Mutations in Ran system affected telomere silencing in Saccharomyces cerevisiae

Mutations in Ran system affected telomere silencing in *Saccharomyces cerevisiae.*

Naoyuki Hayashi [1, 2, *], Masahiko Kobayashi[1], Hiroko Shimizu[1],

Ken-ichi Yamamoto[1], Seishi Murakami[2] and Takeharu Nishimoto[3]

1:Department of Molecular Pathology, 2:Department of Molecular Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan and 3:Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan

*: Corresponding author

e-mail: naoyuki@kenroku.kanazawa-u.ac.jp

Abastract

The Ran GTPase system regulates the direction and timing of several cellular events, such as nuclear-cytosolic transport, centrosome formation, and nuclear envelope assembly in telophase. To gain insight into the Ran system's involvement in chromatin formation, we investigated gene silencing at the telomere in several mutants of the budding yeast *Saccharomyces cerevisiae*, which had defects in genes involved in the Ran system. A mutation of the RanGAP gene, *rna1-1*, caused reduced silencing at the telomere, and partial disruption of the nuclear Ran binding factor, *yrb2-Δ2*, increased this silencing. The reduced telomere silencing in *rna1-1* cells was suppressed by a high dosage of the *SIR3* gene or the *SIT4* gene. Furthermore, hyperphosphorylated Sir3 protein accumulated in the *rna1-1* mutant. These results suggest that RanGAP is required for the heterochromatin structure at the telomere in budding yeast.

Keywords: Gsp1, heterochromatin, Ran, Rna1, telomere silencing, Sir3, Sit4, Snf2, budding yeast, Yrb2

Introduction

Heterochromatin is cytologically detected in nuclei as a condensed part of the genome. It is present in telomeric and centromeric areas, and interacts with the nuclear matrix to maintain the chromosomal stability. Repetitive sequences are often found in heterochromatic regions, and transcription within these regions is strongly repressed. In *Saccharomyces cerevisiae*, the telomere, subtelomere, rDNA region on Chromosome XII, and the silent mating loci on Chromosome III have heterochromatic features [1, 2]. These regions in chromatin are highly condensed, replicate in the late S phase, and bind to nuclear periphery [3, 4]. The silent information regulator proteins, Sir2/3/4, are essential for establishing silencing in these regions [2].

The Sir3 protein interacts with histone H3 that is methylated at lysine 4, Rap1, and the Ku complex, and none of these interactions is dispensable for telomere silencing [5, 6, 7]. Sir3 could be the limiting factor for silencing at the telomere, because a high dosage of the *SIR3* gene extends the transcriptional silencing region farther from the ends of the chromosome [8]. Furthermore, Sir3's silencing function at the telomere is regulated by its phosphorylation by MAP kinase and protein phosphatase 2A (PP2A),

under control of the target of rapamycin (TOR) system in response to cellular stress [9, 10].

The Ras-like nuclear small G protein, Ran plays a key role in nuclear-cytosolic transport, mitotic spindle formation, and nuclear envelope assembly in the late M phase [11]. Ran predominantly localizes to the nucleus. Some nuclear Ran is binds to chromatin, which it does by different mechanisms, either dependent on or independent of RCC1, which catalyzes guanine nucleotide exchange on Ran [12]. The chromatin-bound Ran induces chromosomal decondensation, nuclear envelope assembly, and formation of the nuclear pore [13]. During interphase, the GTPase-activating protein for Ran, RanGAP, mainly localizes to the cytosol, but in mitosis, it functions at the kinetochore and mitotic spindles, in higher eukaryotes [14]. The RanGAP protein sequence contains a nuclear localization signal (NLS) and 2 nuclear export signals (NES), and its localization is dependent on Crm1/exportin [15]. In *Schizosaccharomyces pombe*, the *cla4* and *snf2SR* genes, which encode histone H3 methyltransferase, which is involved in heterochromatin formation and chromatin remodeling factor homologue, respectively, were isolated as multicopy suppressors for *rna1* temperature-sensitive (Ts)

mutants [16]. The Rna1 protein in *Sch. pombe* interacts directly with histone H3 in vitro, and this interaction stimulates the methylation of histone H3 on K9 by Cla4 in vitro [17]. In *S. cerevisiae*, the overproduction of Prp20, an RCC1 homologue, weakens telomere silencing, and Gsp1, a Ran homologue, bind to Sir4, in which the N-terminus is deleted [18]. Together, these findings indicate that the Ran system might be involved in the regulation of heterochromatin structure.

To investigate the possible role of the Ran system in chromatin regulation at the telomere, we examined telomere silencing in several *S. cerevisiae* mutants with defects in Ran system genes. A silencing deficiency and hyperphosphorylation of Sir3 were found in an *rna1-1* mutant, and the silencing deficiency was suppressed by high dosages of the *SIR3* gene or the *SIT4* gene, which encodes silencing information regulator, or PP2A. Furthermore, the disruption of *SNF2*, which encodes a chromatin remodeling factors, enhanced the telomere-silencing deficiency of *rna1-1*. On the other hand, *gsp1* and *yrb2-Δ2* mutants showed increased gene silencing at the telomere compared with wild-type cells. These results suggest that the Ran system controls the formation of heterochromatin at the telomere in *S. cerevisiae*.

Materials and methods

Strains and media

The *S. cerevisiae* strains used are listed in Table 1. *Escherichia coli* strain DH5α was used for plasmid construction. The culture media, transformation techniques, and genetic manipulations for yeast cells were as described previously [19]. The *URA3* marker that was designed to be inserted at the *ADH4* locus in the vicinity of the telomere of the left arm of chromosome VII and the *SIR3-Flag* fusion gene were genetically introduced from previously constructed strains [10]. The gene disruption of *SNF2*, *snf2::kanMX4*, was genetically derived from yeast strain BY4742 (EUROSCARF).

Plasmid construction

The *RNA1* DNA was amplified from genomic DNA prepared from *the S. cerevisiae* wild-type strain W303-1A [20] using the primers, 5'-AACATGAGCTCCTTAGGTGC-3' and 5'-ACCCTTATTATCGGGAGCTC-3'. The resulting 2.4-kb PCR product was digested with *Sac*I (Takara Bio Inc., Japan), and cloned into YEplac181 [21] to produce plasmid YEpLRNA1. The DNA fragment isolated from YEpLRNA1 by digestion with *Eco*RI and *Sal*I (Takara Bio Inc., Japan),

containing *RNA1*, was cloned into pRS316 [22] to yield plasmid pRSRNA1. The DNA fragment isolated from YCpSIT4 (kindly provided by Drs. Sakumoto and Harashima) by digestion with *Bam*HI (Takara Bio Inc., Japan), containing *SIT4*, was cloned into YEplac181 and YEplac112 [21] to produce plasmids YEpLSIT4 and YEpTSIT4, respectively. The DNA fragment isolated from p195YRB2 [23] by digestion with *Bam*HI and *Hin*dIII (Takara Bio Inc., Japan), containing *YRB2*, was cloned into YEplac112 to produce plasmid YEpTYRB2. The DNA fragment isolated from pTKS-yrb2-Δ2 [34] by digestion with *Sal*I and *Eco*RI (Takara Bio Inc., Japan), containing the promoter and N-terminus of *YRB2* in *yrb2-Δ2*, was cloned into YEplac112 to yield plasmid YEpy2D2.

To examine dosage effect of the *SNF2* gene to expression of *URA3* at the telomere in the *rna1-1* cells, we disrupted the *URA3* gene on pSY286 [24] by homologous recombination of the mutated PCR product, which was synthesized with the primers 5’-GATAAATCTGTCGAAAGCTACATATAAGG-3’ and 5’-CAAAAGGCCCTAGGTTCCTTTGTTAC-3’ introducing lack of translational initiation codon and frame shift to *URA3*. Wild-type strain NBW5 [25] harboring

pSY286, which contained 2μ, *URA3, SNF2-myc*, and *Saccahromyces kluyveri HIS3*, was transformed with the PCR product described above, and selected on histidine drop-out and 5FOA plate. The plasmid DNA, which was harvested from the survived transformants, was confirmed His^+ Ura^- phenotype in the re-transformants, and named pSY286u.

