Elucidation of novel budding yeast separase mutants

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The mitotic separase cleaves Scc1 in cohesin to allow sister chromatids to separate from each other upon anaphase onset. Separase is also required for DNA damage repair. Here, we isolated and characterized 10 temperature-sensitive (ts) mutants of separase ESP1 in the budding yeast Saccharomyces cerevisiae. All mutants were defective in sister chromatid separation at the restricted temperature. Some esp1-ts mutants were hypersensitive to the microtubule poison benomyl and/or the DNA-damaging agent bleomycin. Overexpression of securin alleviated the growth defect in some esp1-ts mutants, whereas it rather exacerbated it in others. The Dro sophila Pumilio homolog MPT5 was isolated as a high-dosage suppressor of esp1-ts cells. We discuss various features of separase based on these findings.

Key words: DNA repair; MPT5; Pumilio; securin; separase

In eukaryotic cells, DNA replication produces two sister chromatids linked by a protein complex called cohesin. Cohesin, consisting of two SMC (structural maintenance of chromosomes) proteins (Smc1 and Smc3), the alpha-kleisin Scc1/Mcd1/Rad21, and Scc3, is proposed to form a ring-like structure that embraces the two DNA duplexes. Scc1 of cohesin is cleaved by the mitotic protease separase at anaphase onset to allow sister chromatids to separate from each other (Fig. 1(A)). Separase is inhibited by its binding partner securin until metaphase. Therefore, lack of the securin protein Pds1 (precocious dissociation of sister chromatids) in the budding yeast Saccharomyces cerevisiae causes precocious dissociation of sister chromatids because of abolishment of its inhibitory action against separase. In addition, securin also promotes separase import from the cytoplasm to the nucleus in G1-S phases. Thus, securin functions as both the activator and repressor of separase. Actually, in fission yeast, Schizosaccharomyces pombe, like separase/Cut1, securin/Cut2, shows the “cut” (cell untimely torn) phenotype (impairment of chromosome separation). In addition, separase is also required for DNA damage repair. DNA double-strand breaks (DSBs) promote separase-dependent dissociation of cohesin loaded during the previous S phase in budding yeast, which is required for effective DSB resection and DNA repair. A non-cleavable Scc1 mutation by separase reduces resection at DSBs and compromises the efficiency of repair. In fission yeast, Separase/Cut1 mutants are hypersensitive to DNA damage.

Furthermore, the budding yeast separase Esp1 has another role in early anaphase. Activation of Cdc14 phosphatase, which reverses phosphorylation of cyclin-dependent kinase (Cdk), is required for condensation and segregation of the highly repeated rDNA (coding rRNA) region in early anaphase. Cdc14 is tethered in the nucleolus by the nucleolar protein Net1 until metaphase, but it is released from the nucleolus by phosphorylation of Net1 by Cdk and polo kinase/Cdc5. This pathway is called as the Cdc14 early anaphase release (FEAR) network. Esp1 promotes the FEAR network and rDNA segregation in early anaphase. Interestingly, this role of Esp1 in the FEAR network is independent of its protease activity (Fig. 1(A)).

Here, we report characterization of newly isolated temperature-sensitive (ts) esp1 mutants that show various interesting aspects of Esp1 function. In addition, we isolated MPT5 as a high-dosage suppressor of esp1-ts mutants. We discuss novel features of yeast separase based on our findings.

Materials and methods

Strains and media. Saccharomyces cerevisiae strains (all W303 background) used are listed in Supplementary Tables S1. Glucose-based YPAD (YPD containing 0.01% adenine) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared in standard ways.

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Abbreviations: APC/C, Anaphase-promoting complex/cyclosome; FEAR, Cdc14 early anaphase release; Cdk, cyclin-dependent kinase; DAPI, 4',6-diamidino-2-phenylindole; DSB, double-strand break; GFP, green fluorescent protein; SAC, spindle assembly checkpoint; ts, temperature sensitive.

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Screening for esp1-ts mutants.

