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Yeast *tom1* mutant exhibits pleiotropic defects in nuclear division, maintenance of nuclear structure and nucleocytoplasmic transport at high temperatures

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Abstract

A *tom1-1* mutant was isolated from *Saccharomyces cerevisiae*. At high temperatures, 60% of the cells were arrested as dumbbell forms with a single large nucleus containing duplicated DNA and a short spindle. Electron-microscopy showed electron-dense structures scattered within the nucleus. Indirect immunofluorescent microscopy revealed these structures to be fragmented nucleoli since the dotted structures were stained with anti-Nop1(fibrillarin) antibody in large regions of the nuclei. Fluorescent in situ hybridization analysis using oligo(dT) revealed nuclear accumulation of poly(A)$^+$RNA. We cloned *TOM1* which encodes a large protein (380 kDa) with a hect (homologous to E6-AP C terminus)-domain at its C terminus. Deletions of either this hect-region or the entire gene made cellular growth temperature-sensitive. Site-directed mutagenesis of the conserved cysteine residue (*tom1*^{C3235A}) in the hect-domain, supposed to be necessary for thioester-bond formation with ubiquitin, abolished the gene function. When a functional glutathione *S*-transferase (GST)-tagged hect protein was overproduced, it facilitated the protein conjugation with a myc-tagged ubiquitin^{RA}, while this was not seen when GST-hect^{C3235A} was overproduced. The protein conjugation with a hemagglutinin-tagged Smt3 was not affected by the overproduction of GST-hect. Taken together, we suggest that Tom1 is a ubiquitin ligase. As a multi-copy suppressor of *tom1*, we isolated *STM3/NPI46/FPR3* which encodes a nucleolar nucleolin-like protein. We discuss possible functions of Tom1 with respect to the pleiotropic defects of nuclear division, maintenance of nuclear structure, and nucleocytoplasmic transport.

Keywords: Budding yeast; hect-domain; Protein-ubiquitination

1. Introduction

Protein ubiquitination is a system for tagging proteins in eukaryotic cells. Multi-ubiquitinated conjugates are known to be recognized and degraded by 26S proteasomes in an energy-dependent way (Hershko and Ciechanover, 1998). Since proteolysis is an irreversible process, the target proteins should be recognized with high specificity and proper timing; thus strict and sophisticated regulatory systems are needed. Ubiquitination involves the concerted action of the E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and additional E3s which function as 'docking proteins' to recognize substrate proteins (Hershko and Ciechanover, 1998). Recently, another type of E3 was found: ubiquitin ligases containing a 30 kDa hect (homologous to E6-AP C-terminus)-domain form a thioester bond with a ubiquitin molecule (Huibregtse et al., 1995), so that it is transferred through an E1–E2–E3 enzyme thioester cascade to a substrate (Scheffner et al., 1995). In *Saccharomyces cerevisiae*, there are five hect-genes: *RSP5/NPI1/MDP1* is an essential gene, involved in endocytosis of some membrane proteins (Galan et al., 1996), protein import into mitochondria (Zoladek et al., 1997), and stability of the large subunit of RNA poly-

Abbreviations: FACS, fluorescence-activated cell sorter; FISH, fluorescent in situ hybridization; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; hect, homologous to E6-AP C-terminus; NLS, nuclear localization signal; *SMT3*, suppressor of *mif2*; *STM*, suppressor of *tom1*; *TOM1*, temperature-dependent organization in mitotic nucleus.

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merase II (Wang et al., 1999). *UFD4* is involved in the UFD (ubiquitin fusion degradation) pathway (Johnson et al., 1995). *TOM1* is not an essential gene, as described below and by others (Saleh et al., 1998), but is required for growth at high temperatures (Utsugi et al., 1995). The other non-essential genes, *HUL4* and *HUL5*, have been reported recently (Wang et al., 1999).

Several ubiquitin-like proteins have been described. For example, mammalian RanGAP1 is modified with a ubiquitin-like protein called SUMO-1, and the conjugate can target RanBP2, a nuclear pore complex protein (Matunis et al., 1996). In *S. cerevisiae* there are two ubiquitin-like genes, *RUB1* and *SMT3*. In the Rub1 pathway, Uba3 and Ula1 form a heterodimer to function as E1, and Ubc12 functions as E2, but this system is not essential in mitotic growth. In contrast, the Smt3 pathway is essential in mitotic growth: Uba2 and Aos1 form a heterodimer as E1, and Ubc9 functions as E2, which is required for G2/M transition in the cell cycle (Johnson et al., 1997). These protein conjugation systems may not be involved in proteasome-dependent proteolysis, as is the case for ubiquitinated substrates.

At the G2/M boundary of the cell cycle, anaphase promoting complex (APC or cyclosome) functions as E3 in the ubiquitination pathway (Lamb et al., 1994). APC/cyclosome ubiquitinates a mitotic inhibitor, Pds1, to enter mitosis and B-type cyclins like Clb2 to exit from the mitosis. In addition, various checkpoint controls operate at the G2/M boundary, to determine whether replication, including telomere formation, is complete, DNA damage is repaired, spindle pole bodies (SPBs) are duplicated, normal spindles are formed, or bud formation occurs.

The nucleus contains a crescent-shaped nucleolus, where rRNAs are transcribed and pre-ribosomes are formed from many ribosomal proteins, which are imported from the cytoplasm. Large pre-ribosomes are exported through nuclear pore complexes (NPCs) to the cytoplasm. It has been suggested that the nucleolus has some role in mRNA export (Schneiter et al., 1995). mRNAs are covered with RNA-binding proteins which are likely to be recognized by export machinery after processings such as capping, splicing, and poly(A) addition.

In this paper we report the isolation and characterization of a *tom1* (Temperature dependent Organization in Mitotic nucleus) mutant of *S. cerevisiae*. At high temperatures the mutant cells were arrested at the G2/M phase boundary in the cell cycle, and exhibited nucleolar fragmentation and nuclear accumulation of poly(A)$^+$RNA. The isolated *TOM1* gene encoded a large protein of 3268 amino acids containing a hect-domain. Overproduction of a functional GST-hect fusion protein facilitated the protein-ubiquitination. Thus Tom1 is essential for growth at high temperatures (Utsugi et al., 1995) and is involved in many important cellular processes. Recently Saleh et al. (1998) reported that Spt7 of ADA/SAGA complex is ubiquitinated in a Tom1-dependent way.