Analysis of Sir3 phophorylation

Yeast cells bearing a *SIR3-Flag* fusion gene [10] were grown to late logarithmic phase in 5 ml of YPDA liquid medium, and approximately 10^7 cells were harvested. Cell extracts were prepared as described previously [26], and subjected to immunoblot analysis using the anti-Flag monoclonal antibody M2 (Sigma, USA). The proteins were visualized with the enhanced chemiluminescence (ECL) kit, according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed as described previously [10].

The precipitated DNA was extracted with phenol-chloroform, and concentrated by ethanol precipitation. Prior to the fixation, aliquots equal to half the sample volume were removed and treated as described above as the “input” control samples. Eighteen cycles of PCR were performed in 50-μl volumes using EX-Taq (Takara Bio Inc., Japan) with primers for the Y’ subtelomere and control gene locus, without reaching a plateau phase, as described previously [10]. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide.

Results and discussion

Telomere silencingability in mutants of the Ran-RCC1 genes

Ran localizes to chromatin by different mechanisms, either dependent on or independent of RCC1, but little is known about the purpose Ran's chromatin localization. To examine Ran's possible functions in heterochromatin formation, we investigated gene silencing at the telomere in *S. cerevisiae* mutants in Ran, RCC1, and RanGAP. As a marker gene, *URA3-TEL*, *URA3* attached to telomere sequence as described in Materials and methods [27], was genetically introduced into each mutant. The expression level of *URA3* was estimated by the efficiency of cell growth on a uracil drop-out plate or with 1 mg/ml 5FOA. Slower growth on the uracil drop-out plate represents greater silencing of *URA3* gene expression at the telomere, and slower growth on the 5FOA plate represents a silencing deficiency, because *URA3*-expressing cells are sensitive to 5FOA. Only the *rna1-1* mutant, harboring a Ts mutation in the RanGAP gene, showed a silencing deficiency, and greater silencing was observed in the *gsp1-1268* mutant compared with wild-type (Fig. 1A). The observation that a Ts mutation of the RanGAP gene caused a silencing deficiency are consistent with the recent finding that the overproduction of

Prp20 (RanGEF) weakens telomere silencing [18], although we did not observe any alterations in telomere silencing in the *prp20-1* cells under our assay conditions.

The Sir complex plays a crucial role in gene silencing in *S. cerevisiae* [6, 8]. Telomere silencing requires the Sir complex to bind with Rap1, the Ku complex, and K4-methylated histone H3 [5, 26], and the amount of Sir3 protein on the telomere influences the level of silencing [8]. In growth condition of this experiment, nuclear localization of Gal4-GFP fusion protein was not altered in the *rna1-1* cells (data not shown). We therefore used a ChIP assay to investigate the amount of Sir3 protein on the subtelomeric region in *rna1-1* cells. To compare strains on an identical genetic background, the empty vector or pRSRNA1 was introduced into the N619-7A strain (*rna1-1 SIR3-Flag::LEU2*). We found that the amount of Sir3 protein on the Y' subtelomere was lower in *rna1-1* cells transformed with empty vector than in those transformed with pRSRNA1 (Fig. 1B). This ChIP assay result suggest that the heterochromatin structure at the subtelomere is altered in *rna1-1* cells.

Multi-copy suppression of the silencing defect in *rna1-1* cells

Hyperphosphorylation of Sir3, which is a rate-limiting factor for telomere silencing, was recently observed in *sit4* cells, which lack one of the PP2As. Moreover, the *sit4* cells also exhibit a silencing deficiency and a slightly shortened telomere [10]. Therefore, we examined whether high-dosages of the *SIR3* or *SIT4* gene would affect the silencing in *rna1-1* cells. Transformants of strain N585-5A (*rna1-1 adh4::URA3-TEL*), harboring empty vector, *SIR3,* or *SIT4* on a multicopy plasmid, were examined for their ability to grow on 5FOA or below the restrictive temperature. Either *SIR3* or *SIT4* on a multicopy plasmid partially suppressed the silencing deficiency at the telomere of *rna1-1* cells, but neither rescued the temperature sensitivity of the *rna1-1* mutation (Fig. 2A). This pleiotropy suggests that the cause of the temperature-sensitivity was distinct from that causing the silencing deficiency at the telomere in *rna1-1* cells. Highly phosphorylated Sir3 is released from the telomere and reduces telomere silencing [9], and also *sit4* cells, which lack one of the PP2As, accumulate highly phosphorylated Sir3 protein and have a silencing deficiency [10]. We therefore examined the phospohorylation of Sir3 protein by immunoblot analysis. In *rna1-1* cells, most of the Flag-tagged Sir3 migrated more slowly through the gel (Fig. 2B, lane 2).

Recently, our colleagues found that the *rna1* mutation in *Sch. pombe* is suppressed by overexpression of the *SNF2* homologue, which encodes a chromatin-remodeling factor [16], and RanGAP1 in *Sch. pombe* interacts with globular domain of histon H3 [17]. On the other hand, Snf2 in *S. cerevisiae* loses chromatin association in *hht2-11* mutant, which has point mutaion in globular domain in histone H3 [28], and it is required for gene silencing [29]. To investigate whether Rna1 is involved in the silencing machinery of Snf2, we examined dosage effect of the *SNF2* gene to the *rna1-1* mutant. High dosage of *SNF2* did not suppress Ts phenotype in the *rna1-1* mutant unlike in *Sch. pombe*, but rescued silencing deficiency (Fig. 3A). On the other hands, we constructed a double mutant of *rna1-1* and *snf2* and examined its silencing ability at the telomere (Fig. 3B). In our strain's background, the silencing deficiency in the *snf2* disruptant was not significant, but it showed a tendency toward slower growth on the 5FOA plate. However, a drastic growth defect on the 5FOA plate was observed in *rna1-1 snf2* double mutant compared with the *rna1-1* mutant. Thus, the *SNF2* gene encoding a chromatin remodeling factor took a crucial role for silencing at the telomere in *rna1-1* cells.

In Ts *rna1* mutants of *Sch. pombe*, a silencing deficiency is observed at the centromere but not at the telomere, in contrast to *S. cerevisiae* [16]. Since there are great differences in the structural features of the centromere, subtelomere, and telomere between *S. cerevisiae* and *Sch. pombe*, the regulatory aspects of heterochromatin formation are likely to be different. However, similar phenomena regarding chromosomal segregation are observed in these two yeasts. In *S. cerevisiae*, *rna1-1* cells show more frequent aneuploidy [30], while mitotic chromosomal segregation is deficient in *rna1* mutants in *Sch. pombe* [16].

The nuclear factor *YRB2*, in the Ran-RCC1 system, controls telomere silencing

Yrb2, which possesses a Ran-binding domain at its C-terminus, is located inside the nucleus, and its localization is dependent on its N-terminus [31]. In vitro experiments showed that Yrb2 enhances the RanGAP activity of the *RNA1* gene product [23]. Therefore, we examined silencing in *YRB2* mutants (Fig. 4A). Although a complete null mutation of *YRB2*, *yrb2-Δ1*, did not affect *S. cerevisiae* growth on any medium, *yrb2-Δ2*, which lacked only the Ran-binding domain at the C-terminus, showed

slower growth on the uracil drop-out plate. This result suggests that the N-terminal portion of Yrb2 might have a regulatory function in telomere silencing. To investigate this possibility further, we introduced a plasmid harboring the *yrb2-Δ2* gene into wild-type cells (Fig. 4B). Interestingly, the cells transformed by the *yrb2-Δ2* plasmid showed significantly slower growth on the uracil drop-out plate. In addition, introduction of a plasmid harboring the entire *YRB2* gene did not restore the slowed growth of the *yrb2-Δ2* cells. These observations indicated that the phenotype of increased silencing at the telomere in *yrb2-Δ2* was dominant. The dominant phenotype of the N-terminus of Yrb2 in silencing regulation suggests that the N-terminus acts to open the heterochromatin structure, and the C-terminus has a suppressive role in that function.

Acknowledgement

We thank Drs. Sakumoto and Harashima (Osaka University, Japan) for the plasmids harboring *SIR3* and *SIT4*, Dr. Hinnebusch (NIH, USA) for the plasmid harboring *SNF2*, and Drs. Seino and Nishijima (National Institute of Genetics, Japan) for technical advice and helpful discussions.

References

[1] P. Laurenson, J. Rine, Silencers, silencing, and heritable transcriptional states. Microbiol. Rev. 56 (1992) 543-560.

[2] L. N. Rusche, A. L. Kirchmaier, J. Rine, Establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. Annu. Rev. Biochem. 72 (2003) 481-516.

