# Suppression of a mitotic mutant by tRNA-Ala anticodon mutations that produce a dominant defect in late mitosis

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## Summary

Cold-sensitive dominant mutants *scn1* and *scn2* of *Schizosaccharomyces pombe* were isolated by their ability to suppress temperature-sensitive *cut9-665* defective in an essential subunit (human Apc6/budding yeast Cdc16 ortholog) of anaphase promoting complex/cyclosome (APC/C). APC/C mutants were defective in metaphase/anaphase transition, whereas single *scn* mutants showed the delay in anaphase spindle elongation at 20°C. The *scn* mutants lost viability because of chromosome missegregation, and were sensitive to a tubulin poison. To understand the *scn* phenotypes, mutant genes were identified. Surprisingly, *scn1* and *scn2* have the same substitution in the anticodon of two different tRNA-Ala (UGC) genes. UGC was altered to UGU so that the binding of the tRNA-Ala to the ACA Thr codon in mRNA became possible. As *cut9-665* contained an Ala535Thr substitution, wild-type Cut9 protein was probably produced in *scn* mutants. Indeed, plasmid carrying tRNA-Ala (UGU) conferred cold-sensitivity to wild-type and suppressed *cut9-665* in a dominant fashion. The previously identified *scn1*+ (renamed as *scn3*+) turned out to be a high copy suppressor for *scn1* and *scn2*. These are the first tRNA mutants that cause a mitotic defect.

Key words: Mitosis, Anaphase, tRNA mutant, Anticodon, Dominant mutation

## Introduction

APC (anaphase promoting complex)/cyclosome (hereafter APC/C) is a 20S protein complex that plays a pivotal role in the transition from mitotic metaphase to anaphase in eukaryotic cells by promoting ubiquitin-mediated anaphase proteolysis (King et al., 1995; Sudakin et al., 1995; Yanagida et al., 1999; Peters, 2002). The complex is evolutionarily conserved, present in yeast and human, consists of more than ten different subunits and acts as a ubiquitin ligase (E3) for the polyubiquitination of mitotic cyclins and securin (Funabiki et al., 1996; Yu et al., 1998; Zachariae et al., 1998; Yoon et al., 2002). Fission yeast Cut9 (orthologue of human APC6 and budding yeast Cdc16) is an essential subunit of APC/C (Yamashita et al., 1996; Yamada et al., 1997). Its temperature-sensitive (ts) mutant *cut9-665* produces the mitotically hypercondensed chromosomes with the short metaphase spindle and the high Cdc2 kinase activity. Sister chromatid separation did not take place at all in *cut9-665* cells at the restrictive temperature (Hirano et al., 1986; Samejima and Yanagida, 1994).

Cold-sensitive (cs) mutations designated *scn* (*s*uppressors for *c*ut *n*ine) were isolated as extragenic suppressors for the ts phenotype of *cut9-665* (Samejima and Yanagida., 1994). Genetic analyses enabled them to be classified into two genetic loci, *scn1* and *scn2*. The *cut9 scn1* and *cut9 scn2* double mutants produced colonies at 33°C (a restrictive temperature for *cut9* mutant) but not at 22°C (a restrictive temperature for *scn* mutants). Single mutant segregants of *scn1* and *scn2* obtained by crossing with the wild-type showed the cs phenotype but not the ts phenotype. These single *scn1* and *scn2* mutants revealed notable properties. First, all of the *scn1* and *scn2* mutants obtained were dominant: heterozygous diploids showed the cs phenotype. Second, the cellular phenotypes of *scn1* and *scn2* mutants cultured at 22°C revealed an apparent chromosome segregation defect in late mitosis.

To understand how these dominant cs mutations could suppress the ts phenotype of *cut9-665*, we attempted to characterize *scn* mutant phenotypes in detail and to identify mutant genes and the mutation sites. The results presented in this report clearly showed late mitotic defects particularly in spindle elongation. The resulting chromosome segregation defect seemed to be the cause of cell lethality at the restrictive temperature. Synchronous analysis indicated that cell viability remained high in G1, S and G2. Both *scn1* and *scn2* mutants were hypersensitive to a tubulin inhibitor, thiabendazole (TBZ) (Umesono et al., 1983) at the permissive temperature, which is consistent with the defect in spindle elongation. The destruction of cyclin observed in living cells occurred apparently at a normal timing in *scn* mutant cells so that the destruction of mitotic cyclin was normal in single *scn* mutants.

Fine genetic analyses followed by nucleotide sequencing established that the mutated genes in *scn1* and *scn2* strains were actually the tRNA genes that carried Ala with the anticodon UGC. There are two Ala-tRNA genes with the identical sequence containing anticodon UGC at different map locations in the *Schizosaccharomyces pombe* genome. These *scn1* and *scn2* mutants were found to contain single substitutions in the anticodon sequence that led to UGU from

UGC so that the amino acid codon in mRNA for this tRNA[ala] would be changed to Thr from Ala. The resulting mischarged Ala for the Thr codon seemed to be the true cause for the ability of *scn* mutations to suppress the phenotype of *cut9-665*. The previously identified *scn1*+ was actually a high copy suppressor for *scn1* and *scn2* mutations so that it was renamed as *scn3*+. We will discuss the implication of these unexpected findings on the mitotic defects of dominant tRNA mutations.

## Materials and Methods

### Strains, media and genetic method

Haploid and diploid fission yeast strains were used. *scn1, scn2* and *cut9-665* were isolated as described previously (Samejima and Yanagida, 1994). Deletion mutants Δ*mad2* and Δ*scn3* were made previously (Samejima and Yanagida, 1994). The culture media used were YPD (1% yeast extract, 2% polypeptone, and 2% glucose) and EMM2 (minimal medium; Mitchison, 1970). The YPD and EMM2 Media containing 1.5% agar were used for plating.

### Plasmids and yeast transformation procedure

pSK248 was used as the vector carrying the *S. pombe* genes. About 500 bp long *S. pombe* genomic DNA fragments containing only Ala-tRNA(UGC) gene located near the *ryh1*+ gene (*SPAC4C5.02C*) were amplified by PCR from the wild-type and *scn2-7* mutant, and those PCR products were inserted into pSK248 vector, and were named as pTRNAala.02 or pTRNAala.02(UGC>UGU). Transformation of *S. pombe* was performed by the lithium method (Ito et al., 1983). To isolate multicopy suppressors of *scn2-7* mutant, an *S. pombe* genomic DNA library containing the *Saccharomyces cerevisiae LEU2* gene as the selection marker (a gift from C. Shimoda, Osaka City University, Japan). Plasmids were recovered from Cs+ Leu+ transformants of *scn2-7*. pTRNAthr.06 and pTRNAthr.10 were respectively constructed by PCR amplification and insertion into pSK248 vector.

### Light microscopy and time-lapse imaging

DAPI staining was carried out as previously described (Adachi and Yanagida, 1989). Indirect immunofluorescence microscopy was performed as previously described (Hagan and Hyams, 1988). Anti-tubulin (TAT-1) and anti-Sad1 antibodies were used as primary antibodies (Woods et al., 1989; Hagan and Yanagida, 1995). The secondary antibodies employed were the CY3-tagged anti-mouse antibody and Alexa488-tagged anti-rabbit antibody (Molecular probes). For observing living cells, confocal scanning system CSU21 (Yokogawa) was used. Cells were cultured in the liquid EMM2 media supplemented with leucine on glass base dishes (Iwaki). Images were taken every 15 seconds, 30 seconds or 1 minute. To visualize the SPB movement or the Cdc13 protein degradation in live cells, the pSad1-GFP plasmid or the strain containing GFP-tagged *cdc13*+ gene were used (Nabeshima et al., 1997; Tatebe and Yanagida, 2000).

