



Research Article

The Loss of Rad3 Induces the Diploidization of *Tor2-287* Mutant to Promote the Cell Survival

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Abstract

Maintenance of genomic integrity is essential for all organism that help to maintain the number of chromosomes during cell division. In yeast and other organisms, the diploidization of genome serves as a key survival strategy under changing environmental conditions. Fission yeast ATR homolog Rad3 and TOR complex protein Tor2 are phosphatidylinositol 3-kinase-related protein kinases (PIKKs) and are involved in various functions. Rad3 is a key regulator of DNA damage signaling pathway and is involved in the maintenance of genomic integrity. The TOR complex, TORC1 and TORC2, contain Tor2 and Tor1 protein kinase respectively as their major component are involved in regulating eukaryotic cell growth, metabolism, and survival, in response to nutrient, energy, and environmental cues. In this study, we observed the suppression of temperature sensitive phenotype of a *tor2-287* mutation in *rad3Δ* background. The presence of azygotic asci and tetrad dissection analysis revealed the diploid nature of *rad3Δ tor2-287* double mutant cells, which was further confirmed by flow cytometry (FACS) analysis. In addition, the *tor2-287/tor2-287* homozygous and *tor-287/tor2+* heterozygous diploid exhibit temperature sensitive and resistant phenotypes, respectively. The tetrad dissection analysis revealed that a wild type copy of the *tor2+* and *rad3+* genes was present in *tor2-287 rad3Δ* strain due to the diploid nature of these cells, thereby suppressing the temperature sensitive phenotype. Overall, our results demonstrate that the absence of *rad3* suppresses the temperature sensitivity of *tor2-287* mutant by maintaining the diploid nature of cells.

Keywords

Rad3, Tor2, Ploidy, *S. pombe*

1. Introduction

Eukaryotic cells maintain their number of chromosomes (ploidy) during cell division to prevent genomic instability. Under normal growth conditions, the fission yeast *S. pombe* grows as a haploid cell but can also be maintained as a diploid cell under specialized growth conditions. In yeast and various other organisms, diploidization serves as a key survival and adaptive strategy

under stressful conditions or changing environments. This strategy helps the organism to increase genetic redundancy, boost the DNA repair mechanisms in order to avoid genomic instability, and the potential for sexual reproduction. Changes in ploidy through gain or loss of chromosome also results in aneuploidy that leads to chromosome instability, which is associated with

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Received: 4 February 2026; Accepted: 26 February 2026; Published: 16 March 2026



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cancer and drug resistant in fungi [1, 2]. In yeast, the polyploidy has been proposed to derive the cells for rapid adaptation through beneficial mutations and helps for the cell survival [3]. Fission yeast Rad3 and Tor2 are phosphatidylinositol 3-kinase-related protein kinases (PIKKs) and are involved in diverse functions. ATR homolog Rad3 is a key regulator of DNA damage signaling pathway and is involved in the maintenance of genomic integrity [4, 5]. The fission yeast *S. pombe* contain two TOR complexes, TORC1 and TORC2, that contain Tor2 and Tor1 protein kinase respectively as their major component [6, 7]. Wat1/Lst8, a conserved protein, is present in both the complexes and has the ability to interact with the kinase domain of mTOR or Tor1 independently of the other factors [6, 8]. Tor2 kinase is an important component of TORC1 complex that is primarily involved in promoting cell growth and metabolism in response to nutrient availability [9]. TORC1 also regulates ribosomal biogenesis and is involved in cell differentiation and in the maintenance of mitochondrial integrity [10-12]. Disruption of *wat1* leads to accumulation of reactive

oxygen species that results in chromosomal breakage, leading to activation of checkpoint kinase Chk1 [13]. The *tor2-287* mutant cells exhibit temperature sensitivity, with a phenotype similar to that of nitrogen-starved cells [7]. Here, we demonstrated that the absence of *rad3* suppresses the temperature sensitivity of *tor2-287* mutant by maintaining the diploid nature of cells and hence promote cell survival.