[3] E. D. Andrulis, A. M. Neiman, D. C. Zappulla, R. Sternglanz, Perinuclear localization of chromatin facilitates transcriptional silencing. Nature 394 (1998) 592-595.

[4] S. Gasser, Positions of potential: nuclear organization and gene expression. Cell 104 (2001) 639-642.

[5] J. E. Haber, Sir-Ku-itous routes to make ends meet. Cell 25 (1999) 829-832.

[6] A. Hecht, T. Laroche, S. Stahl-Bolsinger, S. M. Gasser, M. Grunstein, Histone H3 and H4 N-termini interact with SIR3 and HECHT SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell 80 (1995) 583-592.

[7] H. Santos-Rosa, A. J. Bannister, P. M.Dehe, V. Geli, T. Kouzarides, Methylation of H3 lysine 4 at euchromatin promotes Sir3p association with heterochromatin. J. Biol.

Chem. 279 (2004) 47506-47512.

[8] A. Hecht, S. Stahl-Bolsinger, M. Grunstein, Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature 383 (1996) 92-96.

[9] W. Ai, P. G. Bertram, C. K. Tsang, T. –F. Chan, X. F. S. Zheng, Regulation of subtelomeric silencing during stress response. Mol. Cell 10 (2002) 1295-1305.

[10] N. Hayashi, T. Nomura, N. Sakumoto, Y. Mukai, Y. Kaneko, S. Harashima, S. Murakami, The *SIT4* gene, which encodes protein phosphatase 2A, is required for telomere function in *Saccharomyces cerevisiae.* Curr. Genet. 47 (2005) 359-367.

[11] S. Sazer, M. Dasso, The ran decathlon: multiple roles of Ran. J. Cell Sci. 113 (2000) 1111-1118.

[12] D. Bilbao-Cortes, M. Hetzer, G. Langst, P. B. Becker, I. W. Mattaj, Ran binds to chromatin by two distinct mechanisms. Curr. Biol. 12 (2002) 1151-1156.

[13] C. Zhang, M.W. Goldberg, W. J. Moore, T. D. Allen, P. R. Clarke, Concentration of Ran on chromatin induces decondensation, nuclear envelope formation and nuclear pore complex assembly. Eur. J. Cell. Biol. 81 (2002) 623-633.

[14] J. Joseph, S. –T. Liu, S. A. Jablonski, T. J. Yen, M. Dasso, The RanGAP1-RanBP2

complex is essential for microtubule-kinetochore interactions in vivo. Curr. Biol. 14 (2004) 611-617.

[15] W. Feng, A. L. Benko, J. H. Lee, D. R. Stanford, A. K. Hopper, Antagonistic effects of NES and NLS motifs determine *S. cerevisiae* Rna1p subcellular distribution. J. Cell Sci. 112 (1999) 339-347.

[16] A. Kusano, T. Yoshioka, H. Nishijima, H. Nishitani, T. Nishimoto, *Schizosaccharomyces pombe* RanGAP homolog, SpRna1, is required for centromeric silencing and chromosome segregation. Mol. Biol. Cell 15 (2004) 4960-4970.

[17] H. Nishijima, J. Nakayama, T. Yoshioka, A Kusano, H. Nishitani, K. Shibahara, T. Nishimoto, Nuclear RanGAP is required for the heterochromatin assembly and is reciprocally regulated by histone H3 and Clr4 histone methyltransferase in *Schizosaccharomyces pombe*. Mol. Biol. Cell 17 (2006) 2524-2536.

[18] M. Clement, F. Deshaies, L. De Repentigny, P. Belhumeur, The nuclear GTPase Gsp1p can affect prper telomeric function through the Sir4 protein in *Saccharomyces cerevisiae*. Mol. Microbiol. 62 (2006) 453-468.

[19] A. Adams, D. E. Gottschling, C. A. Kaiser, T. Stearns, Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. (1997).

[20] R. J. Rothstein, One-step gene disruption in yeast. Methods Enzymol. 101 (1983) 202-211.

[21] G. T. Drivas, A. Shih, E. Coutavas, M. G. Rush, P. D'eustachio, Characterization of four novel RAS-related genes expressed in a human teratocarcinoma cell line. Mol. Cell. Biol. 10 (1990) 1793-1798.

[22] R. S. Sikorski, P. Hieter, A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122 (1989) 19-27.

[23] E. Noguchi, N. Hayashi, N. Nakashima, T. Nishimoto, Yrb2p, a Nup2p-related yeast protein, has a functional overlap with Rna1p, a yeast Ran-GTPase-Activating Protein. Mol. Cell. Biol. 17 (1997) 2235-2246.

[24] S. Yoon, H. Qiu, M. J. Swanson, A. G. Hinnebusch, Recruitment of SWI/SNF integrity, SRB mediator, and SAGA. Mol. Cell. Biol. 23 (2003) 8829-8845.

[25] H. Matsuzaki, R. Nakajima, J. Nishiyama, H. Araki, Y. Oshima, Chromosome

engineering in *Saccharomyces cerevisiae* by using a site-specific recombination system of yeast plasmid. J. Bacteriol. 172 (1990) 610-618.

[26] I. M. Fingerman, C. -L. Wu, B. D. Wilson, S. D. Briggs, Global loss of Set1-mediated H3 Lys^4 trimethylation is associated with silencing defects in *Saccharomyces cerevisiae*. J. Biol. Chem. 280 (2005) 28761-28765.

[27] M. S. Singer, D. E. Gottschling, TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. Science 266 (1994) 404-409.

[28] A. A. Duina, F. Winston, Analysis of a mutant histone H3 that perturbs the association of Swi/Snf with chromatin. Mol. Cell. Biol. 24 (2004) 561-572.

[29] V. Dror, F. Winston, The Swi/Snf chromatin remodeling complex is required for ribosomal DNA and telomeric silencing in *Saccharomyces cerevisiae.* Mol. Cell. Biol. 24 (2004) 8227-8235

[30] N. S. Atkinson, R. W. Dunst, A. K. Hopper, Characterization of an essential *Saccharomyces cerevisiae* gene related to RNA processing: cloning of *RNA1* and generation of a new allele with a novel phenotype. Mol. Cell. Biol. 5 (1985) 907-915.

[31] T. Taura, G. Schlenstedt, P. A. Silver, Yrb2p is a nuclear protein that interacts with Prp20p, a yeast Rcc1 homologue. J. Biol. Chem. 272 (1997) 31877-31884.

[32] J. M. Ivy, A. J. Klar, J. B. Hicks, Cloning and characterization of four *SIR* genes of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6 (1986) 688-702.

Table1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
NBW5	*MATα trp1 leu2 his3 ade2 ura3 can1*	[25]
N309-1C	*MATα trp1 leu2 his3 ade2 ura3 lys2 can1 adh4::URA3-TEL DIA5-1**	[10]
N585-5A	*MAT***a** *rna1-1 trp1 leu2 his3 ade2 ura3 can1 adh4::URA3-TEL DIA5-1**	This study
N586-6B	*MATα prp20-1 trp1 leu2 his3 ade2 ura3 can1 adh4:: URA3-TEL DIA5-1**	This study
N587-2A	*MAT***a** *gsp1::HIS3::gsp1-1268::LEU2 trp1 leu2 his3 ade2 ura3 can1 adh4::URA3-TEL DIA5-1**	This study
N588-4A	*MATα gsp1::HIS3::gsp1-1757::LEU2 trp1 leu2 his3 ade2 ura3 can1 adh4::URA3-TEL DIA5-1**	This study
N589-1D	*MAT***a** *gsp1::HIS3::GSP1::LEU2 trp1 le2 his3 ade2 ura3 can1 adh4::URA3-TEL DIA5-1**	This study
N619-7A	*MATα rna1-1 SIR3-5FLAG::LEU2 tp1 leu2 his3 ade2 ura3 can1*	This study
N619-7B	*MATα SIR3-5FLAG::LEU2 tp1 leu2 his3 ade2 ura3 can1*	This study
N688-1B	*MAT***a** *yrb2-Δ2::LEU2 tp1 leu2 his3 ade2 ura3 can1 adh4::URA3-TEL DIA5-1**	This study
N689-5A	*MATα yrb2-Δ1::LEU2 tp1 leu2 his3 ade2 ura3 can1 adh4::URA3-TEL DIA5-1**	This study
BY4742	*MATα snf2::kanMX4 lysΔ0 leu2Δ0 his3Δ1 ura3Δ0*	EUROSCARF
N752-7B	*MATα snf2::kanMX4 rna1-1 trp1 leu2 his3 lys2 ura3 can1 adh4::URA3-TEL*	This study
N752-7C	*MATα snf2::kanMX4 trp1 leu2 his3 lys2 ura3 can1 adh4::URA3-TEL*	This study
N755-15C	*MATα rna1-1 trp1 leu2 his3 ade2 ura3 can1 adh4::URA3-TEL*	This study

* *DIA5-1* refers to the directed integration of *ADE2* adjacent to the telomere of the right arm of chromosomeV [27].

