Rvb2/reptin physically associates with telomerase in budding yeast

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▶ **To cite this version:**

Nathalie Grandin, Michel Charbonneau. Rvb2/reptin physically associates with telomerase in budding yeast. FEBS Letters, 2011, 585, pp.3890-3897. 10.1016/j.febslet.2011.11.013 . hal-01154308

HAL Id: hal-01154308

https://univ-tours.hal.science/hal-01154308

Submitted on 4 Nov 2015

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Elsevier Editorial System(tm) for FEBS Letters
Manuscript Draft

Manuscript Number: FEBSLETTERS-D-11-01347R1

Title: Rvb2/reptin physically associates with telomerase in budding yeast

Article Type: Research Letter

Keywords: telomerase; telomere length; Rvb2; budding yeast

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Manuscript Region of Origin: FRANCE

Abstract: Telomerase is a reverse transcriptase that maintains linear telomeres at a constant length. Here, we report that in the budding yeast Saccharomyces cerevisiae, Rvb2, a highly conserved member of the AAA+ family of ATPases, physically associates with telomerase/Est2 in vivo, both expressed from their endogenous promoter. Importantly, in genetic settings leading to a failure to recruit telomerase at telomeric ends, Rvb2 still associated with Est2. On the other hand, Rvb2 was present in immunoprecipitates of crosslinked telomeric chromatin even in the presumed absence of telomerase at the telomeres. Finally, we could also isolate RVB2 mutant alleles conferring slight, but stable, telomere shortening.

Suggested Reviewers:

Opposed Reviewers:

Rvb2/reptin physically associates with telomerase in budding yeast

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Running head: Rvb2/reptin and telomerase

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4,715 words

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Abstract

Telomerase is a reverse transcriptase that maintains linear telomeres at a constant length. Here, we report that in the budding yeast *Saccharomyces cerevisiae*, Rvb2, a highly conserved member of the AAA+ family of ATPases, physically associates with telomerase/Est2 *in vivo*, both expressed from their endogenous promoter. Importantly, in genetic settings leading to a failure to recruit telomerase at telomeric ends, Rvb2 still associated with Est2. On the other hand, Rvb2 was present in immunoprecipitates of crosslinked telomeric chromatin even in the presumed absence of telomerase at the telomeres. Finally, we could also isolate *RVB2* mutant alleles conferring slight, but stable, telomere shortening.

***Highlights**

S. cerevisiae telomerase (Est2) and Rvb1 and Rvb2 physically associate. Rvb2 was present in telomeric chromatin following immunoprecipitation under cross link conditions. Rvb2 still associated with Est2 in cells in which telomeras/Est2 could not be recruited at telomeric ends. *rvb2* mutant alleles conferring slight, but stable, telomere shortening could be isolated.

*Manuscript text (starting from the introduction)
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1. Introduction

Telomerase is a reverse transcriptase with a built-in RNA template that is essential for maintaining functional telomeres at a constant length in most eukaryotic organisms. Maintenance of a constant telomere length is essential to protect the chromosomes from undergoing degradation (1, 2). In mammals this protection is provided mainly by the so-called shelterin complex, a complex of six telomeric proteins (TRF1, TRF2, POT1, TIN2, TPP1 and RAP1) that prevent inappropriate fusion between telomeres as well as unwanted telomeric recombination (3). A rather similar complex exists in the fission yeast *Schizosaccharomyces pombe* (4), while in the budding yeast *Saccharomyces cerevisiae*, a somewhat simpler protection complex, consisting mainly of the Cdc13, Stn1 and Ten1 proteins (sometimes referred to as the CST complex) is present (5). However, very recently the orthologs of *S. cerevisiae* CST were found in humans as well as in *S. pombe* and *Arabidopsis thaliana* (6). A number of accessory proteins are required for the assembly of telomerase (7). Subsequently, distinct proteins are required for the loading of telomerase onto the telomeres as well as for its activation. It is not currently known in detail how telomerase, which is present at the telomeres even when it is not performing its telomere elongation function, can attain its activation state.

