with β -alanine (0.5 mg/l⁻¹), thiamine–HCl (0.2 mg/l⁻¹), biotin (2 μ g l⁻¹), calcium pantothenate (0.4 mg/l⁻¹) and inositol (2 mg/l⁻¹), and time-lapse movies were made¹⁸. At 4-min intervals, a focal series of bright-field and fluorescence images was collected. At cell separation, the daughter shifted its position relative to the mother in the bright-field image. We performed five experiments with temperature-sensitive-*act5 YEB1* cells and seven with temperature-sensitive-*act5 yeb1*Δ cells.

Synchronized cell assay

Temperature-sensitive-*act5* cells grown at 28 °C were synchronized with α -factor alone or with α -factor followed by hydroxyurea¹⁹, shifted to 37 °C for 20 minutes, and released from treatment at 37 °C. α -factor was readded either after 30 min or immediately. Samples were fixed and stained with 4,6-diamidino-2-phenylindole. More than 200 cells were counted for each time point. Viability was determined by plating on YPD medium. Immunofluorescence using anti-tubulin antibodies or GFP–TUB1 confirmed that mitotic spindles were intact in the large-budded cells with both nuclei in the mother.

Cell forms as described in Table 1 were counted at each time point. With use of hydroxyurea, unbudded uninucleate cells at $t = 0$ (8–11%) were not synchronized and therefore assumed not to participate in the experiment. This percentage was subtracted from values obtained at subsequent time points. As one normal mitosis produces two unbudded uninucleate cells, the number of such cells was divided by two to calculate number of mitoses.

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Figure 1.

Movie assay for cell-cycle delay. **a**, Possible outcomes of mitosis in *act5* mutant cells. The upper of each pair of diagrams shows a normal mitosis. The lower shows a mitosis with a misaligned spindle. **b**, Experimental results. Time from spindle elongation to cell separation was measured in (temperature-sensitive) *ts-act5 YEB1* and *ts-act5 yeb1Δ* cells. Groups drawn at left include cells with properly aligned spindles, cells with misaligned spindles that corrected these spindles, and cells with misaligned spindles that did not correct. *N*, number of cells. Some *ts-act5 YEB1* cells did not correct their misorientated spindles (asterisk) and cell separation was not observed. These movies ended at 200–320 min. A time to cell separation of 200 min is significantly different from that of 55 min for normally dividing *ts-act5 YEB1* cells, as is the value of 91 min for corrected misaligned *ts-act5 YEB1* cells ($P < 0.01$).

Figure 2.

Synchronized cell assay for cell-cycle delay. Synchronization was achieved with hydroxyurea and α -factor in **a–c**, and with α -factor alone in **d**. **a, d**, Percentage of mitoses that produce binucleate large-budded cells and binucleate unbudded cells against time following release from arrest. **a**, One representative experiment. Two independent experiments gave similar results. **b, c**, Viability (**b**) and cell number (**c**) for these and additional control strains are plotted against time. Table 1 shows extra data from experiments in which hydroxyurea was used. Wt, wild type; ts, temperature sensitive.

Figure 3. Spindle formation and elongation. **a**, Formation of the short pre-anaphase spindle over time. Cells were synchronized with α -factor and released. **b**, Elongation of the spindle, from short to long, over time. Cells were synchronized with hydroxyurea and released. More than 200 cells were counted for each time point.

Figure 4.

Effect of cold temperature on nuclear segregation. Wild-type and *yeb1Δ* cells grown to late log phase ($>10^7$ cells ml^{-1}) at 30 °C were shifted to 14 °C for 36 h or 80 h. Control cells were kept at 30 °C. Data are shown only for abnormal cell forms: binucleate large-budded cells, binucleate unbudded cells, and multi-nucleate unbudded cells with three or more nuclei. More than 200 cells were counted for each sample. Error bars indicate s.e.m. Cells at 14 °C divided slowly, reaching saturation during the time course of the experiment. Nuc, nucleus.

Table 1

Cell-cycle-delay assay

Time after hydroxyurea release (min)	Strain	Unbudded cells			Budded cells		
		No nucleus	One nucleus per cell	Two nuclei	Both nuclei in mother	One nucleus in mother, one in bud	One nucleus in mother, none in bud
0	WT	0	11	0	1	1	87
	<i>act5-ts</i>	0	10	0	0	0	88
	<i>yeb1Δ</i>	0	9	0	0	0	91
	<i>yeb1Δact5-ts</i>	0.5	8	1	2	1	87
	<i>yeb1-29act5-ts</i>	0.5	8	1.5	2.5	1.5	81
30	WT	0	14	0	0	7	79
	<i>act5-ts</i>	0	13	0	4	5	78
	<i>yeb1Δ</i>	0	11	0.5	0.5	6	81
	<i>yeb1Δact5-ts</i>	0.5	11	1	3	5	79
	<i>yeb1-29act5-ts</i>	1	9	1	2	6	81
60	WT	0	15	0	1	26	58
	<i>act5-ts</i>	0	15	0	8	23	54
	<i>yeb1Δ</i>	0	18	1	1	20	60
	<i>yeb1Δact5-ts</i>	1	17	3	8	19	52
	<i>yeb1-29act5-ts</i>	3	14	4	9	21	49
75	WT	0	36	0	1	45	18
	<i>act5-ts</i>	0	32	0	14	35	19
	<i>yeb1Δ</i>	0	34	1	1	41	23
	<i>yeb1Δact5-ts</i>	8	21	11	8	34	18
	<i>yeb1-29act5-ts</i>	9	22	12	7	35	15
90	WT	0	66	1	1	24	8
	<i>act5-ts</i>	0	44	1	19	25	11
	<i>yeb1Δ</i>	0	59	1	1	26	13
	<i>yeb1Δact5-ts</i>	7	39	15	7	23	9
	<i>yeb1-29act5-ts</i>	10	35	19	6	20	10
105	WT	0	77	0	1	16	6
	<i>act5-ts</i>	0	57	0	16	23	4
	<i>yeb1Δ</i>	1	69	1	1	21	7
	<i>yeb1Δact5-ts</i>	8	55	16	4	16	11
	<i>yeb1-29act5-ts</i>	9	48	18	4	13	8
120	WT	0	84	1	0	13	2
	<i>act5-ts</i>	0	63	0	14	19	3
	<i>yeb1Δ</i>	0	83	1	1	9	6
	<i>yeb1Δact5-ts</i>	15	51	21	2	8	3
	<i>yeb1-29act5-ts</i>	13	55	19	3	6	4
150	WT	0	89	1	0	6	4
	<i>act5-ts</i>	0	77	0	11	9	3
	<i>yeb1Δ</i>	0	87	1	1	8	3
	<i>yeb1Δact5-ts</i>	11	60	17	3	5	4
	<i>yeb1-29act5-ts</i>	12	63	16	3	3	3

Data in the last six columns indicate the percentages of different cell forms resulting over time in the indicated strains. A subset of these data is plotted in Fig. 2. WT, wild type; ts, temperature sensitive.