Figure legends

Fig. 1. Silencing ability of Ran-RCC1 mutants. (A) Expression of *URA3* at the telomere was altered in some of Ran-RCC1 mutants. The wild-type (N589-1D), *rna1-1* (N585-5A), *prp20-1* (N586-6B), *gsp1-1268* (N587-2A), and *gsp1-1757* (N588-4A) strains, which all harbored the *URA3* marker at the end of chromosome VII, were cultured in YPDA liquid medium and spotted on uracil drop-out, complete synthetic, and 5FOA plates. The culture of each strain was diluted to an $OD_{660\,nm}$ of 1.0. The four patches in each row represent 10-fold serial dilutions. The cells were incubated for 3 days at 27^{o}C. (B) The binding of Sir3 to the Y' subtelomere was reduced in *rna1-1* cells. Transformants derived from the *rna1-1* (N619-7A) strain harboring empty vector (pRS316) or pRSRNA1 were compared in a ChIP assay with Flag-tagged Sir3. The co-precipitated DNA was analyzed as described previously [10]. The PCR products of the Y' subtelomere and *YGL213c/SKI8* were 0.9 kbp and 0.4 kbp in length, respectively.

Fig. 2. Multicopy suppression of the silencing deficiency in *rna1-1* cells. (A) The silencing deficiency in *rna1-1* cells was suppressed by the introduction of *SIT4* or *SIR3*

on a multicopy plasmid, but the temperature sensitivity was not. The *rna1-1* (N585-5A) strain was transformed with vector (YEplac181), *RNA1* (YEpLRNA1), *SIR3* (pKAN63; [32]), or *SIT4* (YEpLSIT4) plasmid. Each transformant was cultured in leucine drop-out liquid medium, and spotted on leucine drop-out plates with and without 5FOA, as described in Fig. 1. The control and 5FOA plates were incubated at 27 ^{o}C for 3 days, and temperature sensitivity was examined at 35 ^{o}C. (B) Modification of Sir3 protein in Ran-RCC1 mutants. The slower migrating bands of Flag-tagged Sir3 immunoblotted with an anti-Flag M2 monoclonal antibody represent phosphorylated Sir3. Extracts derived from the wild-type (N619-7B, lane 1), *rna1-1* (N619-7C, lane2) were separated by 6% SDS PAGE, and visualized as described in Materials and methods.

Fig. 3. Interactions between *RNA1* and *SNF2* in telomere silencing. (A) High dosage of the *SNF2* gene suppressed silencing deficiency, but not Ts growth in *rna1-1*. The *rna1-1* (N755-15C) strain harboring vector (pRS313), or *SNF2* (pSY286u) plasmid, and wild-type (N309-1C) strain harboring vector plasmid were cultured in histidine drop-out liquid medium, and spotted on histidine drop-out plates with and without 5FOA, as

described in Fig. 1. The control and 5FOA plates were incubated at 27 °C for 3 days, and temperature sensitivity was examined at 35 °C. (B) The silencing deficiency of *rna1-1* was enhanced by the lack of the *SNF2* gene. The wild-type (N309-1C), *rna1-1* (N585-5A), *rna1-1 snf2::kanMX4* (N752-7B), and *snf2::kanMX4* (N752-7C) strains, which all harbored the *URA3* marker at the end of the left arm of chromosome VII, were cultured in YPDA liquid medium and spotted on complete synthetic and 5FOA plates, as described in Fig. 1. The spotted plates were incubated at 27 °C for 3 days.

Fig. 4. Increased silencing at the telomere in a *yrb2* mutant, which lacked the Ran-binding domain but retained the N-terminal portion of Yrb2. (A) The wild-type (N309-1C), *yrb2-Δ1* (N689-5A) and *yrb2-Δ2* (N688-1B) strains were cultured and spotted on uracil drop-out, complete synthetic, and 5FOA plates, as described in Fig. 1. The spotted plates were incubated at 27 °C for 3 days. Bottom panel represents the structural features of the *YRB2* locus in each mutant, which were described in detail previously [23]. *LEU2* DNA was inserted into the deleted region in *yrb2*. (B) The increased silencing phenotype of the *yrb2-Δ2* mutation was dominant. The wild-type

(N309-1C) and *yrb2-Δ2* (N688-1B) strains were transformed with vector (YEplac112) or *yrb2-Δ2* (YEpy2D2) plasmid, and vector (YEplac112) or *YRB2* (YEpTYRB2) plasmids, respectively. Each transformant was cultured in tryptophan drop-out liquid medium, and spotted on tryptophan and uracil drop-out and tryptophan drop-out plates, as described in Fig. 1. The spotted plates were incubated at 27 ^{o}C for 3 days.

A

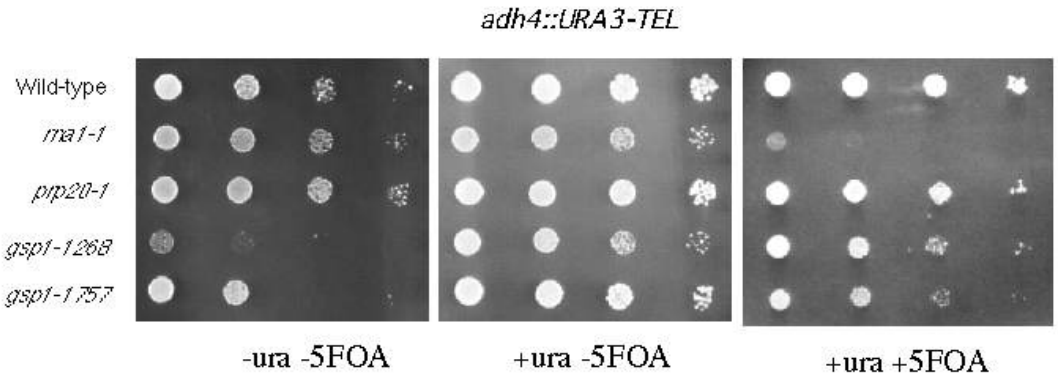

adh4::URA3-TEL
Wild-type
rna1-1
prp20-1
gsp1-1268
gsp1-1757
-ura -5FOA
+ura -5FOA
+ura +5FOA

Fig.1A

B

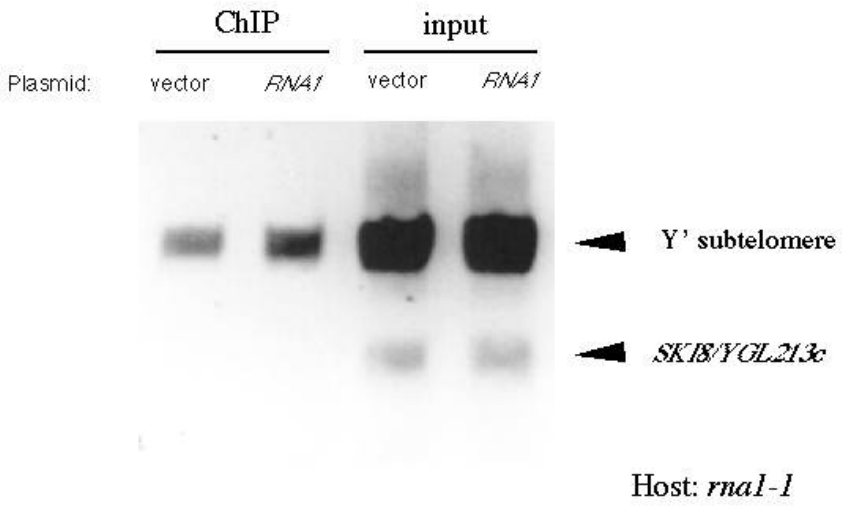

ChIP
input
Plasmid:
vector
RNA1
vector
RNA1
Y' subtelomere
SKI8/YGL213c
Host: rna1-1