Very recently, pontin and reptin, two highly conserved members of the AAA+ family of ATPases (putative DNA helicases), initially named RUVBL1 and RUVBL2 after the bacterial RuvB-Holliday-junction migrating helicase, were found to associate with both subunits of human telomerase (8). In both *S. cerevisiae* and humans, Rvb1/pontin and Rvb2/reptin are parts of several complexes involved in chromatin modification and also play major roles in the regulation of transcription (9-12). In *S. cerevisiae*, there has been so far no interaction, physical, genetic or other between Rvb1 or Rvb2 and telomerase reported in the literature. We report here the existence in *S. cerevisiae* of a physical association between Rvb2 and telomerase and the isolation of *RVB2* mutants with shortened telomeres.

2. Materials and methods

2.1. Yeast strains, plasmids and inducible overexpression by galactose

Yeast strains used in this study, derivatives of BF264-15Daub (*ade1 his2 leu2-3,112 trp1-1a ura3Dns*), were cultured as described previously (13). All strains were made isogenic by back crossing at least five times against our genetic background. Strain origins, prior to back crossing, were as follows. The *rvb2*::*KanMX4*/*RVB2*, *est2*::*KanMX4*/*EST2*, *tel1*::*KanMX4* and

yku70::*KanMX4* strains were from Euroscarf (Frankfurt, Germany). The *tlc1*::*TRP1* strain was from the Gottschling laboratory.

All constructs were made by using Polymerase Chain Reaction (PCR) to adapt the relevant restriction sites to the sequence of the genes and details of the constructs can be available upon request. Induction of genes placed under the control of the *GAL1-10* promoter, in a high-copy vector (episomal, 2μ), was done by transferring cells growing in glucose-containing liquid medium into galactose-containing liquid medium after several washes by centrifugation and allowing overexpression for 2 hr at 29°C. In experiments involving expression of protein parts under *GAL1-10* promoter control a supplementary methionine residue was added in front of the truncated sequence (if starting from other places than the natural initiating ATG) to initiate efficient translation.

2.2. Immunoprecipitation and immunoblotting

Mouse monoclonal anti-HA raw ascites fluid 16B12 (BabCO) and mouse monoclonal anti-HA 12CA5 antibody (Roche Diagnostics) were used for immunoprecipitation and imunoblotting, respectively. Myc-tagged proteins were visualized after immunoprecipitation and Western blotting with monoclonal anti-Myc antibody 9E10 (Roche Diagnostics).

Techniques for cell extract preparation, immunoprecipitation and immunoblotting (analyzed using an Odyssey Imager), as well as for block and release experiments, have been described previously (13, 14).

To perform immunoprecipitation-Western blotting experiments following conditions of protein-DNA crosslink, cell cultures were treated with 1% formaldehyde for 15-30 min at the selected temperature for growth. Cell extracts were then prepared as usual but, here, chromatin was shredded by sonication. The rest of the protocol for immunoprecipitation and Western blotting was as usual.

2.3. Generation of RVB2 *mutant alleles and measurement of telomere lengths on teloblots*

To mutagenize *RVB2*, the 1413 base pair (bp)-long *RVB2* ORF plus ~ 310 bp upstream of the ATG and ~ 300 bp downstream of the stop codon was amplified by error-prone PCR under the following conditions. The concentration of dNTPs was either kept as in standard conditions (200 μM each) or one of the four dNTP concentration changed to 0.5-1.0 mM, those of the other three being kept at 200 μM, and, in both cases, the concentration of $MgCl_2$ was changed from 1.5 to 3.0 mM. Standard *Taq* polymerase and PCR buffer (Promega) were used. Following a 30-cycle amplification, the mutated fragments were transformed into an

rvb2::*KanMX4* strain carrying wild-type *RVB2* on a *CEN-URA3* plasmid, together with a centromeric, *TRP1*-based (YCplac22), plasmid carrying *RVB2* ORF plus the same flanking sequences and made linear by digestion with *Xba*I and *Nco*I, endogenous sites located ~ 90 base pairs post-initiating ATG and ~ 115 base pairs downstream of the stop codon, respectively. After counter-selection on 5-FOA medium, transformants having extruded wild-type *RVB2* were picked out randomly from the plates, propagated for ~ 20 days at 24°C to attain telomere length equilibrium.