### The G1 arrest by nitrogen starvation

Nitrogen source starvation was done according to the procedures described previously (Saka and Yanagida, 1993). Cells ($7.5 \times 10^6$ cells/ml) were cultured in EMM2 medium at 33°C, washed four times with the EMM2-N media (EMM2 without $NH_4Cl$) and then cultured in the EMM2-N media for 36 hours at 33°C (the cell concentration, $6 \times 10^7$ cells/ml). The FACS analysis confirms that the cells were arrested in the G1 phase. Arrested cells were released into the YPD medium at 20°C. Progression of DNA replication was monitored by FACS, and mitotic progression was monitored by determining the septation index.

## Results

### Cellular phenotypes of single *scn1-17* and *scn2-7* mutants

As shown in Fig. 1A, ts *cut9-655* mutant defective in the mitotic transition from metaphase to anaphase failed to produce colonies at 33°C, whereas the double mutants *cut9-665 scn1-17* and *cut9-665 scn2-7* could form colonies at 33°C but not at 22°C. Single *scn1* and *scn2* mutants obtained as tetrad segregants after crossing with the wild-type strain also showed the cs phenotype. Thus, *scn1-17* and *scn2-7* were extragenic suppressors for *cut9-665* mutant (Samejima and Yanagida, 1994). It was previously shown that all of the isolated *scn1* and *scn2* were dominant cs mutations.

Wild-type, *scn1-17* and *scn2-7* grown at 33°C were shifted to 20°C, and their increase in cell number was measured. Approximately two rounds of cell division occurred before the cease of cell number increase in the cultures of *scn1* and *scn2* (Fig. 1B). In addition, cell elongation was observed at 20°C (Fig. 1C). The average cell length of *scn1* mutant cells after 24 hours at 20°C increased from 10.7 μm to 14.1 μm, 32% longer than that of wild-type. Basically the same cell elongation was observed for *scn2* mutant cells (data not shown).

A prominent feature of *scn1* mutant cells cultured at 20°C and stained by DAPI was the accumulation of cells revealing late mitosis (Fig. 1D). Divided daughter nuclei often displaced from the middle of cell were seen. Chromosomes appeared to be still condensed. These results showed late mitotic defects with some pleiotropic effects in *scn1* mutant. Similar results were obtained for *scn2* mutant cells (data not shown).

### Defects in spindle elongation, nuclear position and cell division

Immunofluorescence microscopy using anti-tubulin antibodies (red) for the spindle and DAPI (blue) for nuclear chromatin staining was done for *scn1-17* mutant cells that were cultured at 20°C for 9-12 hours, and shown in Fig. 2A. Cells designated A are those in late anaphase. Divided daughter nuclei were often displaced from the middle of cell (indicated as B). The spindle that was not fully extended was frequently seen in the mutant cells, suggesting that mutant cells might be retarded in late anaphase.

The frequency of late anaphase cells increased from ~3% in exponentially growing wild-type cells and 5.9% in mutant cells at the permissive temperature to 13.7% in mutant cells after 9 hours at the restrictive temperature (Fig. 2B). In Fig. 2C, quantitative data for those containing the displaced daughter nuclei and those lacking the nucleus are shown. Cells lacking the nucleus increased from a negligible level at the permissive temperature to approximately 20% after 24 hours at 20°C in mutant cells. The displaced nuclei increased to ~7% in mutant cells, while they were at a negligible level in the wild-type cells. The temporary decrease of late anaphase cells after 3 hours in the wild-type and mutant cells was probably due to the shift to 20°C. The displaced daughter nuclei, mostly connected with the spindle, were frequently seen and seemed to be characteristic of *scn* mutant cells at 20°C, as the same cellular phenotypes were seen in *scn2* mutant cells. These results indicated that sister chromatid separation took place but full segregation of the resulting daughter nuclei failed. Because nuclear positioning was abnormal, cytokinesis would produce
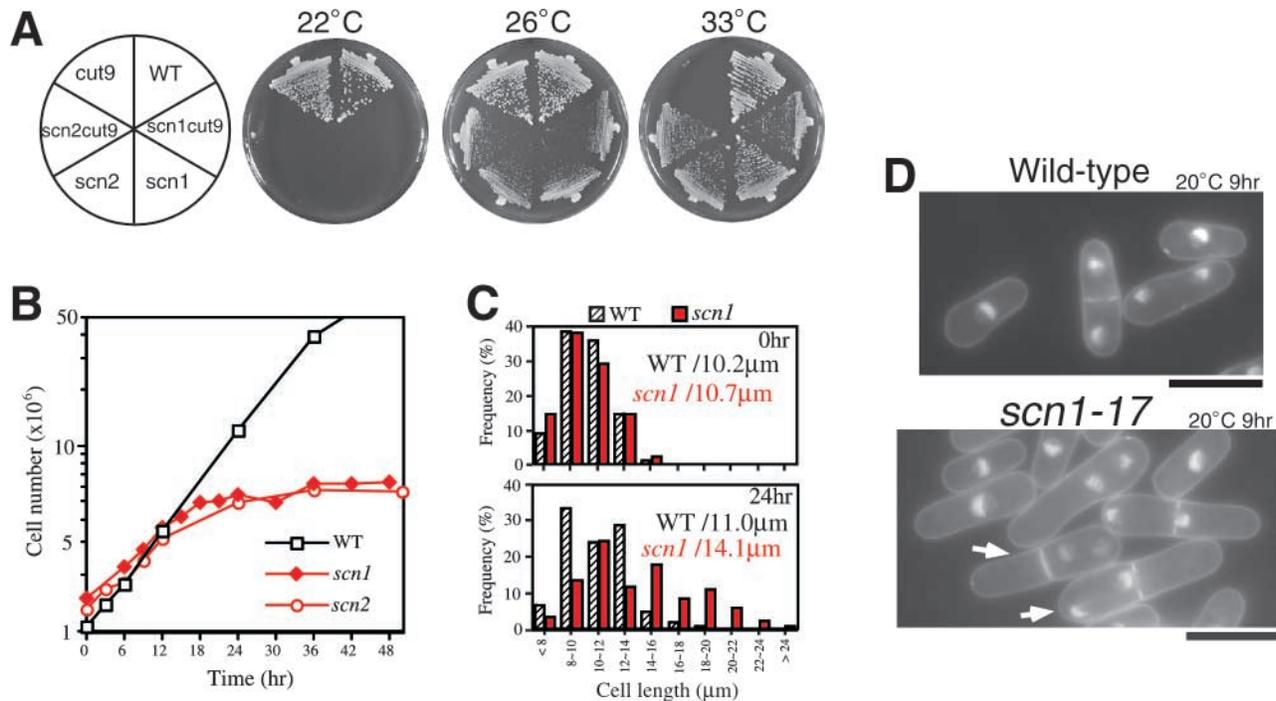
**Fig. 1.** Phenotypes of cs *scn1* and *scn2* mutants. (A) Fission yeast *scn1* and *scn2* are extragenic suppressors for ts *cut9-665* that causes the assembly defect in APC/C (Samejima and Yanagida, 1994; Yamada et al., 1997). Single *cut9* mutant fails to form colonies at 33°C, whereas double mutants *cut9 scn1* and *cut9 scn2* form colonies at 33°C. Single *scn1* and *scn2* mutants are unable to form colonies at 22°C. (B) Wild-type, *scn1* and *scn2* grown at 33°C were transferred to 20°C and cultured for 50 hours in the complete YPD medium. The cell number of wild-type (WT), *scn1* and *scn2* mutants was scored. (C) The cell length of wild-type (hatched columns) and *scn1* mutant (red columns) was measured after 0 and 24 hours culture at 20°C. The average cell length of wild-type and *scn1* mutant is indicated. (D) Light micrographs of wild-type and *scn1* mutant cells cultured at 20°C for 9 hours. Wild-type and *scn1* mutant cells were cultured at 20°C, fixed with glutaraldehyde and stained with DAPI. The arrows indicate the displaced divided nuclei abundantly present in *scn1* and *scn2* mutant cells. Bar, 10 μm.

two cells, one of which lacked the nucleus. The loss of nucleus was hence another characteristic of *scn* mutant cells.