2. Materials and methods

2.1. Strains and genetic manipulations

Escherichia coli K12 strain DH5α [*supE44ΔlacU169 (ø80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi1 relA1*] was used for amplification of plasmids. A strain YA21 was used to prepare a GST-Tom1 fusion protein. Yeast strains 15Dau (*MATa ade1 his2 ura3 leu2 trp1 bar1*; S. Reed), W303-1A (*MATa ade2 his3 ura3 leu2 trp1 can1-101*), RAY-3A (*MATa his3 ura3 leu2 trp1*; Uesono et al., 1997) and RAY (a homozygous diploid strain of RAY-3A) were used as wild-type strains. A *tom1-1* mutant (TUY024; *MATα tom1-1 ura3 leu2 trp1*His$^-$) *was isolated as follows: when we intended to disrupt the HTR1/MPT5* gene (Kikuchi et al., 1988, 1994) of 15Dau, we isolated a mutant with abnormal morphology. When this mutant was crossed with RAY-3A, another temperature-sensitive mutation was segregated out as a single mutation, which was recessive to the wild type. TUY044 (*MATa/MATα tom1-1/tom1-1ura3/ura3 leu2/leu2 trp1/trp1* His$^-$) was a homozygous diploid strain and its isogenic TUY059 (*MATa/MATα tom1-1/tom1-1::TOM1::TRP1 ura3/ura3 leu2/leu2 trp1/trp1* His$^-$) was constructed by integrating a linearized pTOM11 plasmid into the genome of TUY044 by homologous recombination. Deletion mutants, TUY101 (*tom1-2::LEU2*), TUY112 (*tom1-3::LEU2*) and TUY211 (*tom1-10::LEU2*) were constructed from RAY-3A. All the deletions were verified by the standard Southern hybridization (Sambrook et al., 1989). YT02L (*smt3::TRP1* pYES-*HA-SMT3-LEU2*) given by Y. Takahashi, had a deletion in *SMT3* of W303-1A and contained a YES-based plasmid expressing a HA-tagged Smt3 from *GAL1* promoter. TUY030 was constructed by disrupting *RAD9* of TUY024, using pRR330 (Schiestl et al., 1989) and the disruption was verified as its UV sensitivity. Media and methods for mating, sporulation, and tetrad analysis were described previously (Kaiser et al., 1994).

2.2. Plasmids and transformation

All the vectors such as YEp13 (*LEU2*), YEplac195 (*URA3*), pRS316 (*URA3*) and pJJ-series were described previously (Yashiroda et al., 1996). pTOM1 was isolated from a YEp13-based genomic library, provided by Dr Ohya of The University of Tokyo. The following plasmids were constructed by standard DNA manipulations (Sambrook et al., 1989). pTOM5 (pRS316-*TOM1*); the 14 kb *Bam*HI–*Pvu*II fragment of pTOM1 was inserted

into pRS316. pTOM6 (for *tom1-2* deletion); the 4 kb *Hin*dIII–*Xba*I fragment from pTOM1 was cloned into Bluescript SK$^+$ in which the 0.9 kb *Pst*I–*Bgl*II fragment was replaced by the 2 kb *Pst*I–*Bam*HI fragment of *LEU2*. pTOM6 was digested with *Sac*I and *Xho*I (both sites were in the multi-cloning sites of the vector) and used for transformation. pTOM10 (pRS316-*tom1*^{C3235A}) contained the 13 kb *Bam*HI–*Eco*RI fragment of pTOM5, but carrying a *tom1*^{C3235A} mutation on pRS316. The mutation was generated using a primer 5′-ATC/ACA/TAC/CGC/ATT/CAA/TCA/AC-3′ and oligonucleotide-directed in vitro mutagenesis system (Amersham). The mutated gene contained a new *Bsm*I site. pTOM11 (YIp-*TOM1*) was generated by replacing the 5.9 kb *Bam*HI–*Xho*I fragment (carrying 2μ replication origin and *LEU2*) of pTOM1 with the 0.9 kb *Bam*HI–*Sal*I fragment containing *TRP1* from pJJ246. pTOM11 was digested with *Mlu*I to be linearized and used for transformation. pTOM13 (for *tom1-3* deletion); the 2 kb *Xba*I–*Pst*I fragment (*LEU2*) from pJJ250 was inserted between the 0.6 kb *Hin*dIII–*Xba*I fragment and the 0.9 kb *Pst*I–*Bgl*II fragment from pTOM1 on pJJ246. The plasmid was digested with *Bgl*II and *Hin*dIII and used for transformation. pTOM15 (GST-Tom1 for an antigen); the 0.6 kb *Hin*dIII–*Cla*I fragment from pTOM1 was inserted into pGEX-KG to express a fusion protein in *E. coli* YA21. pTOM21 (for *tom1-10* gene disruption) carried the 1.8 kb *Bam*HI–*Xba*I fragment from pTOM1, the 2 kb *Sal*I–*Xba*I fragment of *LEU2* from pJJ252, and the 1.4 kb *Sal*I–*Xba*I fragment from pTOM1 on Bluescript KS$^+$. The plasmid was digested with *Bam*HI and *Sac*I and used for transformation. pTOM31 (*GST-hect*); the 3 kb *Pst*I–*Xba*I fragment from pTOM1 was inserted into the *Pvu*II site of pKOM1 (a YEp24-based GST-tagged expression vector, constructed by Dr K. Kominami) to express a GST-hect fusion protein under *TDH3* promoter. pTOM32 (*GST-hect*^{C3235A}) contained the corresponding *Pst*I–*Xba*I fragment from pTOM10 on pKOM1. A plasmid containing *STM3*/*NPI46* was isolated from the YEp13-based genomic library. pYS1 (YEplac195-*STM3*); the 5.4 kb *Sal*I–*Sac*I fragment containing *STM3* was subcloned on YEplac195 . pYS2 (YEplac195-*STM3ΔC*); the 2.5 kb *Sal*I–*Eco*RI fragment of pYS1 was cloned on YEplac195. pYS3 (for *STM3* disruption) contained the 5.6 kb *Sal*I–*Pvu*II fragment on Bluescript SK$^+$, in which the 1.4 kb *Spe*I–*Eco*RI fragment was replaced with the 1.5 kb *Bam*HI–*Eco*RI fragment containing *URA3* from YEp24. pYS3 was linearized with *Sal*I and *Sac*I before transformation. pYS4; the 2.5 kb *Sal*I–*Eco*RI fragment of pYS1 was subcloned into YEp356R (Myers et al., 1986). pRR330 was used to disrupt *RAD9*, as described previously (Schiestl et al., 1989). pYL8A containing *RPL6A* was given by Dr K. Mizuta of Hiroshima University (Mizuta et al., 1992). pRS811 carrying *Pgal-GFP-NPL3* was provided by Dr P. Silver of Harvard Medical School. A YES-based plasmid carrying pGAL1-GFP-nucleoplasmin (*Xenopus laevis*) was constructed by inserting the *Hin*dIII–*Not*I fragment of the plasmid pGAL1-GFP-NP, given by Dr Kohno of Nara Institute of Science and Technology. pUB204 (*pCUP1-UBI*^{RA}*-TRP1-CEN*) and pUB223 (*pCUP1-UBI*$^{HIS\text{-}MYC\text{-}RA}$*-TRP1-CEN*) were given by Dr M. Tyers of University of Toronto (Willems et al., 1996). Yeast transformation and gene disruption were performed as described by Kaiser et al. (1994).