To analyze telomere length by Southern blotting, genomic DNAs were prepared, as described previously (14). Following digestion of genomic DNA with *Xho*I, to cut within the Y' regions of chromosomes, telomere tracts of wild-type cells appear as a broad band of ~ 1.1-1.3 kb which represents the average length of most chromosomes. Results were analyzed using an FLA-5100 Fuji phosphoimager and the ImageGauge software.

3. Results and discussion

3.1. Rvb2 associates with telomerase

The finding that human pontin and reptin (homologues to Rvb1 and Rvb2, respectively), two closely related members of the AAA+ family of ATPases, physically associate with telomerase (8) prompted us to examine whether similar physical interactions existed in budding yeast. In asynchronous cells, a small amount of HA_2-Rvb2 could be detected following immunoprecipitation of Myc_{18}-Est2 using anti-Myc monoclonal antibody, both expressed from their respective genomic locus under native promoter control (**Figure 1A**). Since Rvb2 is much more abundant within the cell than Est2 (15), we verified that the amount of HA_2-Rvb2 immunoprecipitated by Myc_{18}-Est2 was reproducibly higher than the background level of HA_2-Rvb2 (**Figure 1A** and **data not shown**). The *in vivo* interaction between HA_2-Rvb2 and Myc_{18}-Est2 was observed throughout the cell cycle, in cells previously synchronized in G1 phase with alpha-factor (**Figure 1B**). This indicates that in budding yeast, unlike in humans (8), the Est2-Rvb2 interaction does not appear to peak in S phase. In the converse experiment, we could not detect Myc_{18}-Est2 in the HA_2-Rvb2 immunoprecipitate (**data not shown**). Since Rvb2 has been found in physical association with several other proteins in distinct complexes (12), this tends to support the idea that a small fraction only of Rvb2 is in complex with Est2. This conclusion should however be toned down by the fact that Myc_{18}-Est2 being much less abundant than HA_2-Rvb2, the amount of Myc_{18}-Est2 immunoprecipitated by HA_2-Rvb2 might be too small to be detected.

The *in vivo* interaction between Rvb2 and Est2 was very reproducible but small, and we needed confirmation. Therefore, we next examined Rvb2-Est2 physical interactions under conditions of increased expression by using a *GAL1-10* promoter-controlled inducible system. By controlling the duration of induction of the promoter in galactose-based liquid culture medium, one can limit potentially deleterious effects of heavy overexpression. Such a system is currently used in genome-wide analyses because it presents the advantage compared to other systems of assessing *in vivo* interactions between proteins in their native configuration. Upon induction of the *GAL1-10* promoter for 2 hr at 29°C, the amount of Rvb2-HA_2 co-precipitating with either one of two Est2-Myc_3 parts, one of which, $Est2^{420-740}$-Myc_3, comprising the entire catalytic domain, was higher than the background level of Rvb2-HA_2 in the strain expressing the GAL-*RVB2*-HA_2 construct alone (**Figure 1C**).

Repeated attempts to obtain strains expressing an epitope-tagged *RVB1* construct from its genomic locus remained unsuccessful for unknown reasons. We therefore attempted to detect a possible physical interaction between Rvb1-HA_2 and one of the two Est2-Myc_3 parts using the *GAL1-10* overexpression system described above. As shown in **Figure 1D**, like with Rvb2-HA_2, physical association between Rvb1-HA_2 and $Est2^{420-740}$-Myc_3, corresponding to the catalytic domain, could be detected.

Recently, Ies3, a member of the INO80 complex, was found to undergo a weak physical interaction with Est1 (16). Est1 is a single-stranded telomeric DNA-binding protein that loads telomerase onto the telomeric ends (17, 18). Est1 also binds Est2, through bridging with TLC1, the RNA template subunit of telomerase (19). Since Rvb2 is part of the INO80 complex (12), we asked whether physical interactions between Est1 and Rvb2 could be detected. Est1-Myc_3 failed to bind Rvb2-HA_2 (**Figure 1E**). This demonstrated that, contrary to Est2, Est1 has no apparent affinity with Rvb2, at least when both are overproduced.