Fig. 1B

A

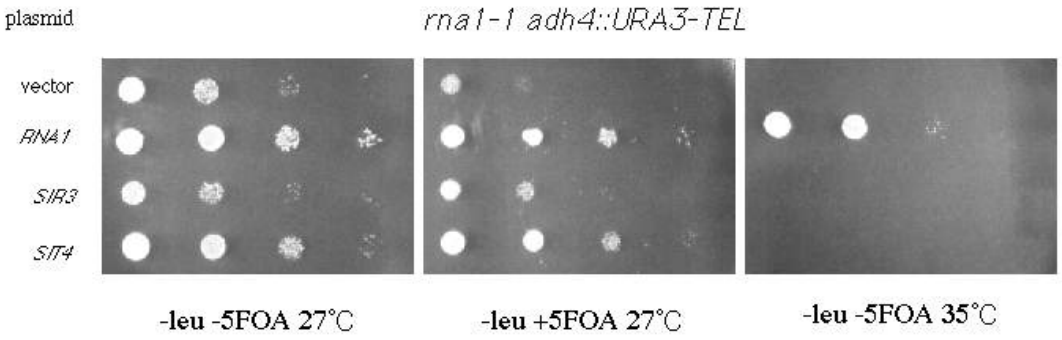

plasmid
rna1-1 adh4::URA3-TEL
vector
RNA1
SIR3
SIT4
-leu -5FOA 27°C
-leu +5FOA 27°C
-leu -5FOA 35°C

Fig. 2A

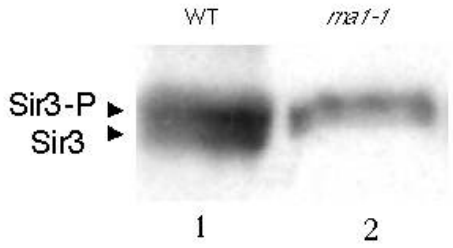


Fig. 2B

A

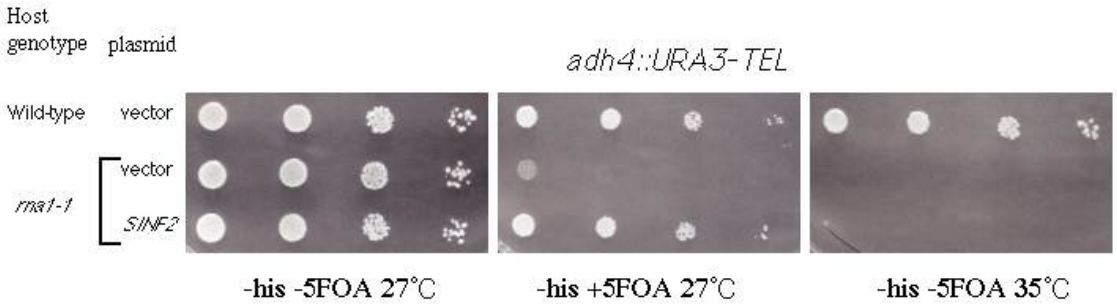

Host
genotype
plasmid
adh4::URA3-TEL
Wild-type
vector
rna1-1
vector
SINF2
-his -5FOA 27°C
-his +5FOA 27°C
-his -5FOA 35°C

Fig. 3A

B

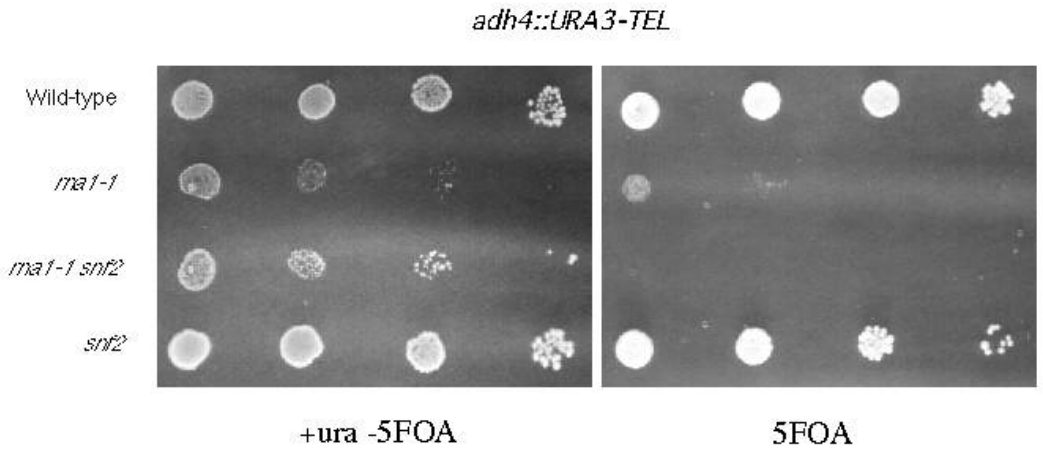


Fig. 3B

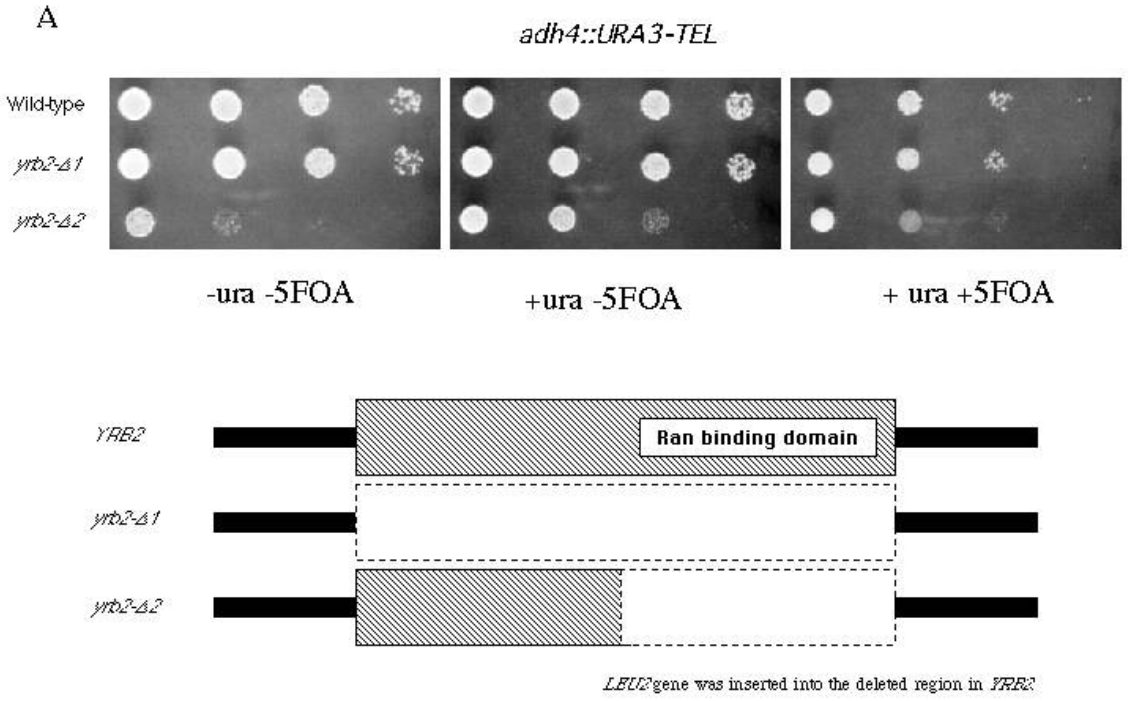

A
adh4::URA3-TEL
Wild-type
yrb2-Δ1
yrb2-Δ2
-ura -5FOA
+ura -5FOA
+ ura +5FOA
YRB2
Ran binding domain
yrb2-Δ1
yrb2-Δ2
LEU2 gene was inserted into the deleted region in YRB2