An URA3-containing plasmid, pSCU750 (p416-ESP1-13MycGFP), was used to transform cells of strain SCU69, and genomic ESP1 was deleted by a one-step PCR-mediated gene disruption method13) to produce the strain SCU1331 (esp1::hphMX [p416-ESP1-13MycGFP]). Detailed methods of the construction of strains are available on request. For mutagenesis, a PCR-amplified ESP1 fragment (promoter plus ORF) was treated with 500 μl of hydroxylamine solution (35 μg of hydroxylamine HCl and 90 μg of NaOH) at 37 °C for 20 h. The DNA purified by ethanol precipitation was used for transformation. SCU1331 cells were transformed with LEU2-containing p415-13MycGFP (cut with BamH1/Sal1) and the hydroxylamine-treated PCR-amplified ESP1 fragment. After 5 days of culture on SD-Leu plates incubated at 25 °C, transformants were replica-plated to SD-Leu plus 5-flouro-otic acid to eliminate the URA3-containing plasmid. As a result, 10 esp1-ts strains (viable at 25 °C and lethal at 37 °C) were isolated, and we designated them esp1-104, esp1-106, esp1-107, esp1-108, esp1-109, esp1-204, esp1-301, esp1-304, esp1-305, and esp1-324.

Microscopic assay. Cells expressing green fluorescent protein (GFP) fused with lacI were fixed with 70% ethanol for 30 s. After washing with distilled water, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml for 1 min. Washed cells were viewed using an Olympus IX71-23FL/S microscope (100 × objective, Tokyo, Japan) with a cooled CCD camera (ORCA-ER-1, Hamamatsu Photonics, Hamamatsu, Japan) connected to a Scanalytics Image Processor LuminaVision (Mitani Corp., Tokyo, Japan). esp1-ts cells possessed GFP-tagged Esp1 proteins but their GFP signals were too weak so that we detected no clear images using this microscopic system.

Results

Isolation of esp1-ts mutants

Several esp1-ts mutants in budding yeast have been isolated, and they are all defective in cohesin cleavage.14,15) Conversely, esp1-ts mutants defective in the FEAR pathway have not yet been isolated. We isolated ten esp1-ts mutants in accordance with an authentic method (“Materials and methods”): esp1-104, esp1-106, esp1-107, esp1-108, esp1-109, esp1-204, esp1-301, esp1-304, esp1-305, and esp1-324. These mutants were all viable at 25 °C, but not at 37 °C (Fig. 1(B)), like previously isolated ts mutants esp1-B3 and esp1-N5.15)
Novel budding yeast separase mutants

Some esp1-ts mutants are hypersensitive to benomyl and bleomycin

The spindle assembly checkpoint (SAC) ensures faithful chromosome segregation during cell division.\(^{19,20}\) Securin is an important target of the SAC. In the presence of insufficient kinetochore–microtubule attachments, the SAC inhibits anaphase onset by the inhibition of the E3 ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome)-Cdc20, which drives cells into anaphase by inducing securin degradation. If securin–separase interaction is attenuated in esp1 mutants, precocious activation of separase might occur in SAC-active conditions, and such mutants might be hypersensitive to microtubule depolymerizers. Most of the isolated esp1-ts mutants (except for esp1-107 and esp1-108) were hypersensitive to the microtubule poison benomyl (Fig. 2 and Table 1). It is likely that these benomyl hypersensitive esp1-ts mutants are defective in securin-mediated repression of separase. This is a first description of microtubule poison-hypersensitive growth phenotypes of esp1-ts mutants.

In addition, separase is involved in DNA repair (see “Introduction”). Most of the esp1-ts mutants (except for esp1-109) were hypersensitive to the DNA-damaging agent bleomycin, which generates DNA DSBs (Fig. 2, Table 1). These mutants might be defective in separase-mediated DNA repair. This is a first report of bleomycin-hypersensitive growth phenotypes of esp1-ts mutants. In fission yeast, separase mutants are hypersensitive to DNA damage caused by ultraviolet, X-ray, and \(\gamma\)-ray irradiation.\(^{7}\)

Carbon-poor conditions alleviate temperature sensitivities of some esp1-ts mutants

In the course of this study, we noticed that the semi-permissive temperature of these esp1-ts mutants changed in different nutrient conditions. Semi-permissive temperatures of esp1-ts mutant cells on nutrient-rich YPD plates were lower than those on synthetic SD plates. Ten-fold serial dilutions of cells of esp1-ts strains were spotted in 1 μl drops onto YPAD plates in the presence or absence of the microtubule poison benomyl (15 μg/ml) or the DNA-damaging agent bleomycin (2 μg/ml) and the plates were incubated at 30 °C for 1 day.