3.2. Rvb2 is present in crosslinked telomeric chromatin

We next asked whether Rvb2 might be part of telomeric chromatin. Chemical crosslinking with formaldehyde and related reagents has been widely used to immobilize protein-DNA contacts for ChIP as well as to fix sub-cellular structures for microscopy (20-22). We decided to apply a crosslink protocol prior to immunoprecipitation using a strain expressing a *CDC13*-Myc_{13} construct from genomic locus under the control of its native promoter (23). Cdc13 is the major single-stranded telomeric DNA-binding protein in budding yeast and has an essential function both in telomere end protection and telomerase recruitment (5, 24). We reasoned that based on ChIP experiments on telomeric proteins in which telomeric DNA is

detected by PCR in the immunoprecipitate, we might be able to detect HA_2-Rvb2 in the telomeric chromatin immunoprecipitated by Cdc13-Myc_{13}. Only under conditions in which the experiment had been performed under crosslink conditions was the association between Cdc13-Myc_{13}-immunoprecipitated telomeric chromatin and HA_2-Rvb2 clearly visible (**Figure 2A**). Bmh1 was used as a control for these experiments because it has a very high intracellular concentration (in fact, Bmh1 is far more abundant than Rvb2, ~ 158,000 and ~ 3,030 molecules/cell, respectively, while a rare telomeric protein such as Cdc13 is present at only ~ 320 molecules/cell; ref. 15) and, therefore, cannot be suspected of being undetected in the anti-Myc immunoprecipitate because of too low an intracellular level. We could not detect any physical association between Cdc13-Myc_{13} and HA_2-Bmh1 whether under crosslink or native conditions, while the HA_2-Rvb2 sample scored positive within the same experiment (**Figure 2A**). The presence of HA-Rvb2 in the crosslinked immunoprecipitate appeared to occur also in the absence of a Myc tag on Cdc13, albeit at a reduced level (**Figure 2A**). Although this cannot be ignored, it should be noted that this background level of HA_2-Rvb2 was constantly observed throughout the course of the present study, whether under native or crosslink immunoprecipitation experiments (**Figure 1A, 2A**). However, the fact is that the increased HA_2-Rvb2 signal observed following immunoprecipitating Cdc13-Myc_{13} necessarily corresponded to a specific signal. This phenomenon most probably results from the intrinsically high levels of intracellular Rvb2 as it was also observed with the highly abundant Bmh1 protein. However, most importantly, with HA_2-Bmh1 the background level did not increase upon Cdc13-Myc_{13} immunoprecipitation, thus strongly suggesting an absence of association between HA_2-Bmh1 and Cdc13-Myc_{13}-bound chromatin, contrary to the case with HA_2-Rvb2 (**Figure 2A**).

The interactions between Rvb2 and telomeric DNA did not change during a synchronous cell cycle obtained after alpha-factor pheromone block and release (**Figure 2B**).

HA_2-Rvb2 was still present in Cdc13-Myc_{13}-immunoprecipitated telomeric chromatin in the absence of functional telomerase, namely in strains deleted for the *TLC1* RNA subunit of telomerase or in the *est1* null mutant (**Figure 2C**). Previous experiments have demonstrated that in the absence of *TLC1*, Est1 and Est2 no longer physically interact (19). Therefore, the present experiments strongly suggest that Rvb2 is present at the telomeres even in the absence of telomere-recruited Est2. However, further experiments using a different approach will be necessary to definitively establish this potentially important finding.