### The loss of cell viability corresponds to the timing of aberrant mitosis

To examine when *scn1* lost the cell viability, mutant cells were first arrested in the G1 phase at the permissive temperature (33°C) by nitrogen source starvation, and then released to the complete YPD medium at 20°C to advance cell cycle progression. The occurrence of DNA replication (Fig. 3A) was slightly delayed in *scn1* mutant cells. The duration of the S phase is indicated by the vertical bar. In this S phase period, however, the cell viability was completely maintained. The loss of viability occurred after 15 hours in *scn1* mutant cells. This decrease corresponded to the passage through the first mitosis and the time when the improper chromosome segregation began to be seen in the *scn1* mutants (Fig. 3B).

In 72% of the *scn1* mutant cells with the septum, sister chromatids were not fully apart. These results showed that the late step of nuclear division in combination with septation and cytokinesis was not properly coordinated in *scn1* mutants. We concluded that *scn1* mutant cells did not lose viability until mitosis, but they had some defects in other events, including the delay in DNA replication. The cause of cell death seemed to be the occurrence of abnormal chromosome segregation followed by cell division.

### Normal timing of cyclin destruction and abnormal spindle dynamics

To determine the defects that would lead to improper chromosome segregation, mitotic phenotypes of *scn1* and *scn2* were further examined. No significant increase of the spindle index was observed after transferring the cells to 20°C, suggesting that scn1 did not arrest in metaphase (Fig. 4A). We then examined the timing of cyclin destruction using the strain of *scn1-17* carrying the chromosomally integrated Cdc13-GFP gene (Tatebe and Yanagida, 2000). Hoechst H33342 was used for staining DNA in order to distinguish anaphase cells. The GFP signal disappeared in all of the anaphase cells (indicated by the arrows in Fig. 4B) observed at the restrictive temperature (20°C). The cyclin destruction thus seemed to be normal in the *scn1-17* mutant.

To monitor the time course of spindle extension in living cells, *scn1-17* cells carrying multicopy plasmid expressing the GFP-tagged Sad1 protein were used. Sad1 is an essential SPB (spindle pole body) component and, when mildly overproduced, distributed in the nuclear envelope (Nabeshima et al., 1997). We found that anaphase spindle elongation was considerably delayed and often compromised in *scn1* mutant cells (Fig. 4C,D). Furthermore, the nuclear envelope breakdown did not seem to take place in mutant cells in the late anaphase stage (Fig. 4C, right panel). The nuclear envelope revealed complex movements during late anaphase, suggesting that the resolution of the nuclear envelope into the two
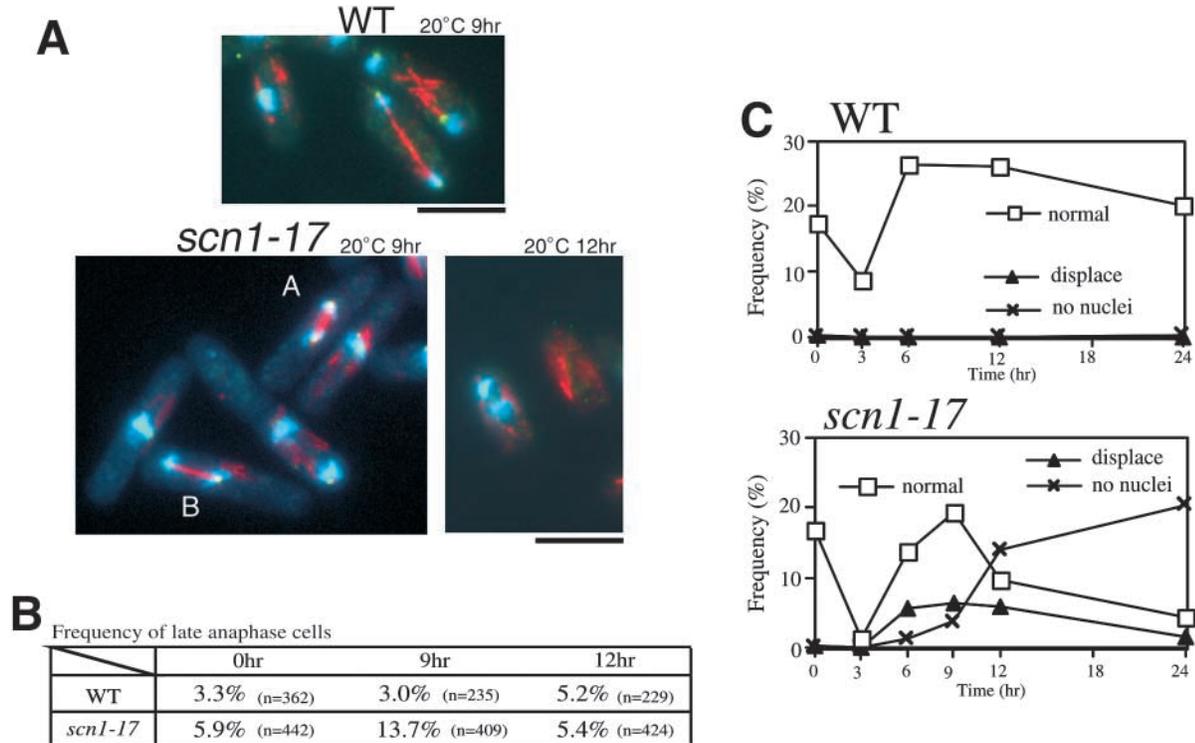
**B** Frequency of late anaphase cells

|  | 0hr | 9hr | 12hr |
|---|---|---|---|
| WT | 3.3% (n=362) | 3.0% (n=235) | 5.2% (n=229) |
| scn1-17 | 5.9% (n=442) | 13.7% (n=409) | 5.4% (n=424) |



**Fig. 2.** Single *scn1* mutant is defective in spindle elongation, nuclear positioning and cell division, producing frequent late mitotic phenotypes at 20°C. (A) Wild-type (WT) and *scn1-17* grown at 33°C were transferred to 20°C in the YPD medium. Anti-tubulin (red), anti-Sad1 (green) and DAPI (blue) staining were done for wild-type and *scn1* mutant cells after 9 hours and 12 hours at 20°C. Late anaphase cells, revealing separated chromatids and a spindle that was not fully elongated, were marked as A. The nuclear displacement indicated as B was often seen in *scn1* mutant cells. Cells lacking the nuclei were also seen (an example indicated as *scn1-17* in right panel). Bar, 10 μm. (B) The frequency of late anaphase cells in the wild-type and *scn1-17* mutant after the transfer to 20°C for 0-12 hours. Cells in late anaphase were significantly accumulated after 9 hours in *scn1-17*. (C) The frequencies of wild-type (upper) and *scn1* mutant (lower) cells showing normal positioning of the separated daughter nuclei (open rectangles), abnormally displaced daughter nuclei (filled triangles) and no nuclei (crosses) are shown.

daughter nuclei might be aberrant in *scn1* mutant cells. The loss of cell viability was possibly due to the cytokinesis-induced breakage of the thin nuclear envelope channel connecting the two daughter nuclei. The rate of spindle extension in anaphase B was greatly reduced in *scn1* mutants. The SPBs did not reach the opposite poles even after 102 minutes (Fig. 4D).