2.3. Indirect immunofluorescent microscopy

Strains TUY044 and TUY059 were grown in YPD for 4 h at 26°C. Then the cultures were shifted to 37°C. Four hours later, approx. 10^8 cells were fixed with 5% formaldehyde for 1 h, washed with 0.1 M KPO_4 buffer (pH 7.5) three times, and samples were prepared essentially as described by Kaiser et al. (1994). For microtubule-staining, an anti-tubulin rat antibody (YOL1/34, Sera-Lab) and fluorescein-labeled goat anti-rat IgG (Cappel) were used. For nucleolar-staining, we used an anti-Nop1 monoclonal antibody (mAb A66), generously supplied by Dr J. Aris of The University of Florida (Aris and Blobel, 1988) and goat anti-mouse IgG Cy3-conjugate (Chemicon). Anti-β-galactosidase monoclonal antibody (Cappel) and anti-mouse IgG rhodamine-conjugate (Boehringer Mannheim) were used to locate the Stm3-lacZ fusion protein. Samples were mounted on a glass-slide with 0.1 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) and 1 mg/ml of *n*-propyl-gallate in 90% glycerol, and observed under an epifluoro-photomicroscope (Olympus BH-2).

2.4. FACS analysis

Cells (TUY059 and TUY044) were grown in YPD at 26°C. Then half of the cultures were shifted to 38.5°C. After 4 h the cells were fixed in 70% ethanol for 15 min, washed with 0.2 M Tris–HCl (pH 7.5), and treated with 1 mg/ml RNase A (Sigma) in the same buffer overnight at 37°C. Cell pellets were resuspended in 100 μl of 0.1% sodium citrate, 10 mM NaCl, and 0.1% Nonidet P-40. Propidium iodide was added to the cell suspensions to a final concentration of 100 μg/ml. Samples were allowed to stand for 30 min at room temperature and diluted with 0.2 M Tris–HCl buffer to adjust the final concentration of 10 μg/ml propidium iodide. After the cell suspensions were briefly sonicated, samples were analyzed using a Becton Dickinson FACScan analyzer.

2.5. Electron microscopy

Samples were prepared by a freeze-substituted fixation method, as described previously (Sun et al., 1992).

Serial sections were viewed on a JEOL 200 CX electron microscope (JOEL USA, Peabudy, MA) at 100 kV.

2.6. In situ hybridization

Cells (TUY044 and TUY059) were grown in YPD at 26°C, then the cultures were shifted to 37°C. Cells were collected at 0 min, 30 min, 1 h, 2 h, and 4 h and fixed in 4% formaldehyde for 15 min. Samples were prepared as described by Kadowaki et al. (1992) and observed under an epifluorophotomicroscope (Olympus BH-2).

2.7. Northern blot analysis

Total cellular RNA was prepared by hot phenol method, as described by Köhrer and Domdey (1991). The 1.2 kb *Bgl*II–*Eco*RI fragment of pYL8A containing *RPL6A* was used as a probe (Mizuta et al., 1992). Northern blotting was performed using ECL direct nucleic acid labeling and detection systems (Amersham), as recommended by the manufacturer.

2.8. Preparation of anti-Tom1 antibody

E. coli YA21 cells harboring pTOM15 were grown in 600 ml LB medium containing 50 μg/ml of ampicillin at 30°C. Then 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the culture for further 5 h cultivation at 30°C. Cells were collected, resuspended in 10 ml of PBS buffer, and broken by sonication. The cell lysate was centrifuged to remove cell debris, and the resultant supernatant was mixed with 4 ml of 50% slurry of glutathione–Sepharose 4B beads (Pharmacia) and kept at 4°C for 1 h. The GST-fusion protein was eluted with 50 mM Tris–HCl (pH 8.0) containing 20 mM glutathione. The GST-Tom1 fusion protein was used as an antigen to raise an antibody in rabbit. The antiserum was run through the GST-conjugated Sepharose 4B column (Pharmacia) first, and its flow through-fraction was run through the second column of GST-Tom1-conjugated Sepharose 4B. Proteins bound to the GST-Tom1 were eluted with 100 mM diethylamine (pH 11.4), and the eluate was dialyzed against PBS overnight and was used as an affinity-purified anti-Tom1 antibody.

2.9. Immunoblot analysis

When ubiquitin genes under *CUP1* promoter were expressed, cells were grown in a SD medium lacking tryptophan and uracil at 26°C overnight and the cultures were diluted 20 times into a fresh medium containing 200 μM $CuSO_4$ and incubated at 26°C for 3 h and transferred to 37°C for another 45 min. Transformants of YT02L were grown in a SGal medium lacking uracil at 26°C and the cultures were shifted to 37°C for 45 min before collecting cells. Preparation of cell lysates and immuno-blotting were performed essentially as described previously (Yashiroda et al., 1996). The protein concentrations of the lysates were determined by the Bio-Rad protein assay system. Samples were subjected to electrophoresis of a 7.5% polyacrylamide–sodium dodecyl sulfate (SDS) gel. A blotted membrane was incubated with the anti-Tom1, anti-myc (Promega), or anti-GST antibody. The alkaline phosphatase-conjugate or horseradish peroxidase-conjugate was used for detection (Promega).

3. Results

3.1. Defective G2-M transition in a tom1 mutant

To characterize a temperature-sensitive *tom1-1* mutant, we examined the terminal phenotype of its homozygous diploid strain (TUY044) with its isogenic $Tom1^+$ strain (TUY059) as a control. The growth of TUY044 was stopped after 4 h incubation at 37°C, and the arrested cells were examined for cellular morphology by phase-contrast microscopy, for nuclear localization by DAPI-staining, and for microtubules by indirect immunofluorescent microscopy. As shown in Figs. 1 and 2A, 60% of the arrested mutant cells had a single or two large buds, and 95% of those cells contained a single large nucleus with a short intranuclear spindle, indicating that they were mainly arrested at the G2/M phase boundary. About 37% of the unbudded cells were anuclear, which were never seen in the control cells. We observed, though, a few well-separated cytoplasmic spindles (asters) extending from spindle pole bodies (SPBs) without an intranuclear spindle, as shown in Fig. 2B. Some SPBs appeared as dot structures. Also, a significant number of the nuclei did not properly locate near the isthmus between mother and daughter cells.