Fig. 4A

B

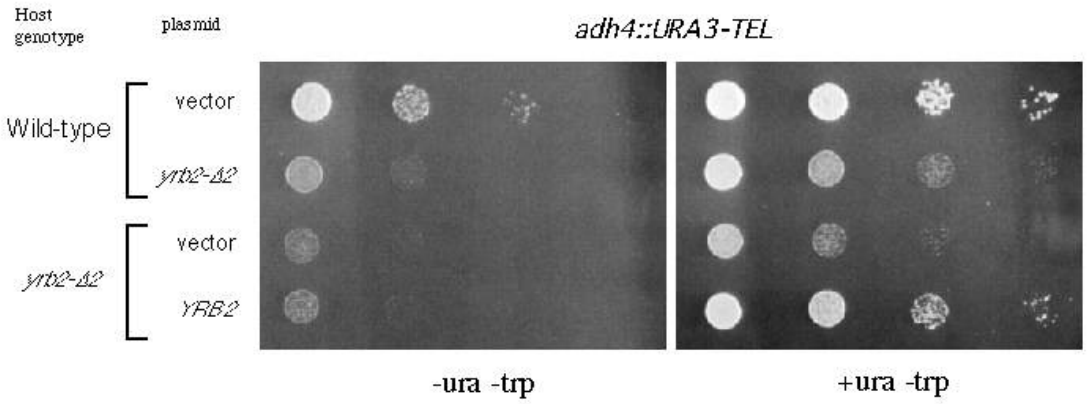

Host genotype
plasmid
adh4::URA3-TEL
Wild-type
vector
yrb2-Δ2
yrb2-Δ2
vector
YRB2
-ura -trp
+ura -trp

Fig. 4B

A

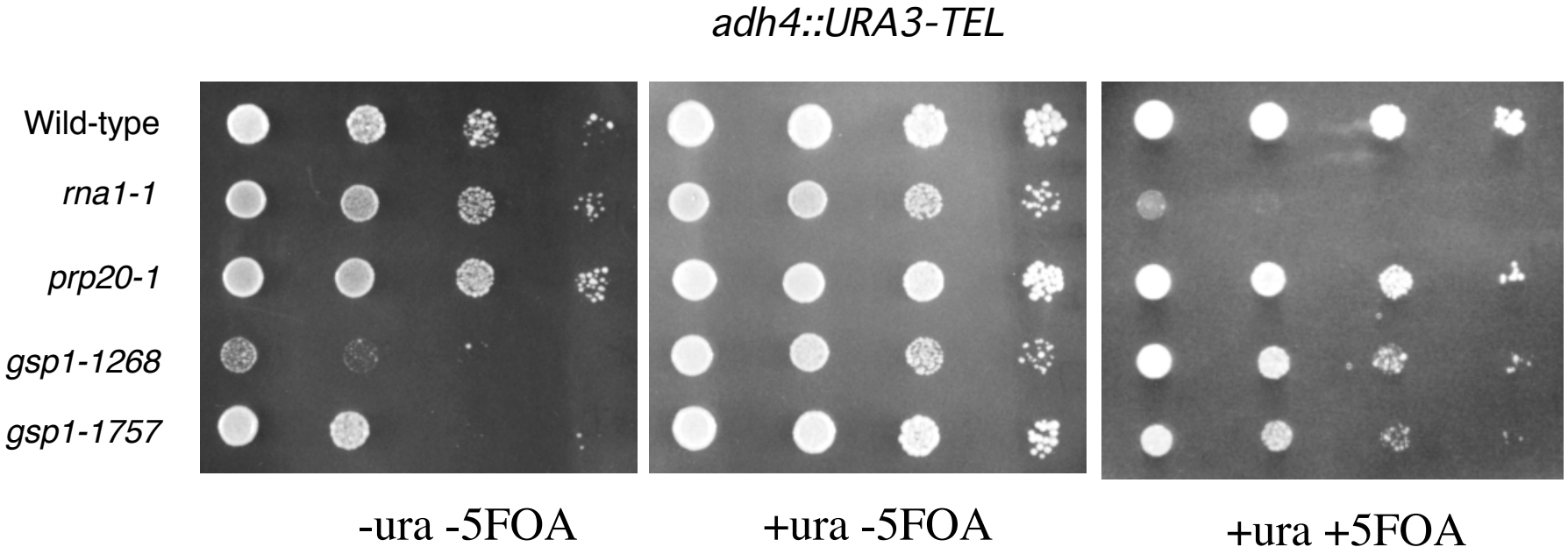

adh4::URA3-TEL
Wild-type
rna1-1
prp20-1
gsp1-1268
gsp1-1757
-ura -5FOA
+ura -5FOA
+ura +5FOA

Fig.1A

B

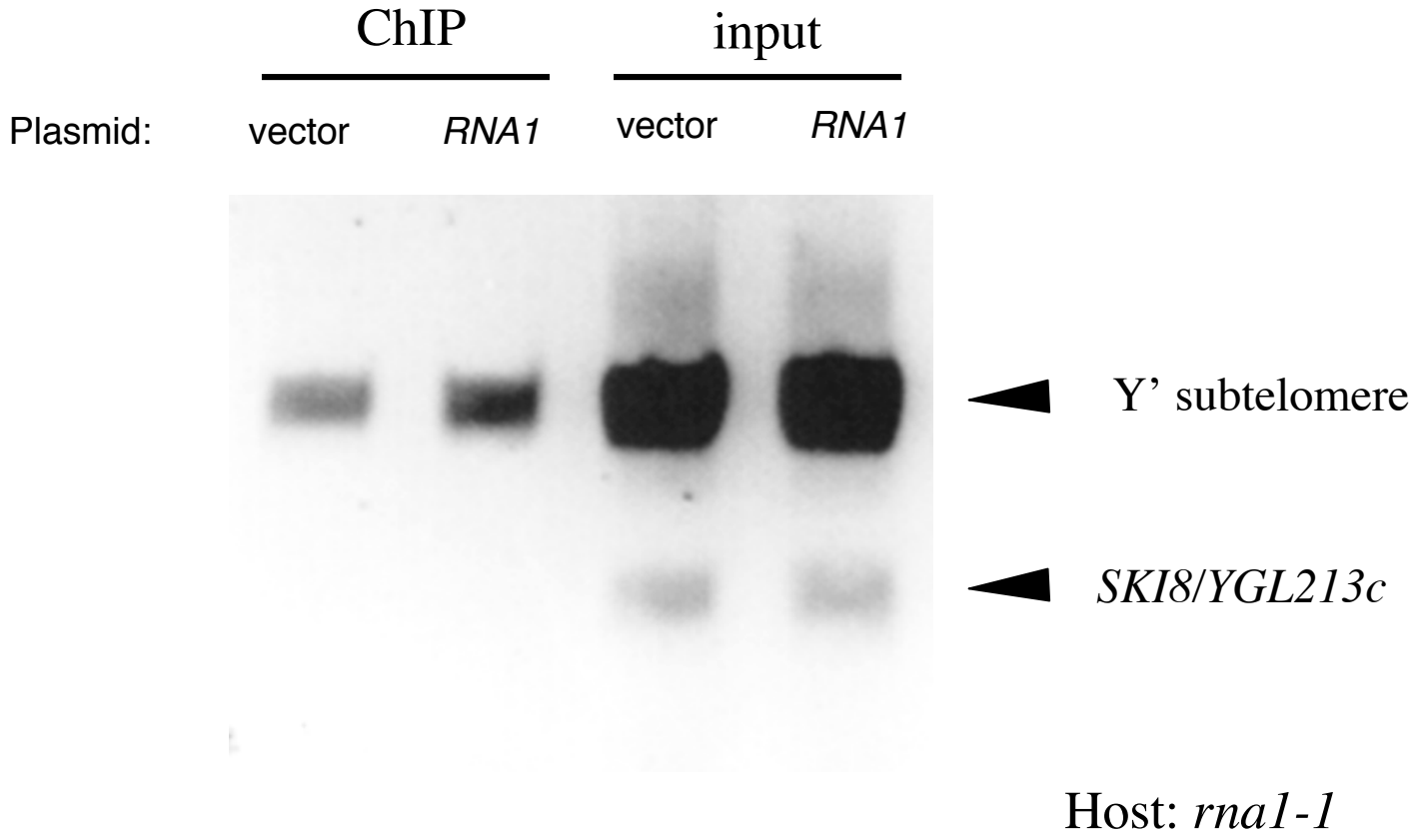

ChIP
input
Plasmid:
vector
RNA1
vector
RNA1
Y' subtelomere
SKI8/YGL213c
Host: rna1-1