2. Methods

Yeast method: The *S. pombe* strains used in this study are listed in Table 1. Yeast Extract Medium (complete medium) and synthetic Edinburgh Minimal Media (EMM) were used for growing the cells. For the spotting assay, approximately 10^7 cells were serially diluted and spotted on a complete media plate. The homozygous and heterozygous diploid strains were constructed by the intragenic complementation of *ade6-210* and *ade6-216* alleles of *ade6* gene as described earlier [14].

Table 1. Strains used in this study.

Strain	Genotype	Source
SP3	<i>h⁺ leu1-32</i>	Lab stock
SH1695	<i>h⁺ leu1-32 ura4D18 rad3::ura4</i>	This study, derived from FY14154 (NBRP)
SH1692	<i>h⁻ ura4D18 tor2-287::hyg^R</i>	This study, derived from FY40338 (NBRP)
SH1701	<i>h⁺ leu1-32 ura4D18 tor2-287::hyg^R rad3::ura4</i> (exist as diploid)	This study
SH1836	<i>h leu1-32 ura4D18 tor2-287::hyg^R rad3::ura4</i> (exist as diploid)	This study
SH2094	<i>h⁺/h⁻ leu1-32/leu1⁺ tor2-287::hyg^R/tor2-287::hyg^R ade6-216/ade6-210</i>	This study
SH2096	<i>h⁺/h⁻ leu1-32/leu1⁺ tor2-287::hyg^R/tor2⁺ ade6-216/ade6-210</i>	This study
SH1658	<i>h⁺/h⁻ leu1-32/leu1-32 ade6-216/ade6-210</i>	Lab stock

Table 2. Percent sporulation in various strains.

S.No.	Genotype	Total no. of cells	Azygotic asci	Zygotic asci	% azygotic asci
1	Wild type	251	0	1	0.4%
2	<i>rad3::ura4</i>	222	0	1	0.45%
3	<i>tor2-287::hyg^R</i>	212	0	0	0
4	<i>tor2-287::hyg^R rad3::ura4</i>	295	56	12	19%
5	Wild type diploid	185	44	9	23.8%

Microscopy: For tetrad analysis, the cells were allowed to sporulate on a minimal media plate, and spores from 16 asci in two independent crosses were dissected on a YES plate using an Axioscope A1 tetrad dissection microscope (Zeiss). The

DIC images of sporulation were captured using an Olympus CKX53 microscope.

Flow cytometry: Cells were grown at 25°C till mid log phase. After centrifugation, the cells were collected and fixed

with 70% ethanol. Samples were processed for Flow cytometry analysis as described earlier [15] and analysed with a Becton-Dickinson FACS Calibur.

3. Result and Discussion

Absence of Rad3 suppresses the temperature sensitive phenotype of tor2-287 mutant with azygotic ascire. n sensitive phenotype of tor2-287 mutant.

In a genetic interaction study involving *rad3* and *tor2*, we observed that the *rad3Δ tor2-287* double mutant cells (in two different strains) were able to form colonies at non-permissive temperature as compared to *tor2-287* single mutant that exhibits a temperature-sensitive phenotype (Figure 1A), suggesting the suppression of temperature sensitive phenotype of *tor2-*

287 mutant in the absence of *rad3*. Further examination of these *tor2-287 rad3Δ* cells revealed the large-sized morphology at permissive and non-permissive temperature. Under minimal media conditions, the *S. pombe* cells form zygotic asci by the conjugation of two haploid cells, whereas pre-existing diploid cells form linear azygotic asci without prior cell conjugation. In order to further evaluate the sporulation, we grew the cells on minimal media plates and monitored them under the microscope. As shown in Figure 1B, approximately 24% of wild type diploid cells exhibit azygotic asci (Table 2). Interestingly, 19% of *tor2-287 rad3Δ* cells also showed azygotic asci (Figure 1B and Table 2). In comparison, the wild type and *rad3Δ* cells showed only 0.4%, 0.45% azygotic asci, respectively, while in *tor2Δ* cells, we could not observe asci (Figure 1B and Table 2). These results indicate the diploid nature of *tor2-287 rad3Δ* cells.