Flow cytometric analysis (FACS) indicated that 67% of the arrested mutant cells (TUY044) contained 4N, 23% had 8N and there were a few cells with 2N population (Fig. 3). In contrast, 41% of the $Tom1^+$ cells (TUY059) had 2N, 59% had 4N and there was no 8N population. At 26°C, both strains showed normal distribution. In order to see whether the replication had been completed, we constructed a *tom1-1 rad9* double mutant (TUY030), which was viable after a 2 day incubation at 37°C (data not shown), indicating that DNA synthesis must have been completed and that the arrest was independent of the Rad9-checkpoint control. These results indicate that the mutant cells were mainly arrested in the G2/M boundary at high temperatures.

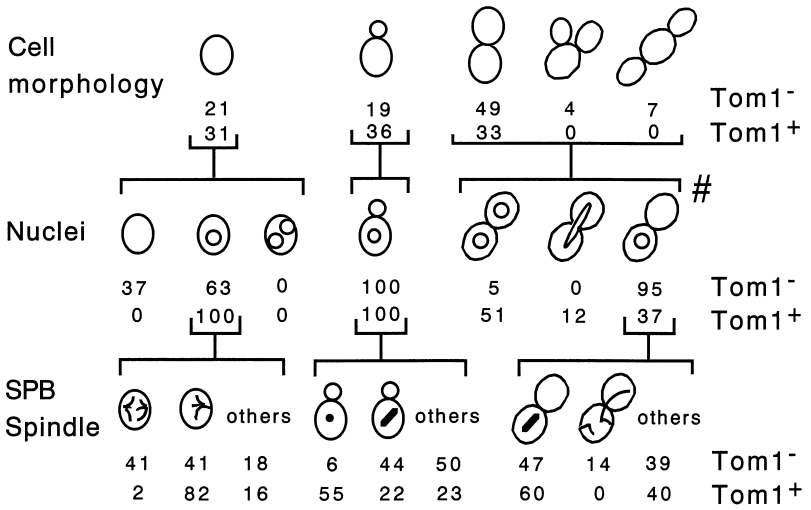


Fig. 1. Morphological distribution of arrested *tom1* cells. The *tom1-1* homozygous diploid (TUY044, Tom1$^-$) cells were incubated at 37°C for 4 h and the terminal morphology was examined. TUY059 (Tom1$^+$) served a positive control. Nuclei were stained with DAPI. Spindles and SPBs were immuno-stained with an anti-tubulin antibody. More than 200 cells were examined and classified. Distribution is expressed as percentages. Only singly nucleated cells are counted for spindles and SPBs. # Multiply-budded cells are also included in this category.

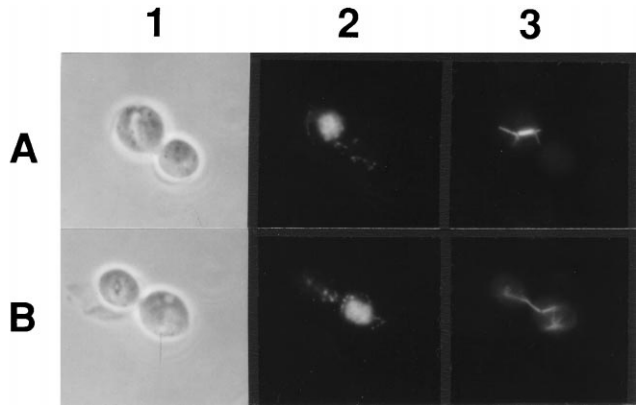


Fig. 2. Micrographs of the arrested *tom1* mutant. (**A**) Represents a typical cell of the *tom1* mutant (TUY044). (**B**) Represents a cell containing unusual microtubules. Examples were taken from the experiment of Fig. 1. 1, phase-contrast; 2, DAPI-staining; 3, tubulin staining.

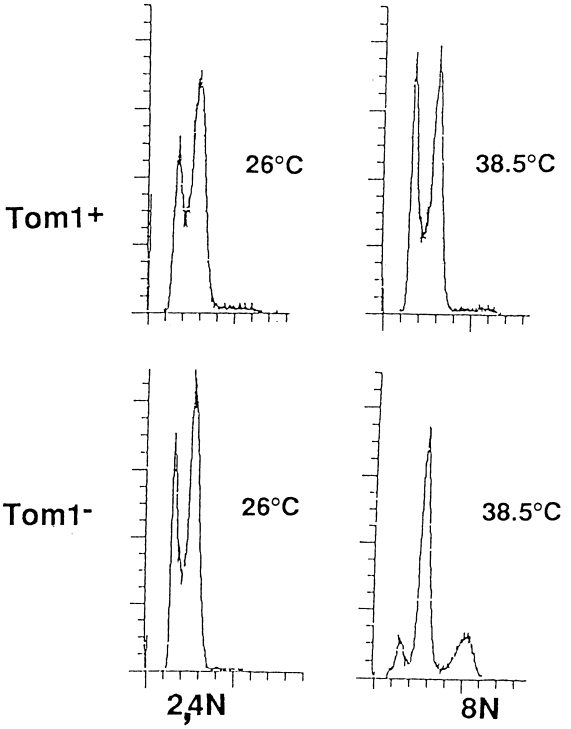
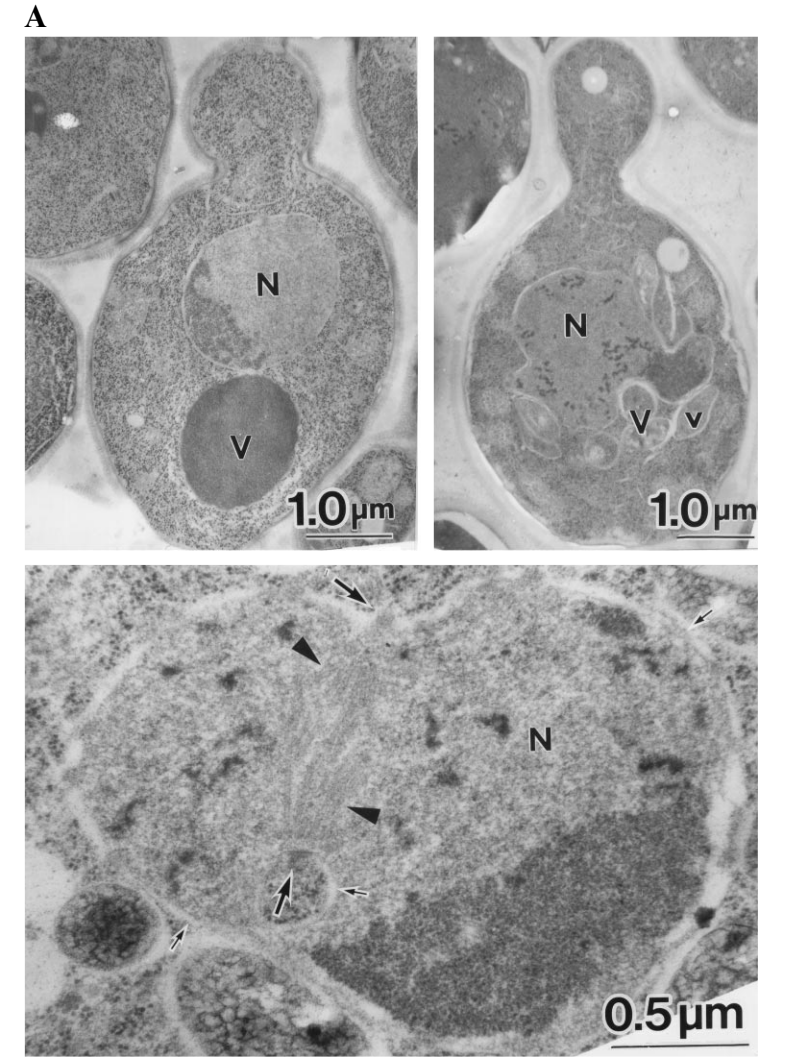