Fig. 1B

A

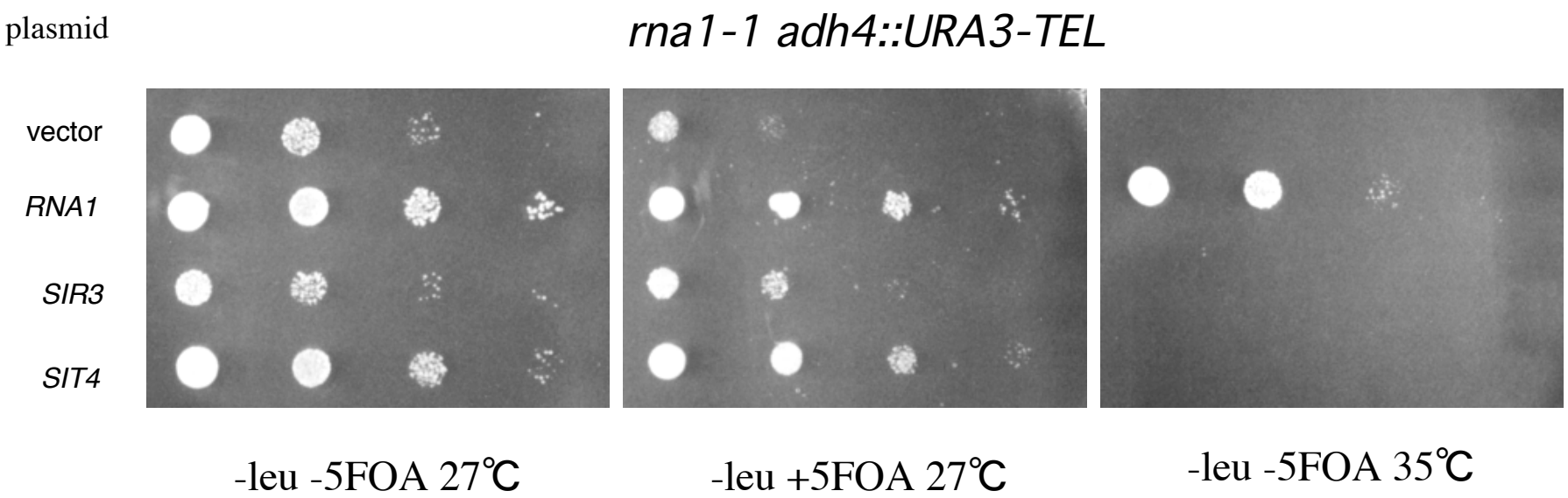

plasmid
rna1-1 adh4::URA3-TEL
vector
RNA1
SIR3
SIT4
-leu -5FOA 27℃
-leu +5FOA 27℃
-leu -5FOA 35℃

Fig. 2A

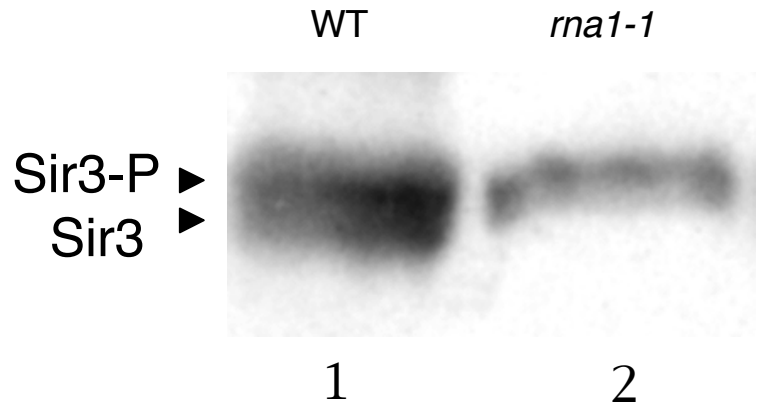


Fig. 2B

A

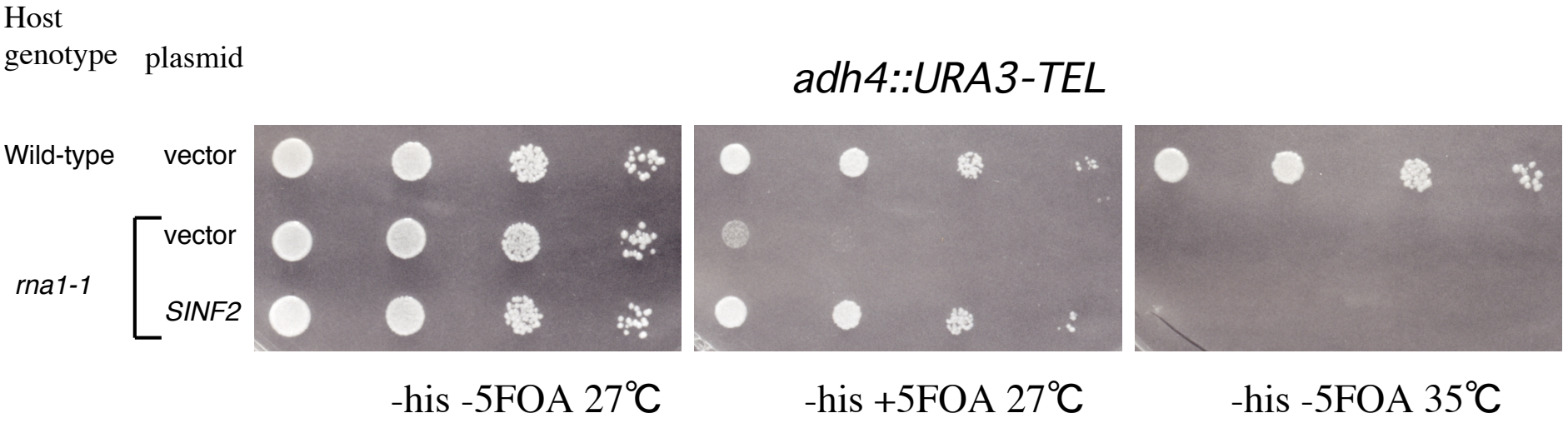

Host genotype
plasmid
adh4::URA3-TEL
Wild-type
vector
rna1-1
vector
SINF2
-his -5FOA 27℃
-his +5FOA 27℃
-his -5FOA 35℃