3.3. The Rvb2-Est2 interaction takes place even when telomerase is not present at the telomeres

The Rvb2-Est2 interaction described above might potentially take place at the telomeres but nothing excluded the possibility that Rvb2 could associate with Est2 at locations other than the telomeres. To explore this possibility, we took advantage of previously reported *in vivo* findings showing that both the YKU complex, during the G1 phase and early S phase (25), and Est1, from the end of S phase to the end of mitosis (17, 26, 27), are required for telomerase loading at the telomeres and, importantly, that inhibiting these two pathways totally prevent the presence of telomerase on the telomeres (26). As expected, combining the *est1* null and *yku70* null mutations led to greatly accelerated senescence, leaving too little time to grow enough cells to conduct the experiments prior to senescence (**data not shown**). Therefore, the experiments were performed on *est1* null *yku70* null cells undergoing post-senescence telomeric recombination. Not only did this not impinge on the desired experimental design but, in addition, confirmed that all telomerase was completely off the telomeres, as cells incapable of loading telomerase at their telomeres undergo telomeric senescence followed, in a small minority of them, by post-senescence survival by telomeric recombination (28, 29). Interestingly, in an *est1* null *yku70* null double mutant (**Figure 3A**) as well as in the *est1* null mutant (**Figure 3B),** HA_2-Rvb2 was found to still physically associate with Myc_{18}-Est2 to levels similar to that in the wild type. We note that the amount of Myc_{18}-Est2 was larger in the *est1 yku70* double mutant than in the wild-type but additional experiments would be required to conclude on this point.

It should be noted that in the *est1* null mutant, *TLC1* RNA was found to mostly accumulate in the cytoplasm (30), thus making unlikely the presence of an abundant and functional Est2-TLC1 complex at the telomeres in that mutant. On the other hand, deletion of *EST1* did not prevent the formation of an Est2-TLC1 complex (31), which would therefore be cytoplasmic in that mutant (30). Altogether, this indicates the physical interaction between Rvb2 and Est2 detected in the present study presumably takes place in the cytoplasm (or possibly but unlikely in the nucleus but off the telomeres) during maturation and/or assembly of telomerase (7).

3.4. rvb2 *mutants with shortened telomeres*

Since Rvb2 and telomerase physically associate, we next set out to identify possible *rvb2* mutants deregulated in telomere length control. Two hundred colonies resulting from the transformation of an *rvb2* null strain with PCR-mutagenized *RVB2* (see Materials and

methods) were picked out randomly from the plates and individually propagated for ~ 20 days at 24°C so that they could attain telomere length equilibrium. Under these conditions, we could select, by Southern blot analysis of the terminal restriction fragments, five *rvb2* mutants with altered telomere length, which all exhibited slight telomere shortening (**Figure 4A**). None of these mutants exhibited signs of cellular senescence, a phenomenon that takes place when telomeres erode beyond a critical threshold (**data not shown**). Upon propagation during additional cell divisions, the decreased telomere length was found to be stable in all five selected mutants (**Figure 4B**). Therefore, telomere shortening in the isolated *rvb2* mutants is stabilized at a shorter than wild type value, just like in the deletion mutants of the YKU or MRX complexes or of *TEL1* (32-34), although the effects of these later mutations are more dramatic (**Figure 4B**). All mutations (Rvb2-2 and Rvb2-3: A50T, T388A; Rvb2-24 and Rvb2-26: T99A; Rvb2-94: T262A, N310D, I370M, all in the *rvb2::Kan-MX4* YCplac22-pro-*rvb2*-tail genetic context) were found to localize to residues that have been conserved between the budding yeast and human proteins, and, in addition, were outside of the Walker A and B ATPase motifs, which serve to bind ATP (**Figure 4C**). Incidentally, both budding yeast Rvb1 and Rvb2 have been recently shown to exhibit ATPase activity, as well as ATP-dependent DNA helicase activity *in vitro* with increased activities when the two proteins were in complex (35).

The relatively modest telomere shortening of the isolated *rvb2* mutants have so far prevented us from uncovering interesting genetic interactions with other mutations previously implicated in telomere maintenance (NG, MC, unpublished data).

3.5. Conclusions

The major finding of the present study is to have uncovered a weak, albeit very reproducible, *in vivo* physical interaction between Rvb2/reptin and the catalytic subunit of budding yeast telomerase, Est2. Given the status of budding yeast *S. cerevisiae* and humans as two of the major biological model systems to study telomeres, this data is of major importance in light of the existence of a similar interaction between human telomerase and reptin (8). Altogether, the present data suggest that Rvb2 may specifically associate with Cdc13-Myc_{13}-bound telomeric chromatin in telomerase-independent transactions. We speculate that the Rvb1/2 helicases (35) may assist in changing the conformation of telomeric ends so as to optimize telomerase recruitment, perhaps also taking advantage of the potentially important Rvb2-Est2 physical association shown here.