Fig. 3. DNA was duplicated in the *tom1* mutant. Exponentially growing cells of TUY059 and TUY044 were incubated either at 26°C or 38.5°C for 4 h. Cells were prepared for FACS analysis, as described in Materials and methods.

3.2. Disorganized nuclear structure and defective export of mRNA

Electron microscopy (EM) revealed that the Tom1$^+$ cells (TUY059) grown at 37°C had a smooth and round nuclear membrane to which a single crescent-shape nucleolus was attached (Fig. 4A). In contrast, the nuclear membranes of the mutant (TUY044) were wavy and invaginated especially at the position of the SPBs, since the length of the intranuclear spindles remained short in huge nuclei. Despite the abnormal membrane shape, the NPCs on the nuclear membrane appeared to be normal. Also the structure of the SPB or spindle itself seemed to be normal. We could not find any cells which had only cytoplasmic spindles without intranuclear ones in the limited number of EM samples. Furthermore, electron-dense materials, which might represent nucleolar fragmentation, were dispersed inside the mutant nuclei (Fig. 4A). To check our hypothesis, we stained cells with an antibody against Nop1, a nucleolar localizing fibrillarin (Aris and Blobel, 1988). Many dotted structures were dispersed in large regions of the mutant nuclei, whereas a single oval structure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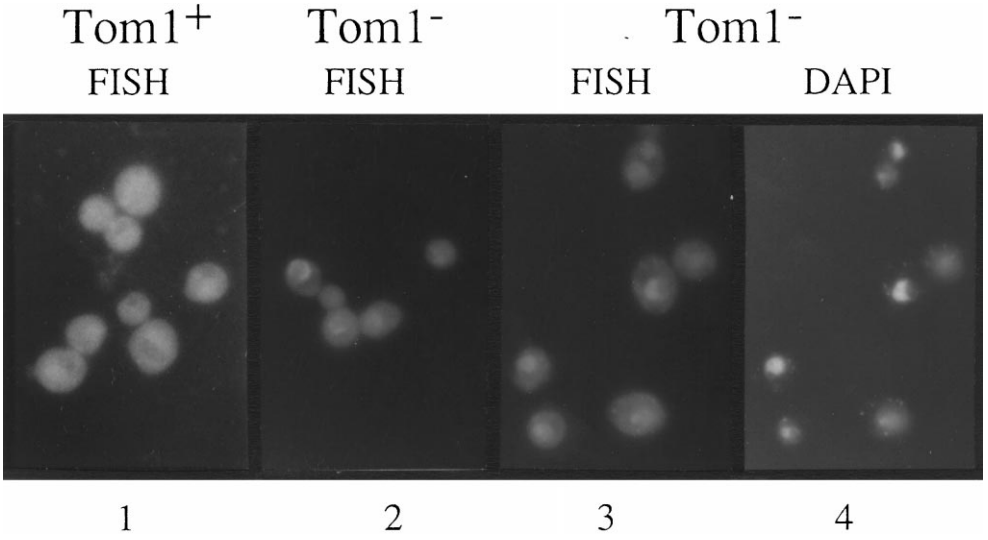

Fig. 5. Nuclear accumulation of mRNA in the *tom1* mutant. After Tom1$^+$ (TUY059) and Tom1$^-$ (TUY044) cells were incubated at 37°C for 4 h, intracellular localization of poly(A)$^+$RNA was examined by FISH analysis, using oligo(dT)-biotin/avidin-FITC. 1, Tom1$^+$ (TUY059); 2, Tom1$^-$ (TUY044); 3, 4, Tom1$^-$ cells were doubly-stained with oligo(dT)/FITC and DAPI, respectively.

was detected in each wild-type nucleus (Fig. 4B). These results suggested that nucleolar fragmentation had occurred in the mutant nucleus.

Since nucleolar fragmentation was reported in some mutants defective in mRNA export, we examined the distribution of mRNA by fluorescent in situ hybridization (FISH) using oligo(dT) as a probe (Kadowaki et al., 1992). As shown in Fig. 5 (columns 2–4), stained poly(A)$^+$RNA, which was sometimes observed in a ring shape, probably around the nuclear periphery, accumulated in the *tom1* nuclei at 37°C. This nuclear accumulation of poly(A)$^+$RNA occurred in 20% of the mutant cells after a 1 hr and 60% after a 2 h temperature-shift. In contrast, poly(A)$^+$RNA of Tom1$^+$ cells was distributed in both the cytoplasm and in the nucleus (Fig. 5, column 1).

Next we tested whether the mutation affects pre-mRNA splicing. Total cellular RNA was isolated from the cells after 1 and 2 h incubations at 37°C and subjected to Northern blot analysis, using a DNA probe of a ribosomal protein gene *RPL6A* (pYL8A; Mizuta et al., 1992). Only a mature-sized mRNA was detected in each wild-type or mutant RNA preparation (data not shown). Furthermore, we examined the nuclear protein import, using the GFP-nucleoplasmin (YES-GFP-NP) and GFP-Npl3 fusion genes, which are under the *GAL1* promoter. When GFP-nucleoplasmin or GFP-Npl3 was expressed by adding galactose to media after the temperature shift, all GFP fluorescence was detected in the *tom1* nuclei, which suggests that the general system for protein import was not affected by the mutation (data not shown). Taken together, the *tom1* mutation caused G2/M arrest, disorganized the nuclear structure, and impaired mRNA export at high temperatures.