## *scn2* is alanine-tRNA(UGC) mutant

In the previous study, the *scn2*+ gene was not isolated because the cs *scn2* mutation was dominant so that plasmids introduced into mutant cells by transformation failed to rescue the cs phenotype (Samejima and Yanagida, 1994). To determine the precise map location in the *S. pombe* genome, we conducted a series of genetic mapping by crossing *scn2-7* with strains carrying various marker genes. Ultimately, it was found that *scn2-7* was tightly linked to the *ryh1* locus (PD:NPD:TT=56:0:0). The *ryh1*+ gene (*SPAC4C5.02C*) in *scn2* mutant was isolated by PCR, but no nucleotide change was found in the *ryh1*+ gene sequence obtained. Then PCR amplification was made for the ~2 kb long genomic DNA around the *ryh1*+ gene in *scn2-7* mutant (corresponding to cosmid c4C5 2425-4520). The resulting PCR product was sequenced (Fig. 5A). Surprisingly, a single nucleotide

substitution was found within the adjacently located Ala-tRNA(anticodon UGC) gene sequence (*SpaTRNAala.02*). In *scn2-7* mutant cells, the anticodon UGC of this tRNA gene was substituted to UGU. It was further found that *scn2-27*, another allele of *scn2*, contained the identical anticodon substitution, and otherwise the same sequence to the wild-type. The *scn2* mutant Ala-tRNA(UGC>UGU) should mis-interact with the Thr codon in mRNA instead of Ala codon (Fig. 5B).

To confirm that the Ala-tRNA(UGC>UGU) mutant gene was the real cause for the phenotype seen in *scn2* mutant cells, the ~500 bp long DNA fragment containing the Ala-tRNA(UGC>UGU) mutant gene was cloned into the vector pSK248. Resulting plasmid (pTRNAala.02 (UGC>UGU)) was transformed into the wild-type or *cut9-665* mutant. Plasmid containing the Ala-tRNA(UGC>UGU) gene suppressed the ts phenotype of *cut9-665* (Fig. 5C). In addition, the same plasmid conferred the cold-sensitivity if introduced into wild-type cells. Control plasmid containing the wild-type Ala-tRNA(UGC) (pTRNAala.02) neither suppressed the ts phenotype of *cut9-665* mutant nor caused any cs effect to wild-type cells (Fig. 5C). The wild-type cells carrying the mutant Ala-tRNA(UGC>UGU) plasmid showed the cellular phenotypes similar to that of *scn1* and *scn2* mutant at 20°C (Fig. 5D). We therefore concluded that *scn2* was an anticodon allele of the Ala-tRNA(UGC).02 gene.
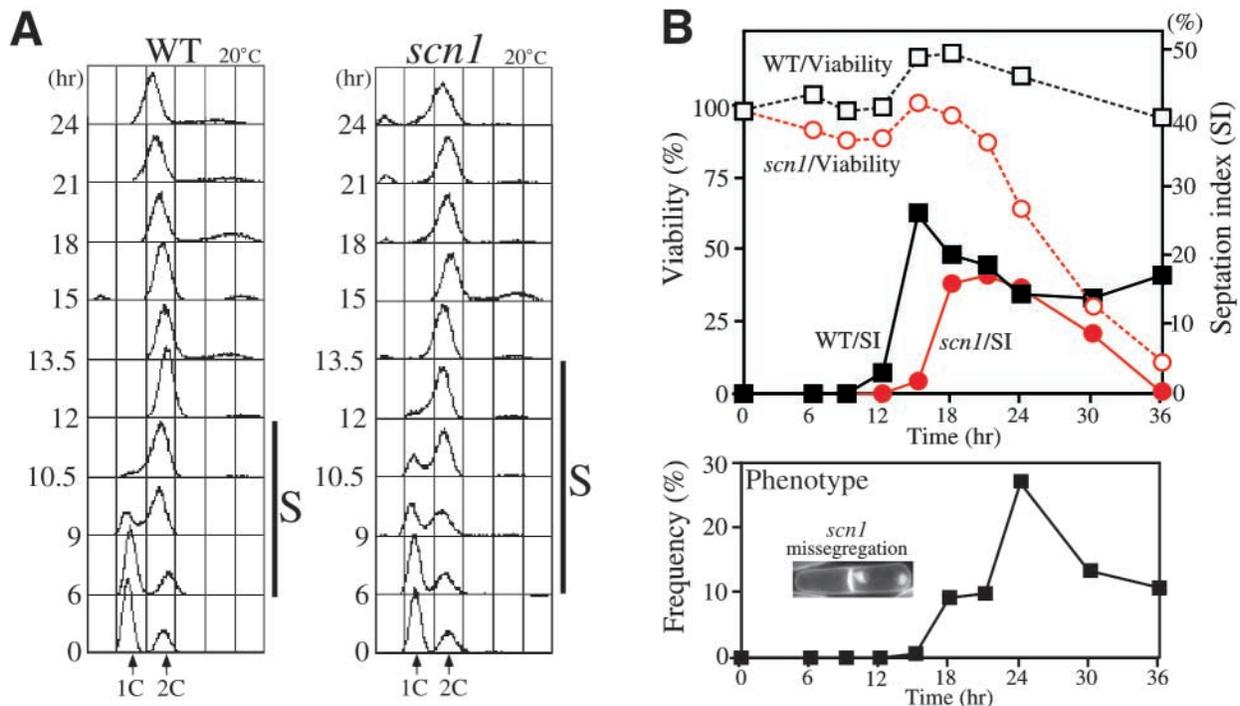
**Fig. 3.** The loss of viability in *scn1-17* coincides with aberrant mitosis. (A) The wild-type and *scn1-17* cells were first arrested at the G1 phase under the nitrogen source starvation at 33°C, and then shifted to the complete YPD medium at 20°C. The DNA contents of wild-type and *scn1-17* were determined by FACS analysis. In wild-type, the S phase (S) occurred from 6 to 12 hours, whereas, in *scn1-17*, the progression of S phase was delayed by 1.5 hours (6-13.5 hours). 1C indicates the DNA content of a haploid cell in G1 that has unreplicated DNA, whereas 2C indicates those of a G2 cell that has already replicated DNA once. (B) Upper panel: the cell viability (Viability%) and septation index (SI%) were measured after the release from the G1 arrest at various time points in wild-type and *scn1* mutant cells. The *scn1* mutant cells lost the viability during the passage through the first mitosis, with the concomitant increase of missegregation. Lower panel: the frequency of mitotic phenotype in *scn1-17* was determined. Frequency (%) of chromosome missegregation in *scn1* (displaced, separated daughter nuclei) was determined (lower panel).

### The strain *scn1* is also Ala-tRNA(UGC) mutant

The genome contains generally multiple copies of the tRNA genes for each amino acid. If there are many copies for the Ala-tRNA(UGC) gene in the *S. pombe* genome, how does a substitution mutation in one copy strongly affect cell multiplication in a dominant fashion? We examined the genomic tRNA database of *S. pombe* (http://rna.wustl.edu/GtRDB/Sp/) to find out how many Ala-tRNA(UGC) genes existed in the *S. pombe* genome and found that *S. pombe* has only two copies of the Ala-tRNA(UGC) gene (there are nine other Ala-tRNA genes but they have different anticodons). Furthermore, we noticed that the second Ala-tRNA(UGC) gene was present only 16.7 kb apart from the *scn1+* (*SPAC688.13*) gene previously identified (Samejima and Yanagida, 1994). This gene was previously isolated by its ability to suppress the cs phenotype of *scn1* mutant cells. The previous study showed that the *LEU2* marker integrated with the *SPAC688.13* gene was tightly linked to the cs *scn1* locus (PD:TT:NPD=16:0:0). This was the reason that the cloned gene was assigned to be the actual *scn1+* gene. However, we failed to find any nucleotide change in the *SPAC688.13* gene cloned from the *scn1* mutant genomic DNAs (corresponding to cosmid c688 27458-28872) so that the gene assignment could be false, although we confirmed that plasmid carrying the *SPAC688.13* gene could suppress the cs phenotype of both *scn1* and *scn2* mutant cells. By searching the conserved domain database (NCBI) we found that the gene product of *SPAC688.13* has a TatD-related nuclease domain (pfam01026) (Holm and Sander, 1997; Wexler et al., 2000).