**Notes:**

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plates (data not shown), suggesting that nutrient-poor conditions suppressed the temperature sensitivity of these esp1-ts mutants. We assessed this possibility using a carbon-poor source (3% glycerol as carbon source instead of 2% glucose) and a nitrogen-poor source (0.1% proline as nitrogen source instead of 2% glucose). The temperature sensitivities of esp1-106, esp1-204, esp1-301, esp1-305, and esp1-324 were clearly suppressed on carbon-poor medium (Fig. 3, Table 1). In contrast, no clear suppression was observed under nitrogen-poor conditions. Thus, carbon-poor conditions alleviated the temperature sensitivity of some esp1-ts mutants. This is a first finding of nutrient-poor-mediated suppression of growth defects in esp1-ts mutants.

Osmotic stress alleviates temperature sensitivities of some esp1-ts mutants

Interestingly, it has been reported that temperature sensitivities of both the esp1-ts mutants examined (esp1-1 and esp1-478) were suppressed by osmotic stress (1.2 M sorbitol).21) In fission yeast, similar features were also observed in all the cut1 mutants examined.21) Thus, osmotic stress mitigates separase defects in both yeasts. We confirmed this: temperature sensitivities of most, but not all, of esp1-ts mutants (except for esp1-204) were alleviated by addition of sorbitol (Fig. 4). Our findings indicated that osmotic stress-mediated growth recovery in esp1-ts mutations is also allele-specific.

Effects of securin overexpression on esp1-ts mutants

Securin regulates separase both positively and negatively (see “Introduction”). If securin-mediated positive functions to separate in esp1-ts mutants were compromised, overexpression of securin should alleviate their growth defect. In contrast, if securin-mediated negative functions against separase were compromised in esp1-ts mutants, securin overexpression should exaggerate their growth defect. Securin overexpression mitigated temperature sensitivity in esp1-104, esp1-107, and esp1-108 mutants, whereas it exacerbated temperature sensitivity in esp1-109, esp1-301, and esp1-304 mutants (Fig. 5, Table 1). These findings suggested that

![Fig. 3. Carbon-poor conditions alleviate the temperature sensitivities of some esp1-ts mutants.](image)

Notes: Fivefold serial dilutions of cells of esp1-ts strains were spotted in 1 μl drops onto three synthetic medium plates containing different carbon and nitrogen sources: control (2% glucose and 0.5% ammonium sulfate), carbon poor (3% glycerol and 0.5% ammonium sulfate), and nitrogen poor (2% glucose and 0.1% proline). The plates were incubated at 34 °C for 2 days for control and carbon-poor plates and for 3 days for nitrogen-poor plates.

![Fig. 4. Osmotic stress alleviates the temperature sensitivities of some esp1-ts mutants.](image)

Notes: Fivefold serial dilutions of cells of esp1-ts strains were spotted in 1 μl drops onto YPAD plates in the presence or absence of 1.2 M sorbitol and the plates were incubated at 34 °C for 2 days.

Table 1. Summary of phenotypes of esp1-ts mutants.

| esp1 mutants | 104 | 106 | 107 | 108 | 109 | 204 | 301 | 304 | 305 | 324 | B3 | N5 |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|
| 25 °C*1      | ++  | ++  | ++  | ++  | ++  | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| 37 °C*1      |     |     |     |     |     |     |     |     |     |     |     |     |
| Benomyl*1    |     |     |     |     |     |     |     |     |     |     |     |     |
| Bleomycin*1  |     |     |     |     |     |     |     |     |     |     |     |     |
| Glycerol*2   | n.d.| +   | NC  | NC  | NC  | +   | +   | NC  | +   | n.d.| n.d.|
| Proline*2    | n.d.| NC  | NC  | NC  | NC  | NC  | NC  | NC  | NC  | n.d.| n.d.|
| Sorbitol*2   | n.d.| +   | +   | +   | +   | +   | +   | +   | +   | n.d.| n.d.|
| PDS1 overexpression*2 | + | +   | +   | +   | +   | +   | +   | +   | +   | n.d.| n.d.|
| MPS5 overexpression*2 | NC | +   | +   | +   | +   | +   | +   | +   | +   | n.d.| n.d.|

*2+, alleviates growth; NC, not changed. n.d., not determined.