3.3. TOM1 *encodes a hect-domain*

From a yeast genomic library, we isolated a multicopy plasmid pTOM1 which complemented the temperature-sensitive *tom1-1* mutation (Fig. 6A). A single-copy pTOM5 containing a 14 kb insert from pTOM1 complemented *tom1-1*. A YIp-type plasmid, pTOM11 was constructed and integrated into the TUY024 genome by homologous recombination. The integrants were able to grow at 37°C, and crossing one of them with the wild-type strain RAY-3A resulted in a sporulated diploid. All the segregants (32 of 32) grew at 37°C, indicating that the integrated gene was tightly linked to the *ts* mutation and was probably the authentic *TOM1* gene. Sequencing analysis of the *TOM1* gene revealed that it encodes a putative ubiquitin ligase domain (hect-domain) (Huibregtse et al., 1995) at its C-terminus (GenBank accession No. D63905) and is identical to YDR457W. Fig. 7 shows the amino acid sequence alignment of the Tom1-hect-domain, with those of other

Fig. 4. Nucleolar fragmentation in the *tom1* mutant. (**A**) Electron micrographs. Tom1$^+$ (TUY059) and Tom1$^-$ (TUY044) cells were incubated at 37°C for 6 h and prepared for EM by a freeze-substituted fixation method (Sun et al., 1992). Upper left: Tom1$^+$ cells. Upper right and bottom: Tom1$^-$ cells. Abbreviations: N, nucleus; V, vacuole. Arrow-heads indicate spindles; large arrows, SPBs; small arrows, nuclear membranes. Bars indicate scales. (**B**) Nucleolar staining of the cells after incubation at 37°C for 6 h. Upper panels show staining patterns with anti-Nop1 antibody. Left: Tom1$^+$ (wild-type RAY). Right: Tom1$^-$ (TUY044). Bottom panels show cellular morphology.

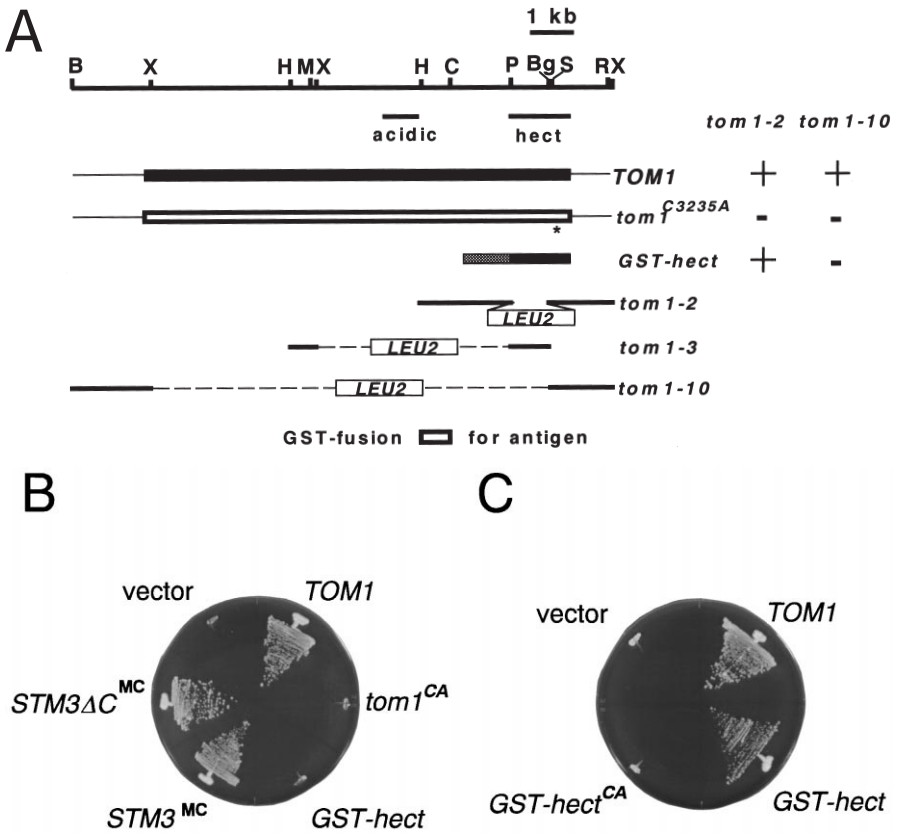

Fig. 6. Restriction map of *TOM1*, complementation activity of its derivatives and suppression by *STM3*. (**A**) Regions subcloned in plasmids and structures of the deletion constructs are shown. Complementation activity of each derivative is shown at the right side of the figure. A GST-fusion protein was used as an antigen to prepare its antibody. A bar indicates 1 kb. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; M, *Mlu*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I. (**B**) A yeast strain TUY211 (*tom1-10*) was transformed with pTOM5 (pRS316-*TOM1*), pTOM10 (pRS316-*tom1*^{C3235A}), pTOM31 (pKOM1-*GST-hect*), pYS1 (YEplac195-*STM3*), pYS2 (YEplac195-*STM3ΔC*) or YEplac195 (vector), and Ura$^+$ transformants were streaked on a YPD plate which was incubated at 37°C for 2 days. (**C**) A strain TUY101 (*tom1-2*) was introduced with each plasmid pTOM5 (pRS316-*TOM1*), pTOM31 (pKOM1-*GST-hect*), pTOM32 (pKOM1-*GST-hect*^{C3235A}) or pKOM1 (vector), and Ura$^+$ transformants were streaked on a YPD plate for 2 days incubation at 37°C.

hect-proteins; Tom1-hect is 33% identical with human E6-AP-hect, and 44% with yeast Rsp5-hect.

We constructed three deletion mutants (*tom1-2, tom1-3* and *tom1-10*), as shown in Fig. 6A. All the deletion mutants were viable and healthy at 26°C, and exhibited temperature-sensitive growth (Fig. 6B, C). When the well-conserved cysteine^{3235} residue in the hect-domain was changed to alanine by site-directed mutagenesis, the mutated gene (*tom1*^{C3235A}) on a single-copy plasmid (pTOM10) did not complement the total deletion, *tom1-10,* at 37°C (Fig. 6B). Thus the cysteine^{3235} residue is essential for its gene function, as reported by others (Saleh et al., 1998).