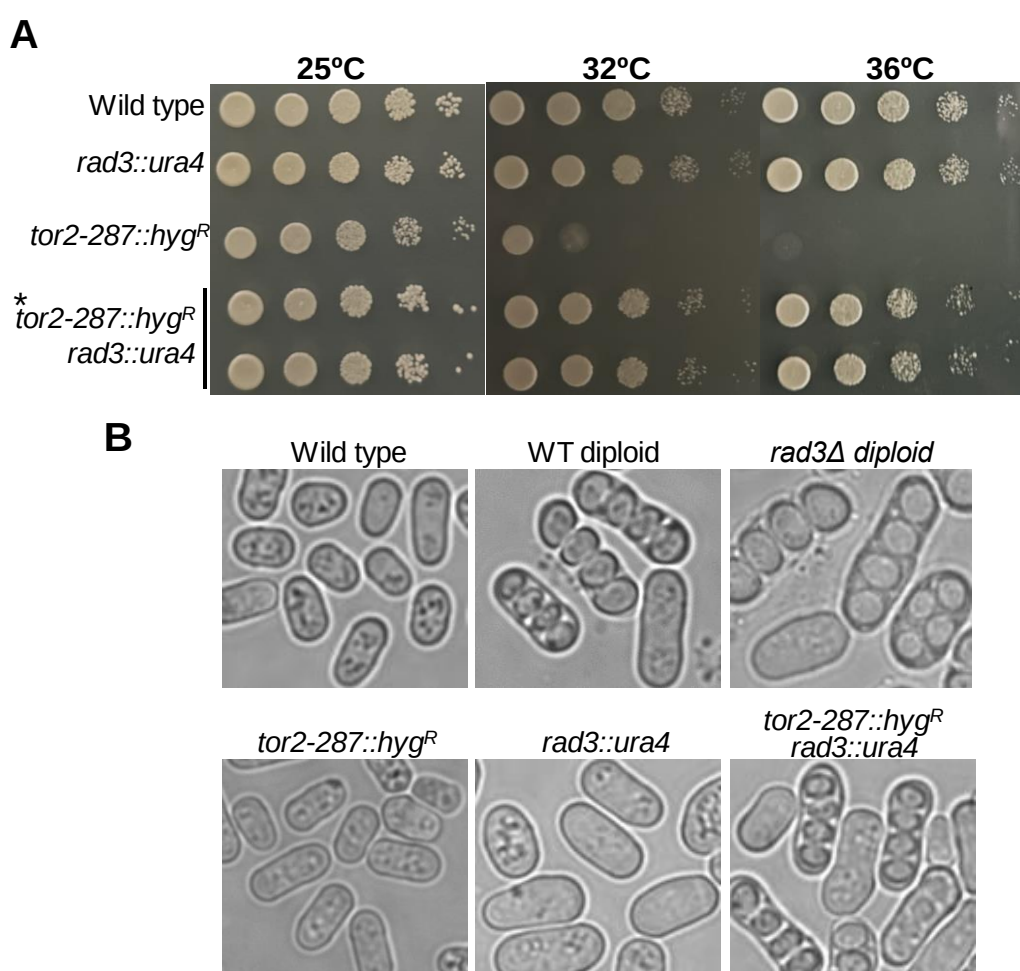


Figure 1. The double mutation of *rad3Δ tor2-287* suppresses temperature sensitive phenotype with azygotic asci. (A) Absence of *rad3* suppresses the temperature sensitivity of *tor2-287* mutant. Indicated strains were grown at 25°C till mid log phase, serially diluted, and spotted on rich media (YES) plates. Plates were allowed to grow at indicated temperature for 3-4 days before taking a photograph. The asterisk denotes a probable diploid nature of cells. The results shown here are from one set of experiment out of three independent experiments (B) Indicated strains were grown on an EMM agar plate, and a DIC image of cells containing spores was monitored under a microscope. For each strain, more than 200 cells were monitored for the calculation of percent azygotic asci.