The second copy of the Ala-tRNA(UGC) (*SpaTRNAala.03*) gene 16.7 kb apart from the *SPAC688.13* gene has the same sequence to the first one (Fig. 5E). If its expression level is similar to that of *scn2* Ala-tRNA(UGC), the same anticodon mutation may lead to the same phenotype as that of *scn2*. Indeed, a single substitution was found in the second Ala-tRNA(UGC) gene in *scn1* mutant cells (Fig. 5E). The anticodon UGC was again changed to UGU, in an identical manner to the *scn2* mutant. The previous gene assignment is therefore clearly false. We propose to call the former *scn1+* as *scn3+*. It acts as a high copy suppressor for both *scn1* and *scn2* mutants. These results established that both *scn1* and *scn2* were the same Ala-tRNA(UGC) anticodon mutants. We found that *scn1* and *scn2* double mutants were synthetic lethal. This was easily explained as the *S. pombe* genome has only two Ala-tRNAs with this anticodon UGC and therefore the GCU codon would not be properly translated at all in the double *scn1 scn2* mutant cells.

### Hypersensitivity to thiabendazole and cycloheximide

As *scn1* and *scn2* mutants were defective in protein synthesis and also in the completion of spindle elongation, we examined whether these tRNA mutants were sensitive to a protein
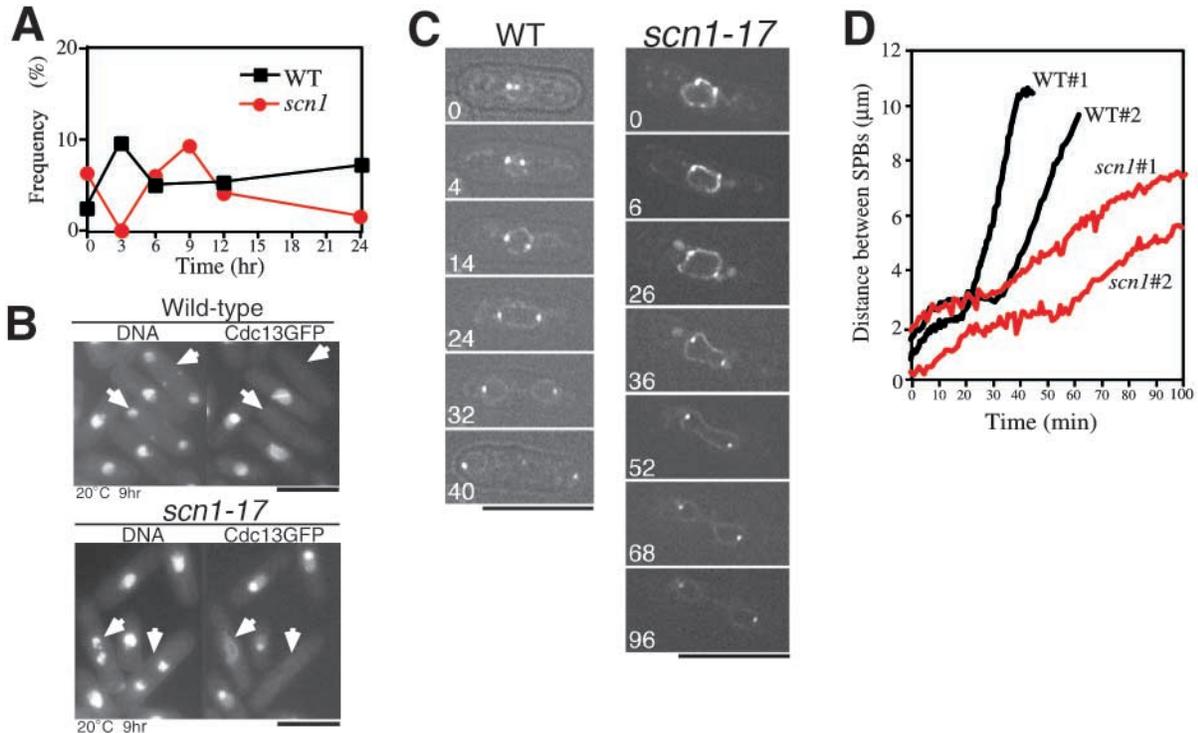
**Fig. 4.** Anaphase spindle elongation was slow and not complete in *scn1-17* mutant. (A) The spindle index of wild-type and *scn1-17* was measured after transfer to 20°C. The short spindle (the metaphase spindle length ~2.5 μm) did not accumulate in *scn1-17*. (B) Cdc13 (mitotic cyclin in fission yeast) is degraded in aberrant anaphase in *scn1*. The *scn1* mutant carrying chromosomally integrated cdc13-GFP gene was cultured at 20°C for 9 hours in the liquid EMM2, harvested and stained with Hoechst H33342 (DNA). The Cdc13-GFP signals (strong in the nucleus) faded in all of the anaphase cells (arrow) examined in *scn1-17*, as well as in wild-type cells. Bars, 10 μm. (C) The wild-type and *scn1-17* mutant cells carrying plasmid pSAD1-GFP (Sad1 is a SPB protein) were observed for the spindle dynamics in living cells. Cells were cultured at 20°C for 9 hours. The white number in each panel shows the time (minutes) after starting the observations using a confocal microscope. Full anaphase spindle extension was complete within 40 minutes in wild-type cells, whereas spindle elongation was slow and incomplete after 96 minutes in *scn1-17* mutant cells. Furthermore, the nuclear envelope in the middle appeared to remain in late anaphase, suggesting that nuclear division was not finished. Bars, 10 μm. (D) Distances between the SPBs were measured in living cells. In the *scn1* mutant cells, the rate of anaphase spindle elongation was strikingly low and the SPBs did not reach the opposite poles of the cells, even after more than 100 minutes.

synthesis inhibitor cycloheximide (CYH) and a tubulin poison thiabendazole (TBZ). As shown in Fig. 6A,B, they were found to be hypersensitive to these drugs. At the permissive temperature (26-30°C), *scn1-17* and *scn2-7* mutants were hypersensitive to the presence of 5-20 μg/ml CYH. They were also sensitive to TBZ. In the presence of 10-15 μg/ml TBZ, *scn1-17* and *scn2-7* grew poorly, whereas wild-type cells grew nearly normally. These are the first RNA mutants sensitive to tubulin inhibitor.

## Scn3 and tRNA-Thr, multicopy suppressors of *scn1* and *scn2* mutants

An *S. pombe* genomic DNA library was used to screen for multicopy plasmids that could suppress the cs phenotype of *scn2* mutant. Plasmids carrying three different genomic DNAs were isolated. One of them contained the *scn3+* gene (*SPAC688.13*, described above). Its product showed sequence similarity to *Escherichia coli* TatD, *S. cerevisae* Ymr262p and *Candida albicans* Orf6.8887p, containing a nuclease motif, and *E. coli* TatD shows DNase activity in vitro (Holm and Sander, 1997; Wexler et al., 2000). Plasmid expressing only the open reading frame of *scn3+* with the inducible promoter nmt1

in the presence of thiamine also suppressed both *scn1* and *scn2* mutants (Fig. 6C). This putative nuclease gene *scn3+* was nonessential for cell viability as gene-disrupted cells grew normally but, unexpectedly, *scn3+*-disrupted cells were resistant to cycloheximide (Fig. 6A). If Scn3 had the ribonuclease activity, the high dosage Scn3 might suppress *scn1* and *scn2* mutant cells by cleaving the mutated Ala-tRNAs.