We prepared anti-Tom1 antibody and used it to detect the gene product by immunoblotting. A faint band of about 380 kDa was detected in a wild-type cell extract (RAY-3A, lane 2 of Fig. 8), while no band was detected at the corresponding position in extracts of the deletion mutant (YUY112; *tom1-3*, lane 1). Overproduction of Tom1 by the multi-copy pTOM1 made the band thicker (lane 3). Thus the band of 380 kDa was identified as the gene product of *TOM1*. As described above, *tom1*^{C3235A} was nonfunctional, but this was not due to its protein instability, since the protein level of *tom1*^{C3235A} was similar to that of the wild-type (Fig. 8, lanes 4 and 5).

We constructed a *GST-hect* fusion gene expressed under a strong promoter (pTOM31). This GST-hect protein was functional, because it complemented *tom1-2* (only the hect-region was deleted) (Fig. 6C). On the other hand, it did not complement the total deletion, *tom1-10* (Fig. 6B), which suggested that a region of the N-terminal other than the hect-domain was also required for its gene function. As expected, *GST-hect*^{C3235A} (pTOM32) did not complement *tom1-2* (Fig. 6C).

3.4. Overproduction of Tom1-hect facilitates protein-ubiquitination

To detect ubiquitin-conjugates easily, the GST-hect protein was over-expressed from the *TDH3* promoter and a myc-tagged mutant ubiquitin (pUB223;

```
E6-AP 532 MIAMENPADLKKQLYVEFEGEQGVDEGGVSKEFFQLVVEEIFNPDIGMF
Tom1 2929 FFKTNDEIK NSKLEITFKGESGVDAGGVTREWYQVLSRQMFNPDYALFL
Rsp5  473 MRQTPEDLK K RLMIKFDGEEGLDYGGVSREFFFLLSHEMFNPFYCLFE

       581 TYDESTKLFWFNPSSFETEGQFTL   IGIVLGLAIYNNCILDVHGPMVV
      2978 PVPSDKTTFHPNRTSGINPEHLSFFKFIGMIIGKAIRDQCFLDCHFSREV
       521 YSAYDNYTIQINPNSGINPEHLNYFKFIGRVVGLGVFHRRFLDAFFVGAL

       628 YRKLMGKKGTFRDLGDSHPVLYQSLKDLLEYVGNVEDDMMITFQISQTNL
      3028 YKNILGRPVSLKDMESLDPDYYKSLVWILE  NDITDIIEETFSV ETDD
       571 YKMMLRKKVVLQDMEGVDAEVYNSLNWMLE  NSIDGVLDLTFSA DDER

       678 FGNPMMYDLKENGDKIPITNENRKEFVNLYSDYILNKSVEKQFKAFRRGF
      3075 YGEHKVINLIEGGKDIIVTEANKQDYVKKVVEYKLQTSVKEQMDNFLVGF
       618 FGEVVTVDLKPDGRNIEVTDGNKKEYVELYTQWRIVDRVQEQFKAFMDGF

       728 HMVTNESPLKYLFRPEEIELLICGSRNLDFQALEETTEYDGGYTRDSVLI
      3125 YALISKD LITIFDEQELLLISGLPDIDVDDWKNNTTY VNYTATCKEV
       668 NELIPED LVTVFDERELELLIGGIAEIDIEDWKKHTDY RGYQESDEVI

       778 REFWEIVHSFTDEQKRLFLQFTTGTDRAP      VGGLGKL KMIIAKN
      3173 SYFWRAVRSFDAEERAKLLQFVTGTSKVPLNGFKELSGVNGVCKFSIHRD
       716 QWFWKCVSEWDNEQRARLLQFTTGTSRIPVNGFKDLQGSDGPRRFTIEKA

       821 GPDTERLPTSHTCFNVLLLPEYSSKEKLKERLLKAITYAK GFGML
      3223 FGSSERLPSSHTCFNQLNLPPYESYETLRGSLLLAINEGHEGFGLA
       766  GEVQQLPKSHTCFNRVDLPQYVDYDSMKQKLTLAVEETI GFGQE
```

Fig. 7. Sequence alignment of each hect-domain of Tom1, yeast Rsp5 or human E6-AP. Tom1-hect and E6-AP-hect are 33% identical and Tom1-hect and Rsp5-hect are 44% identical. The conserved cysteine residue that forms thioester bond with a ubiquitin is in a black box. Residues present in at least two hect-proteins are shadowed. The amino acid numbers are shown at the left side.

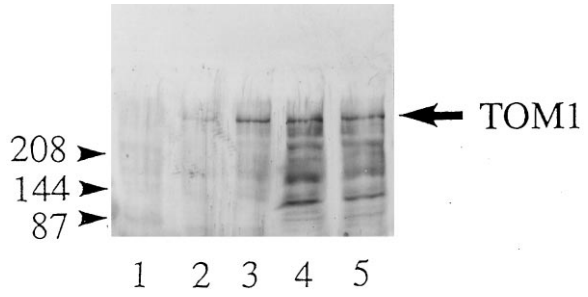

Fig. 8. Identification of wild-type and mutant Tom1 by immunoblotting. Cell lysates were prepared and Tom1 was detected, using anti-Tom1 antibody, as described in Materials and methods. The band of Tom1 is indicated by an arrow. Amounts of total proteins loaded were 4-fold in lanes 4 and 5, compared with lanes 1–3. Standard molecular mass markers are indicated at the left of the figure. Lane 1, TUY112(*tom1-3*); 2, RAY-3A containing YEp13; 3, RAY-3A bearing pTOM1(YEp13-*TOM1*); 4, TUY112 containing pTOM5 (pRS316-*TOM1*); 5, TUY112 carrying pTOM10 (pRS316-$tom1^{C3235A}$).

myc-Ubi^{RA}) was induced in wild-type cells, expression of which should result in accumulation of mono-ubiquitinated target proteins, as described in Willems et al. (1996). By adding 200 μM $CuSO_4$, the myc-Ubi^{RA} was expressed for 3 h at 26°C and the culture was shifted to 37°C for 45 min. Cellular extracts were prepared and total proteins were subjected to immunoblotting. Several strong bands of myc-tagged ubiquitinated conjugates were detected (Fig. 9A). In contrast, the pattern of the myc-tagged ubiquitin-conjugates was similar to the negative control, when the GST-$hect^{C3235A}$ protein was overproduced, even though the protein amount itself was more than that of GST-hect. Thus the overproduction of GST-hect facilitated protein conjugation with ubiquitin.

To exclude the possibility that Tom1 was an E3 in the Smt3-pathway, we prepared cellular extracts of YT02L expressing HA-tagged Smt3 and carrying pTOM31 (*GST-hect*) or pTOM32 (*GST-*$hect^{C3235A}$). As shown in Fig. 9B, overproduction of the GST-hect did not change the total protein conjugation patterns of the HA-tagged Smt3. These results strongly support a notion that Tom1 is a ubiquitin ligase.