The double mutant of tor2-287 rad3Δ cells are diploid in nature

To further confirm the genotype of these haploid spores, we dissected the azygotic asci obtained from *tor2-287 rad3Δ* mutant cells in two independent experiments. Each germinating spores were checked for its genotype by replica plating on uracil deficient plate, hygromycin containing plate, and temperature sensitive phenotype. We observed a 2:2 segregation of *tor2-287::hyg^R* and *rad3::ura4* gene in all the asci except two (Figure 2). Consistently, all the hygromycin resistant colonies exhibited temperature sensitive phenotype and were unable to grow on plates lacking uracil, indicating the *tor2-287* phenotype. These results suggest that the spores might have been generated from diploid cells. The presence of two asci with all four spores having a genotype of *rad3Δ* (*ura4⁺*) could be due to the homozygosing, a process in which a double strand break, followed by recombination, can lead to complete loss of one allelic copy, resulting in asymmetric segregation (4:0 or 0:4) as has been reported earlier [16, 17].

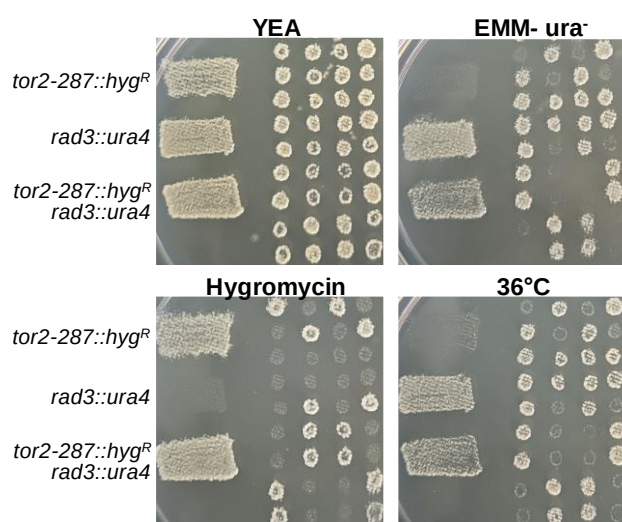


Figure 2. The double mutant of *tor2-287 rad3Δ* cells are diploid in nature and exhibits 2:2 segregation. The asci from *rad3Δ tor2-287* double mutant were dissected on a YES plate, and replica plated on the indicated plates. Parental strains were also patched and incubated at 25°C or 36°C for 3-4 days before taking a photograph. The results shown here are from one set of dissection out of two independent experiments as described in Methods.

In addition, the diploid nature of *rad3Δ tor2-287* double mutant was also checked by FACS analysis. As shown in Figure 3, a peak corresponding to 2C DNA content was observed in 87% of diploid control cells and 66% of *rad3Δ tor2-287* double mutant cells. In comparison, the percentage of cells containing 2C DNA content in *rad3Δ* and *tor2-287* single mutant was 26% and 17%, respectively, while in the wild type, most of the cells showed a single peak corresponding to 1C DNA content, suggesting the diploid nature of *rad3Δ tor2-287* double mutant cells.