The remaining two high copy suppressor plasmids contained the Thr-tRNA(UGU) gene (*SpbTRNAthr.06* and *SpaTRNAthr.10*). This Thr-tRNA(UGU) might be able to suppress *scn2* by competing against the mischarged mutant tRNA. The plasmids could also rescue the *scn1* mutant (pTRNAthr.06 and pTRNAthr.10 in Fig. 6D). Suppression of *scn1* and *scn2* by Thr-tRNA(UGU) expression indicates that the phenotypes of *scn1* and *scn2* are due to misincorporation of Ala to the position of Thr during translation, which could make mal-functional proteins.

## The delay of spindle elongation was independent of Mad2

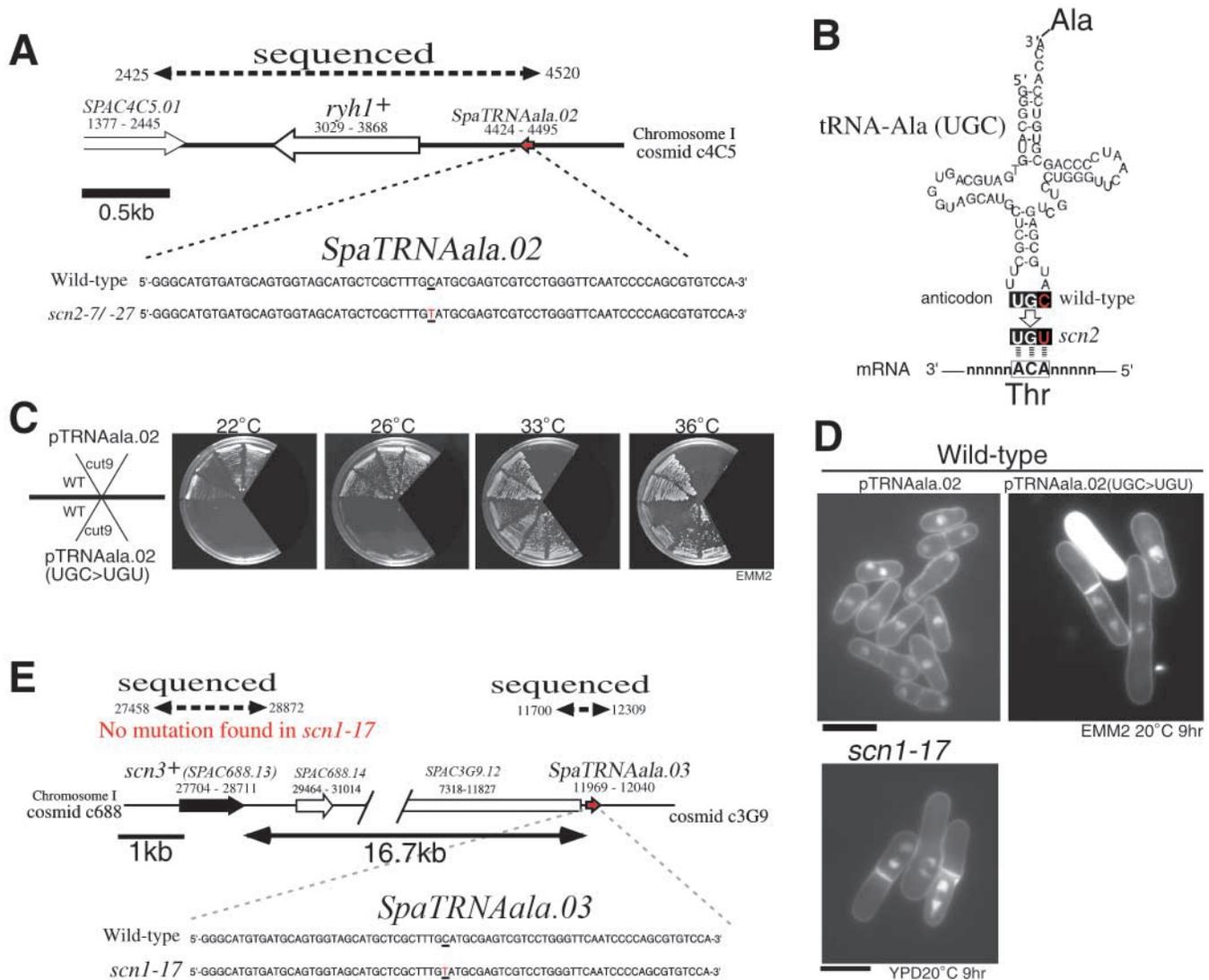To determine whether the spindle elongation delay in *scn*

**Fig. 5.** *scn1* and *scn2* are both Ala-tRNA anticodon mutants. (A) The genomic DNAs were purified from wild-type, *scn2-7* and *scn2-27* cells. The 2 kb long genomic DNA fragments corresponding to the 2425-4520 region of the chromosome I cosmid c4C5 were amplified by PCR, followed by DNA sequencing. Amplified DNAs from the mutant strains *scn2-7* and *scn2-27* showed a single mutation (indicated in red) in alanine-tRNA gene (*SpaTRNAala.02*). Otherwise the sequences were identical to that obtained from the wild-type. (B) The predicted cloverleaf structure of the tRNA-Ala (UGC) in wild-type and *scn2*. The mutated *scn2* tRNA is presumed to recognize the ACA codon in mRNA that encodes threonine. (C) The plasmid containing the mutant tRNA gene of *scn2* conferred cold-sensitivity on wild-type (WT) cells and suppressed the temperature sensitivity of *cut9-665*. Wild-type and *cut9-665* (*cut9*) carrying plasmid of the wild-type tRNA gene (pTRNAala.02) and plasmid of *scn2* mutant tRNA gene (pTRNAala.02(UGC>UGU)) were incubated on the minimal EMM2 plate at 22, 26, 33 and 36°C. (D) Wild-type cells carrying multicopy plasmid containing the mutant Ala-tRNA(UGU) gene showed mitotic phenotypes similar to those of *scn1* and *scn2* mutants. Wild-type cells carrying pTRNAala.02 or pTRNAala.02(UGC>UGU) were cultured in the EMM2 medium at 20°C for 9 hours and fixed with glutaraldehyde. DNA was stained with DAPI. The phenotype of *scn1-17* was also shown. Brightly stained cells were dead and accumulated the dye. Bar, 10 μm. (E) The *scn1* mutant contained a substitution mutation in the second copy of alanine-tRNA gene, but not in the *scn3+* gene. The genomic DNA of wild-type and *scn1-17* was purified, and DNA sequencing of the region containing the second tRNA gene (*SpaTRNAala.03*) was done. The *SpaTRNAala.03* near to the *scn3+* gene was mutated in *scn1-17* in the same position in anticodon as in *scn2*.