3.5. *STM3/NPI46, a multi-copy suppressor of* tom1

In order to gain more insight into the *TOM1* function, we isolated multi-copy suppressors (Suppressors of *tom1*) of *tom1-1*. As reported earlier, *STM1* encoding a G-quartet DNA binding protein was a rather weak suppressor (Utsugi et al., 1995), but *STM3* (pYS1) suppressed even the total *tom1* deletion (*tom1-10*) at

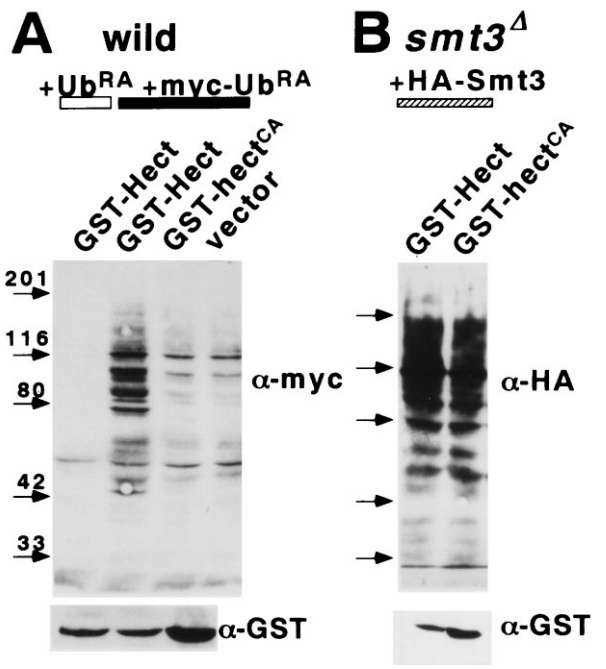

Fig. 9. Facilitated ubiquitin-conjugation by overproduction of the GST-hect protein. (**A**) The wild-type cells (W303-1A) were co-transformed with pTOM31 (*GST-hect*) plus pUB204 (*pCUP1-UBI^{RA}*), *pTOM31 plus pUB223 (pCUP1-myc-UBI^{RA})*, pTOM32 (*GST-hect^{C3235A}*) plus pUB223, or pKOM1(vector) plus pUB223. *UBI^{RA}* was expressed by adding 200 μM $CuSO_4$ for 3 h at 26°C and further 45 min at 37°C. Cellular extracts were prepared and subjected to immunoblotting, using an anti-myc or anti-GST antibody. (**B**) Total cellular extracts of YT02L (*stm3::TRP1* pYES-*HA-SMT3-LEU2*) containing pTOM31 or pTOM32, were subjected to immunoblotting, using an anti-HA or anti-GST antibody. Molecular masses (kDa) of marker proteins are indicated at the left side of the gels.

37°C (Fig. 6B). Subcloning experiments revealed that *STM3* is identical to *NPI46*, whose product has been isolated as a histone H2B-NLS binding protein and was known to be localized in the nucleolus (Benton et al., 1994; Shan et al., 1994). Stm3/Npi46 has two domains: the NH_2-terminal domain contains long stretches of acidic residues alternating with blocks of basic residues, a structure similar to nucleolin. The C-terminal region is a FK506-binding domain as a peptidyl-prolyl-*cis-trans*-isomerase (PPIase). The suppressor activity, however, resided only its NH_2-terminal half, since the 2.4 kb *Sal*I–*Eco*RI fragment entirely lacking a PPIase region (pYS2), was sufficient for the suppressor activity (Fig. 6B). By indirect immunofluorescent microscopy, the N-terminal half-lacZ fusion protein (pYS4) was detected in the nucleus, indicating that the N-terminal half was sufficient to localize in the nucleus (data not shown). As described previously (Benton et al., 1994; Shan et al., 1994), the disruptant (*stm3::URA3*) had no phenotypes, and the double mutant (*tom1-2::LEU2 stm3::URA3*) had no synergistic effect on the growth at high temperatures.

4. Discussion

By using the functional GST-hect protein, we showed that overproduction of the GST-hect facilitated the protein conjugation with ubiquitin (Fig. 9A). In contrast, the protein conjugation pattern of the HA-tagged Smt3 did not change by overproduction of the GST-hect (Fig. 9B). These results strongly support a notion that Tom1 is a ubiquitin ligase.

The arrested phenotype of *tom1* mutant was pleiotropic. Most mutant cells were inhibited from entering mitosis (Figs. 1–3). Since the ubiquitin ligase complex (APC) is required to enter the mitosis, we tested genetic interaction (synthetic lethality or dosage suppression) of *tom1-2* with *cdc23-1* or *cdc16-1* encoding APC components (Lamb et al., 1994), but did not find any between them.

Nucleolar fragmentation has been reported to occur when the constituents were limiting, for example, when RNA polymerase I was depleted or nuclear import was blocked (Loeb et al., 1995). It is also seen upon severe heat-shock (Liu et al., 1996), or in some mutants defective in mRNA export (Schneiter et al., 1995). In the *tom1* mutant, however, protein import appeared to be unaffected, and the defect of mRNA export might be a secondary effect, since the mRNA export was not blocked within 30 min after the temperature-shift like other export mutants.

If Tom1 is directly involved in ribosomal biogenesis, thereby in maintenance of the nucleolar structure, the disorganization of the nuclear structure may impair the export system secondarily. It may also lead to arrest at the G2/M boundary by operating some checkpoint control. In this case, a high dose of the nucleolin-like Npi46/Stm3 protein could help assembly of pre-ribosomes. Furthermore, we have found that mutations in certain ribosomal protein genes are synthetically lethal with the *tom1* mutation (unpublished results).

Other minor defects of the *tom1* mutant are as follows. The second budding occurred prematurely, although it was not very frequent. The checkpoint control for budding might be impaired. Also, the nucleus was not located near the isthmus of the mother and daughter cells. The connection between microtubules from the SPB and a neck structure might be impaired, to dislocate the positioning of the nucleus.

Since the growth of *tom1* mutant cells continues up to 4 h after the temperature shift, it is hard to determine what is the cause and what is the consequence of the mutation, judging from its terminal phenotype. The simplest explanation of those pleiotropic phenotypes of the mutant might be that there are many substrates of the Tom1-ubiquitin ligase. Also, a certain transcription is affected in a Tom1-dependent way (Saleh et al., 1998), so that some terminal phenotype described in this study may be secondary effects. The crucial problem to explain

how Tom1 functions lies in determining the targets of this Tom1-ubiquitin ligase.

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