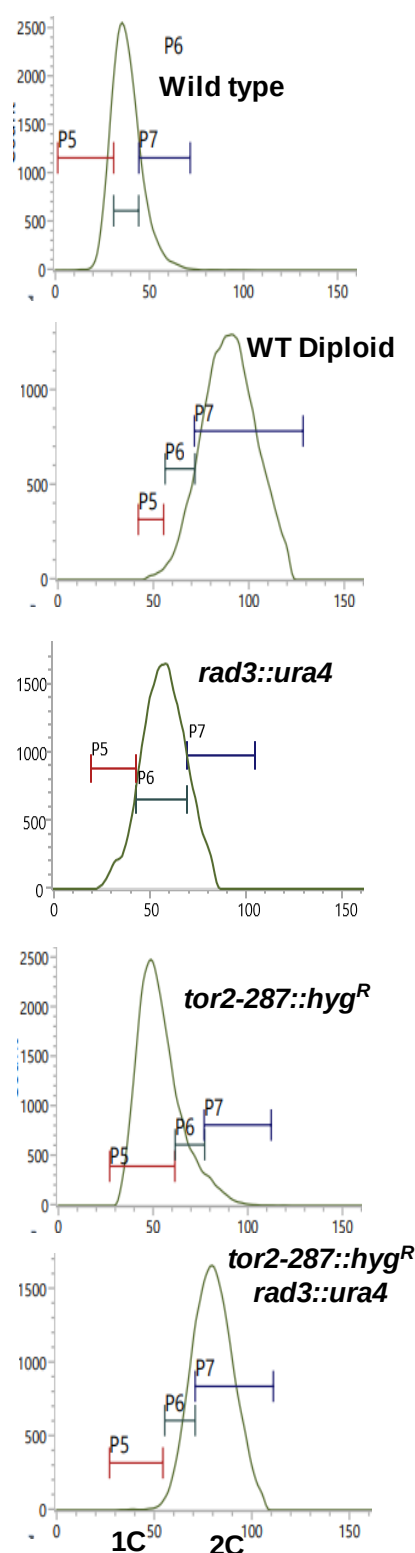


Figure 3. FACS analysis of cells. Indicated strains were grown at 25°C, stained with propidium iodide, and FACS analysis was performed using BD FACS Calibre for the analysis of DNA content. Multiple independent experiments were performed, and the results shown here are from one set of experiments.

The homozygous tor2-287 diploid mutant cells could not suppress the temperature sensitive phenotype

Further to check whether homozygous *tor2-287* diploid cells can also suppress the temperature sensitive phenotype, we constructed homozygous and heterozygous diploids of *tor2-287* mutant by genetic crosses. We observed that, the homozygous diploid of *tor2-287/tor2-287* exhibit temperature sensitivity while heterozygous diploid of *tor2-287/tor2⁺* were able to form colonies at non permissive temperature (Figure

4A). Furthermore, the sporulation analysis revealed azygotic asci in *tor2-287* homozygous and heterozygous strain (Figure 4B). These results suggest that the suppression of temperature sensitive phenotype in *tor2-287 rad3Δ* cells are due to the formation of diploid that also contain a wild type copy of *tor2⁺* and *rad3⁺* and hence gave a 2:2 segregation in our tetrad dissection analysis (Figure 2).