Notes: Fivefold serial dilutions of cells of esp1-ts mutants.
the former mutants are defective in securin-mediated positive functions with separase and that the latter were defective in securin-mediated negative functions. This is a first description of effects of PDS1 overexpression on temperature sensitivities of esp1-ts mutants.

MPT5 genetically interacts with ESP1
To further dissect the features of esp1-ts mutants, we screened high-dosage suppressors of the thermosensitive growth defects in esp1-ts mutants. As a result, we isolated MPT5 as a high-dosage suppressor of esp1-108. Mpt5, a yeast homolog of Drosophila Pumilio and Caenorhabditis elegans FBF, binds to the 3'-untranslated region (3'-UTR) of mRNAs and is involved in mRNA stabilization. \(^{22}\) MPT5 overexpression alleviated growth of esp1-107 and esp1-108 (Fig. 6, Table 1). Interestingly, it resulted in deterioration in growth of esp1-106, esp1-301, and esp1-305 at a semi-permissive temperature. In addition, we failed to obtain cells of esp1-304 transformants with the plasmid overexpressing MPT5 (data not shown), indicating that MPT5 overexpression was lethal in esp1-304 cells. These findings suggested that Mpt5 was a novel positive and negative regulator of separase.

Discussion
Here, we isolated novel esp1-ts mutants. All of the mutants were defective in sister chromatid separation, probably because of a defect in the translocation of separase into the nucleus during G1-S phases. If this scenario is correct, securin overexpression might mitigate sister chromatid separation in some esp1-ts mutants, because securin binds to separase and promotes its nuclear translocation. This was indeed the case as for some esp1-ts mutants, esp1-104, esp1-107, and esp1-108 cells (Table 1). However, in other esp1-ts mutants (esp1-109, esp1-301, and esp1-304), securin overexpression rather aggravated their growth defects. This suggested a possibility that securin also has a negative role in separase regulation in G1-M phases.

Similar features to those found in securin overexpression were observed in MPT5 overexpression, which ameliorated growth defects in some esp1-ts mutants but conversely impeded growth in others (Fig. 5). Considering the fact that Mpt5 promotes mRNA degradation, \(^{22}\) it is most likely that MPT5 overexpression promotes degradation of mRNA of a gene(s) genetically interacting with ESP1, which ameliorates growth defects in some esp1-ts mutants and exacerbates those in others.

Mpt5 physically interacts with Cdk1, and a deletion of MPT5 exacerbates growth defect in the ATP analog-sensitive CDK1/CDCl28 mutant, cdc28-as1. \(^{23,24}\) These findings suggested that Mpt5 is a positive regulator of Cdk1. In addition, Cdk1 phosphorylates securin to promote securin-mediated nuclear import of separase. \(^{25}\) Taken together, there is another possibility that Mpt5 affects separase functions via Cdk1 and securin. Note that MPT5 overexpression alleviated growth defects in esp1-109 and esp1-301 strains, in which the defects were also suppressed by securin overexpression (Table 1). Conversely, MPT5 overexpression exacerbated growth defects in esp1-109 and esp1-301 strains (Fig. 5), in which the defects were also exaggerated by securin overexpression.

Is growth defects in some esp1-ts strains were ameliorated in carbon-poor conditions (Fig. 3). Cohesin SCC1 genetically interacts with PKA, \(^{26}\) which is activated by carbon source. In fission yeast, chromosome segregation defects associated with separase mutations are restored by reduced activity of the target of rapamycin complex 1 (TORC1). \(^{27}\) TORC1 is a protein kinase complex that is activated by nutrient signals. \(^{28}\) It is plausible that suppression of separase defect by carbon-poor conditions is mediated by TORC1 and PKA.

Some esp1-ts mutants were bleomycin hypersensitive (Fig. 3). This supported that separase is involved in DNA repair. DNA damage site-specific activation of separase at DNA damage sites might occur, but its molecular mechanism is largely unknown. DNA damage sensitive esp1-ts mutants may be useful for dissection of the molecular mechanism in the future.

Authors contributions
T.U. designed experiments. Y.S. mainly performed the experiment, and M.N., A.M.Y., N.K., and M.W.T. supported this. T.U. wrote the manuscript.
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Disclosure statement

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Supplemental material

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