mutant cells was due to the activation of spindle checkpoint, the double mutant *scn1-17 Δmad2* was constructed. Mad2 is essential for establishing the spindle checkpoint (Li and Murray, 1991). It was cultured at 20°C for 9 hours, and cells stained by antibodies against tubulin (red) and Sad1 (green) were observed with DAPI (blue) as counterstaining for DNA (Fig. 7A). The appearances and frequencies of late anaphase

cells were not affected in *scn1 Δmad2*. To confirm that the delay in late anaphase was independent of Mad2, the spindle length in the double mutant cells was measured by the pole-to-pole distance in mitotic cells of the wild-type, *scn1* and *scn1 Δmad2* at 20°C for 9 hours (Fig. 7B). The filled arrowheads indicate the position of the wild-type metaphase spindle length. The frequencies of anaphase cells and the size of the anaphase
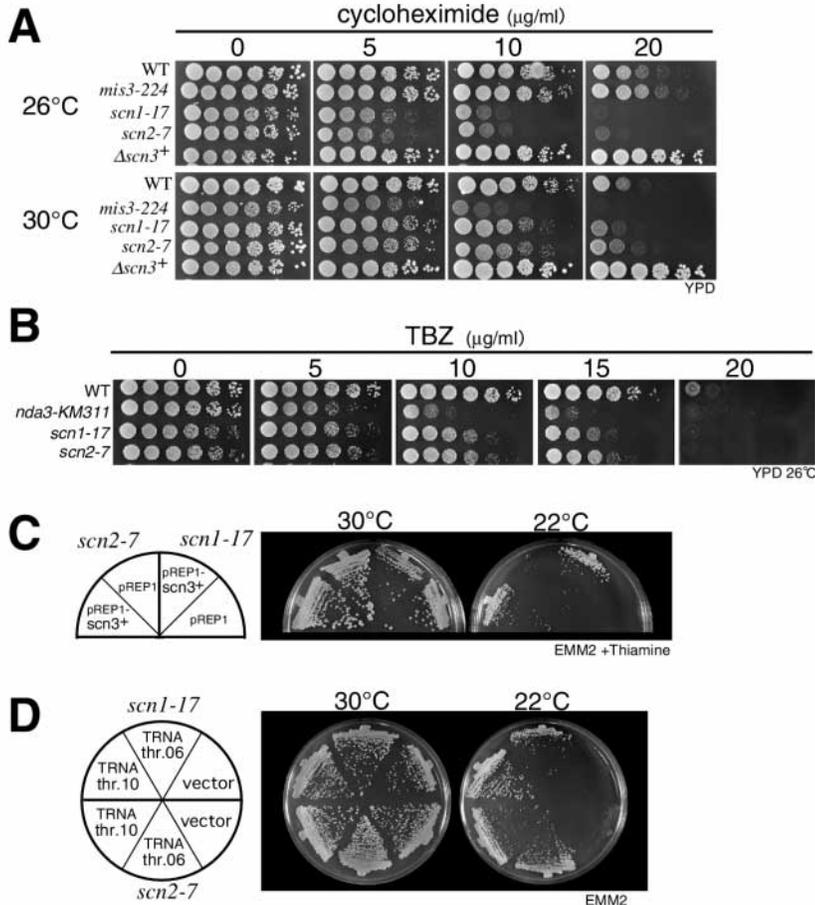
**Fig. 6.** Drug sensitivities of *scn1* and *scn2* tRNA mutants, and high dosage suppression of *scn* mutants by Scn3 and Thr-tRNA. (A) Mutants *scn1* and *scn2* were hypersensitive to cycloheximide, a protein synthesis inhibitor. Wild-type, *mis3-224* (Kondoh et al., 2000), *scn1-17*, *scn2-7* and *scn3*[+]-disrupted cells (Samejima and Yanagida, 1994) were spotted on the YPD plate containing respective concentrations of cycloheximide. (B) Mutants *scn1* and *scn2* are hypersensitive to TBZ, a microtubule destabilizing drug at 26°C, the semi-permissive temperature. Wild-type, β-tubulin mutant *nda3-KM311* (Toda et al., 1983), *scn1-17* and *scn2-7* were spotted on the YPD plate containing respective concentrations of TBZ. (C) The *scn3*[+] gene is a multicopy suppressor for *scn1* and *scn2* mutants. *scn1-17* and *scn2-7* mutants were transformed with plasmid containing the *scn3*[+] gene under the inducible nmt1-promoter (pREP1-scn3[+]). Transformants were plated on the EMM2 containing thiamine (promoter, repressible condition). (D) Plasmid containing the Thr-tRNA(UGU) gene (pTRNAthr.06 and pTRNAthr.10) could suppress the cold-sensitivity of *scn1* and *scn2* mutant cells.
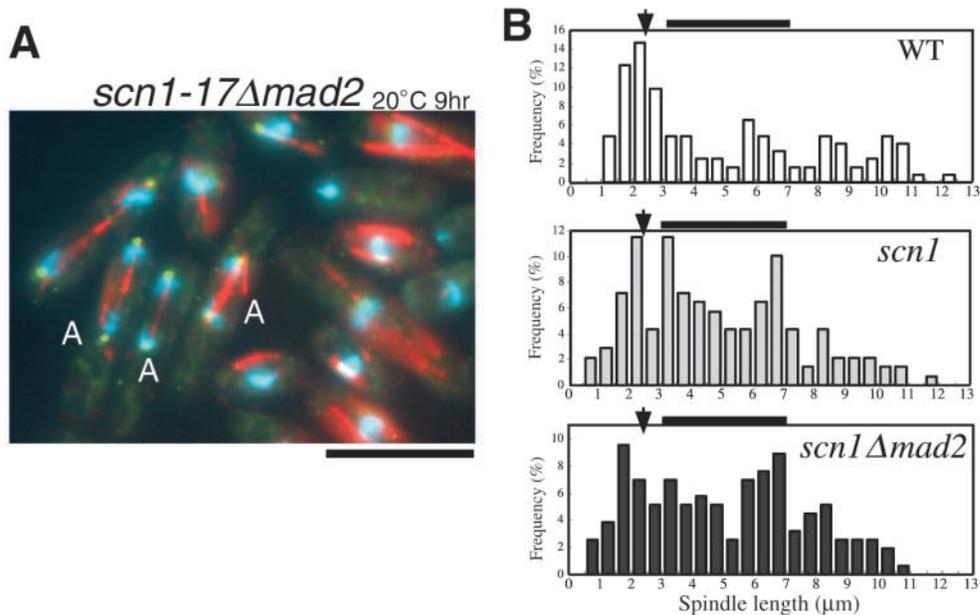


**Fig. 7.** The delay of spindle elongation in *scn1-17* was not dependent on the spindle checkpoint. (A) The double mutant *scn1-17 Δmad2* cells were cultured at 20°C for 9 hours in the YPD medium and cells were fixed for staining by antibodies against tubulin (red) and Sad1 (green). DAPI (blue) staining was done for DNA. The late anaphase cells were designated as A. Late anaphase cells were observed in *scn1 Δmad2* as frequent as in the *scn1* single mutant cells. Bar, 10 μm. (B) The pole-to-pole distance in mitotic cells was measured in the wild-type, *scn1* and *scn1 Δmad2* cells that were cultured at 20°C for 9 hours in YPD. Cells were stained with anti-Sad1 (SPB) and anti-TAT1 (tubulin). Over 100 mitotic cells of each strain were analyzed. The filled arrowheads indicate the size of the wild-type spindle at metaphase. The solid bar indicates the sizes of anaphase spindle that were abundantly seen in *scn1* and *scn1 Δmad2* cells. The cells showing anaphase spindle were accumulated in *scn1 Δmad2* as much as in *scn1* single mutant cells.

spindle were not affected in the double mutant. Hence *scn1* Δ*mad2* double mutant showed the same spindle elongation delay as the single *scn1* mutant cells, suggesting that the spindle elongation delay in *scn1* was not caused by the activation of Mad2-dependent spindle checkpoint.