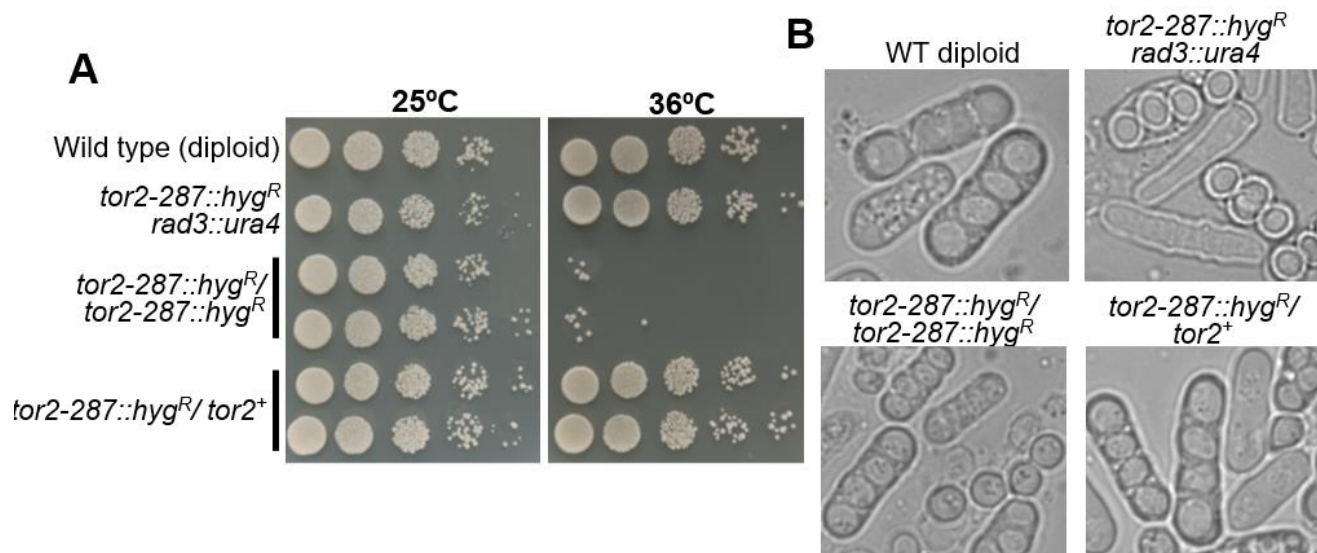


Figure 4. The phenotypic analysis of homozygous and heterozygous diploid strains. (A) The homozygous and heterozygous diploid strains were constructed by genetic crosses as described in Methods, and cells were grown at 25°C till mid log phase, serially diluted, and spotted on rich media (YES) plates. Plates were allowed to grow at the indicated temperature for 3-4 days before taking a photograph. (B) Indicated strains were grown on an EMM agar plate, and a DIC image of cells containing spores was monitored under a microscope.

Spontaneous diploidization in yeast mostly occurs due to endo-reduplication or mating type switching and could be advantageous for growth under certain stressful conditions [18]. In this study, we hypothesize that the cell containing *rad3Δ* in *tor2-287* mutant background undergoes diploidization that also contain a wild type copy of *rad3⁺* and *tor2⁺* gene and hence promotes cell survival. This phenotype was observed only when haploid *rad3Δ* cells were crossed with haploid *tor2-287::hyg^R*, resulting self diploidization. The results presented here cannot rule out the possibility of secondary suppressor mutation or gene conversion event that can also contribute to phenotypic suppression. In a previous study, the spontaneous diploidization was proposed to act as a dosage suppressor to rescue the growth defects associated with spindle pole body mutants that have defective centrosomes [19]. In budding yeast, the high frequency of self diploidization of *def1Δ* cells has also been reported [20]. In a recent study, the SSD1 has been identified as a genetic factor that reduces auto diploidization in *S. cerevisiae* [21].

Abbreviations

FACS Flow Cytometry

YEA Yeast Extract Agar
 EMM Edinburgh Minimal Media
 YES Yeast Extract with Supplement
 DNA Deoxyribonucleic Acid
 DIC Differential Interference Contrast
 Microscopy
 PIKK Phosphatidylinositol 3-Kinase-Related
 Protein Kinase

Acknowledgments

We thank Dr. Jagmohan Singh and lab members for helpful discussions, and yeast resource center NBRP, Japan for providing the strains as mentioned in the strain list. Our special thanks to the Sophisticated Analytical Instrument Facility (SAIF) of CDRI for FACS analysis. The CDRI communication number for this manuscript is 11128.

Funding

The work was supported by the grants from the Council of Scientific and Industrial Research, New Delhi, India

(MLP2044).

Author Contributions

Monika Deoralia: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft

Lalita Panigrahi: Formal Analysis, Methodology, Resources

Shakil Ahmed: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – review & editing

Conflicts of Interest

The authors declare no competing interests.

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