Fig. 3A

B

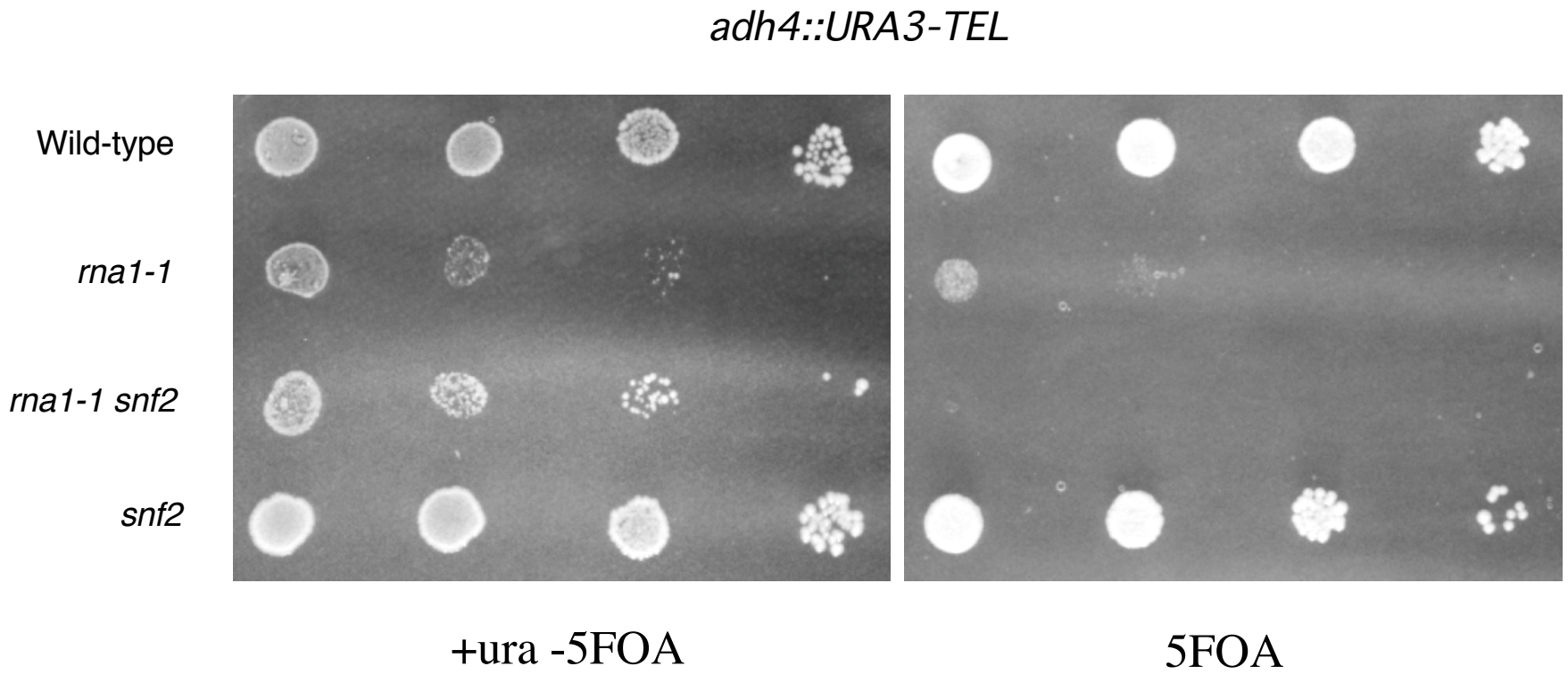
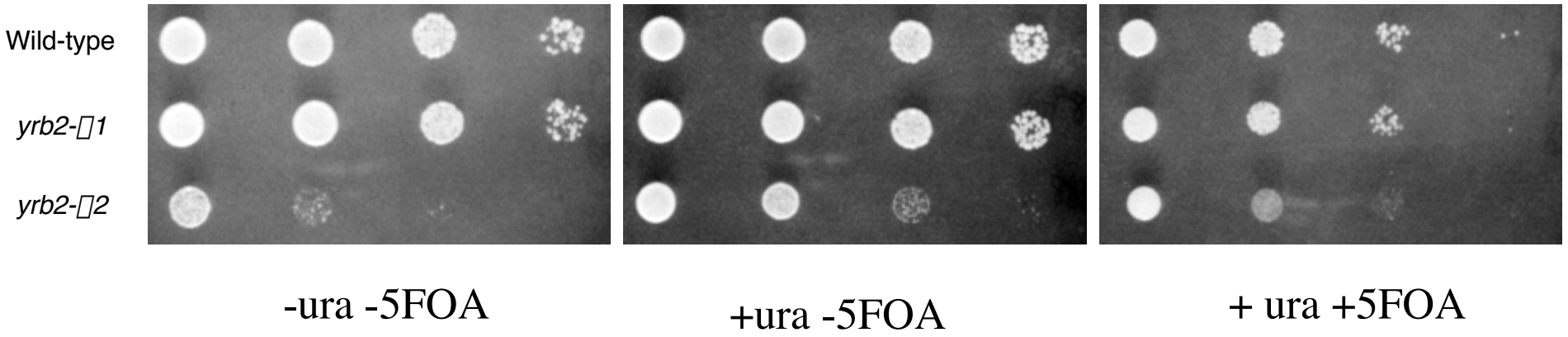
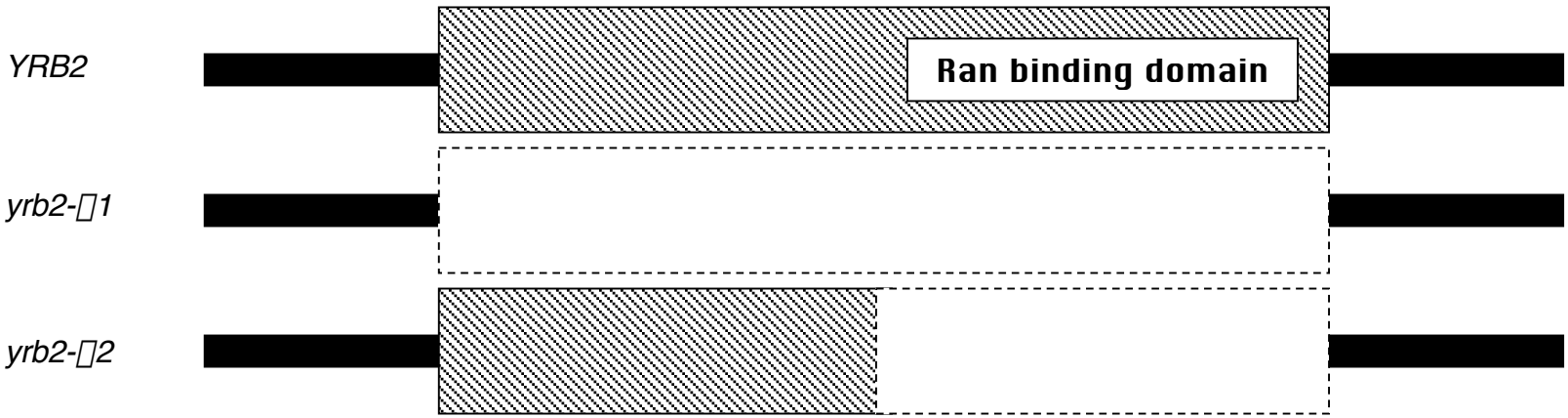


Fig. 3B

A





LEU2 gene was inserted into the deleted region in *YRB2*.

Fig. 4A

B

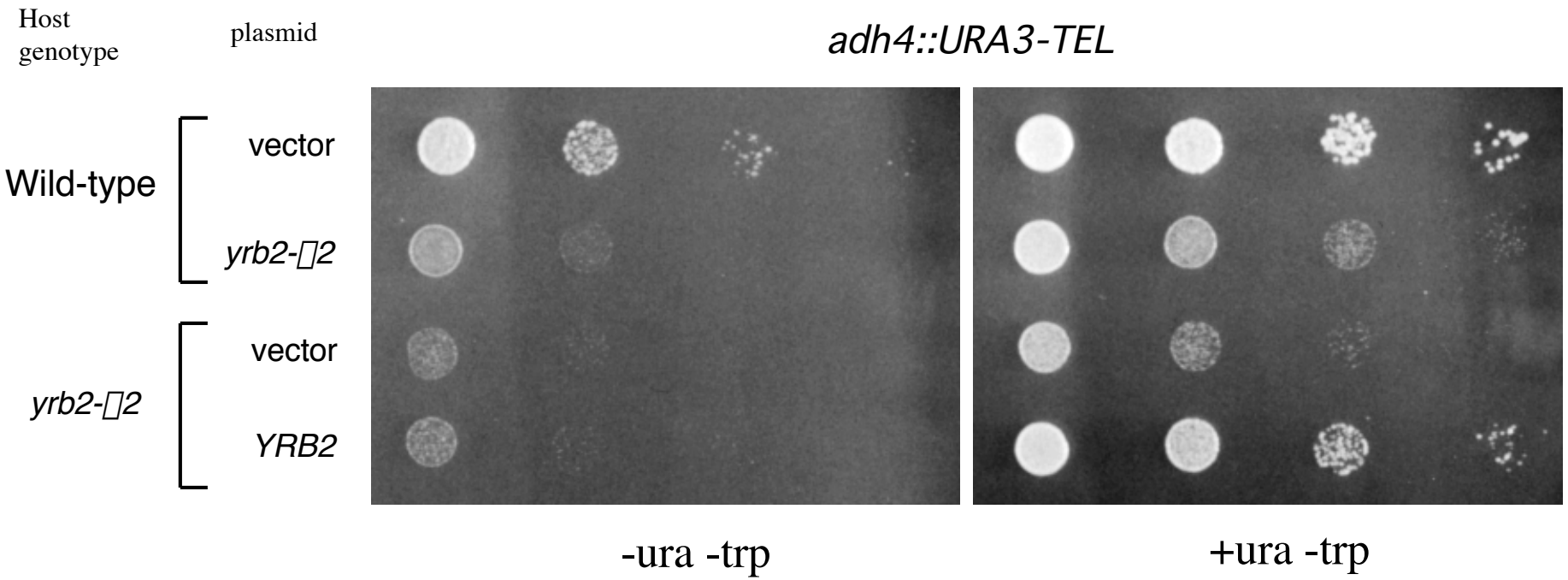

Host genotype
plasmid
adh4::URA3-TEL
Wild-type
vector
yrb2-Δ2
yrb2-Δ2
vector
YRB2
-ura -trp
+ura -trp

Fig. 4B