## Discussion

In this study we show that *scn1* and *scn2*, extragenic suppressors for *cut9-665*, are substitution mutants in the two tRNA genes, *SpaTRNAala.03* and *SpaTRNAala.02*, respectively, both of which contain the UGC anticodon. The *S. pombe* genome has a total of 11 tRNA-Ala genes but only two contain the UGC anticodon. Nucleotide sequences of *SpaTRNAala.03* and *SpaTRNAala.02* were identical, making the same tRNA. The *scn1* and *scn2* mutations, both of which occurred in the anticodon sequence in these two tRNA genes, were also identical (from UGC to UGU), so that the mutant tRNAs should be identical. Although we have no direct evidence, it is most likely that the resulting altered tRNA-Ala made in *scn1* and *scn2* mutants should bind to the ACA (Thr) codons in mRNA and misincorporate Ala into proteins at the site of protein synthesis on the ribosome. Hydrophilic Thr and hydrophobic Ala are distinct in terms of their properties, including the ability to be phosphorylated. It is hence possible that many proteins made in *scn1* and *scn2* mutants have altered properties from the wild-type cells, and some may even become poisonous. Note, however, that one wild-type Ala-tRNA(UGC) gene still exists in mutant cells, producing the normal tRNA-Ala (UGC), and that the activity of aminoacyl-tRNA synthetase often requires the correct anticodon sequence (Ibba et al., 1997; Ibba and Söll, 2000) so that the level of malfunctionally altered proteins made in *scn1* and *scn2* mutants may not be high. Indeed, these mutants normally grew at the permissive temperature. It is hence consistent with the fact that the double mutant *scn1* and *scn2* is synthetic lethal.

It was surprising that the *scn1* and *scn2* mutations in the Ala-tRNA (UGC) genes affect late mitosis rather strongly and that the tRNA mutation can cause improper chromosome segregation and lead to aneuploidy in a dominant fashion. As the mutant tRNA-Ala (UGU) introduced by plasmid into the wild-type cells was also found to cause similar mitotic phenotypes in a dominant manner, the mitotic defects seemed to be a consistent feature by this anticodon mutation. If chromosome missegregation has any implication in evolution through altering the genome organization, the anticodon change in the Ala-tRNA(UGC) gene described in this paper might have contributed to evolution. These tRNA mutants were sensitive to a tubulin inhibitor so that microtubule organization seemed to be somewhat impaired in mutant cells.

Other aspects that should be considered for interpreting the results is that the phenotypes seen in *scn1* and *scn2* mutants at 20°C might be produced by a kind of cell cycle checkpoint (Hartwell and Weinert, 1989). The accumulation of cells delayed in late mitosis might be caused by a checkpoint control that is hitherto unknown. The delay was independent of the presence of Mad2. In an earlier study by Mitchison and his colleagues protein synthesis was shown to be reduced during mitosis and restored after mitosis (Creanor and Mitchison, 1984). In *scn1* and *scn2* mutant cells, the protein synthesis checkpoint that monitored the presence of mutant tRNA might

block cell cycle progression and delay the re-initiation of protein synthesis during late mitosis in *scn1* and *scn2* mutant cells.

The mutation in *cut9-665* was reported to be caused by the nucleotide change from G to A in the 535th codon (Samejima and Yanagida, 1994) that caused the amino acid change from Ala to Thr; the altered codon could be bound to the mutant tRNA-Ala (UGU). As the Ala residue is expected to be incorporated into the 535th position of Cut9 in *scn1* and *scn2* mutant cells, the cause of suppression for *cut9-665* is now resolved. This interpretation is consistent with the failure of suppression for *cut9* null mutant cells and the failure of suppression for other APC/C mutant *nuc2-663* in the background of *scn1* and *scn2* mutations (Hirano et al., 1988; Samejima and Yanagida, 1994). This mode of suppression mediated by tRNA mutations is well known in prokaryotes as the missense suppression, but there have been only a few reports in eukaryotes (Su et al., 1990; Chiu and Morris, 1997; Kim et al., 1999). Dominant tRNA mutation has been reported for the case of PRP2 (see below).

The mitotic phenotypes of *scn1* and *scn2* at 20°C were somewhat reminiscent to that seen in α1-tubulin mutant, *nda2-KM52* (Toda et al., 1983). A hypothesis that tubulin functions in *scn1* and *scn2* mutants may be impaired due to misincorporation of Ala at the Thr positions might be consistent with the result that *scn1* and *scn2* mutants were hypersensitive to TBZ. To estimate how many Thr residues may be affected, we examined the ACA codon usage in the *S. pombe* tubulins. The α1-tubulin, Nda2, has three ACA codons in 455 residues, whereas the α2-tubulin, Atb2/Tub1 has two ACA codons in 449 residues (Toda et al., 1984). The β-tubulin, Nda3, has three ACA codons in 448 residues (Hiraoka et al., 1984). Because the wild-type tRNA-Ala (UGC) is present, the actual misincorporation should be lower than the values indicated here. According to the codon usage database for *S. pombe* (http://www.kazusa.or.jp/codon/), the percentage of ACA codon in all codons is 1.44%, indicating that 6.4 ACA codons are used in a 450 amino acid protein on average. This value is higher than those found in tubulins.

In budding yeast, a dominant cs mutation in the anticodon sequence has been reported (Kim et al., 1999). The *PRP2* gene encodes an RNA-dependent ATPase that activates spliceosomes (Kim et al., 1992). A dominant, allele-specific suppressor of the ts *prp2-1* mutation was identified to be the mutation in the elongation methionine tRNA gene *EMT1*. The *EMT1-201* mutant seemed to suppress *prp2-1* by alleviating the splicing block. *EMT1-201* mutant displayed defects of pre-mRNA splicing and cold-sensitive growth. The mutation in *EMT1-201* exists in the anticodon, changing CAT to CAG, which presumably allowed *EMT1-201* suppressor tRNA to recognize CUG Leu codons instead of AUG Met codons. Curiously, the *prp2-1* allele has a point mutation that changes Gly to Asp, indicating that *EMT1-201* is not a classical missense suppressor. The *scn1* and *scn2* are the first examples of classical missense suppressors in *S. pombe*, although various nonsense suppressor tRNAs of *S. pombe* have been reported by Söll and his colleagues (Kohli et al., 1979; Wetzel et al., 1979). We did not examine whether *scn1* and *scn2* had splicing defects, but it is possible that *scn1* and *scn2* have slight defects in various cellular activities. However, the spindle elongation defects appear to be a most strong phenotype at low

temperature and crucial for maintaining the cell viability of *scn1* and *scn2*.

The *scn3*[+] gene was cloned by transformation followed by tetrad dissection. It was falsely assigned as the *scn1*[+] gene in the previous report – it was actually a high copy suppressor present near the true *scn1*[+] tRNA gene. The ability of *scn3*[+] to suppress both *scn1* and *scn2* mutations suggested that Scn3 has a role in downregulating dominant mutant tRNA. Recently, it was noticed in the conserved domain database that Scn3 has a nuclease motif that is even conserved in bacteria (*E. coli*). We postulate that Scn3 might preferentially cleave *scn1* and *scn2* mutant tRNAs at the restrictive temperature. Note that the level of the Scn3 transcript is stress inducible and increased at the high temperature (Chen et al., 2003).

In summary, the present report is the first to relate tRNA mutations to mitosis and regulation of anaphase. Mutations in the tRNA anticodon should produce a broad effect, but unexpectedly our phenotypic characterizations strongly indicated that mitotic defect was the principal outcome in *scn1* and *scn2* tRNA mutant cells. Translational regulation during mitosis has been poorly understood, and the present study suggests that the late anaphase step may be dependent on the quality control of protein synthesis. It is of considerable interest whether the same mutation causes similar cellular effects on other organisms.

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