Acknowledgements

We are very grateful to Craig Peterson for the gift of the Cdc13-Myc_{13} plasmid. This work was supported by grants from the "LIGUE contre le Cancer, Comité inter-régional Rhône-Alpes Auvergne".

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Legends of figures

Figure 1: Rvb2 physically associates with Est2/telomerase, but not with Est1. **(A)** Cell extracts from asynchronous wild-type cells harboring either HA_2-*RVB2* or Myc_{18}-*EST2* constructs or both. After separation by gel electrophoresis, the immunoprecipitates (IP) were revealed by the corresponding monoclonal antibodies as indicated (West.). **(B)** Cells from a *bar1* mutant expressing both HA_2-*RVB2* and Myc_{18}-*EST2* (from their respective genomic locus) were synchronized in G1 with alpha-factor, released from the block point by washing out the pheromone and analyzed at the indicated positions of the cell cycle (assessed in parallel by FACS analysis) for the existence of a physical interaction between the two proteins. First lane is the control using asynchronous cells from a strain expressing HA_2-*RVB2* alone. **(C)** Immunoprecipitation-Western blotting experiments using strains overexpressing either full length *RVB2*-HA_2 or parts of *EST2*-Myc_3 (amino acids numbers indicated; $Est2^{420-740}$-Myc_3 comprises the entire catalytic domain; ref. 36) ORFs, or both. **(D)** Rvb1-HA_2 physically associates with the fragment of the catalytic subunit of telomerase, $Est2^{420-740}$-Myc_3, corresponding to its catalytic domain. **(E)** Est1-Myc_3 does not physically associate with Rvb2-HA_2. In C-E, transient overexpression under the control of the inducible *GAL1-10* promoter on a high-copy vector (episomal, 2μ) was induced in the liquid cultures for 2 hr at 29°C.

Figure 2: HA_2-*RVB2* is present in Cdc13-Myc_{13}-immunoprecipitated telomeric chromatin. **(A)** Strains expressing either HA_2-*RVB2* or HA_2-*BMH1* (native promoter, chromosomal locus) were processed as described above (no crosslink, **top panels**) or under crosslink conditions with formaldehyde (**bottom panels**). Under crosslink conditions HA_2-Rvb2 but not HA_2-Bmh1 could be detected in Cdc13-Myc_{13} immunoprecipitate. It is important to note that the HA_2-Rvb2 IP-West., HA_2-Bmh1 IP-West and Cdc13-Myc_{13} IP-West. (bottom three rows in each panel) were performed on the same small volume (1/50 of total extract) in order to avoid ending up with saturating amounts of Rvb2 and Bmh1, while, on the opposite, Cdc13 which is far less abundant than Rvb2 and Bmh1 yielded a much weaker signal (see main text). In all crosslink experiments performed, the amount of IP-West. signal for a given protein was always much lower than in the absence of formaldehyde (compare bottom three rows of top panel with bottom three rows of bottom panel). We believe that either the extraction of the proteins by IP is less efficient due to the presence of formaldehyde and/or that a significant number of cells die during the crosslink (prior to the IP). **(B)** Rvb2 associates with telomeric

chromatin throughout the cell cycle. Cell cycle progression was followed by scoring under the microscope for the principal cell cycle stages (in duplicates fixed in parallel) schematically represented here. Unbudded cells were in G1 or G1/S; cells with small buds were initiating S phase and cells with large buds were at G2/M. **(C)** Same experiments as above, but performed in the *tlc1* (*TLC1* codes for the RNA subunit of telomerase) null or *est1* null background, as indicated.

Figure 3: Endogenous Rvb2 and Est2 still physically associate when telomerase access to the telomeres is restricted or even totally prevented. **(A)** Immunoprecipitation-Western blotting experiments (IP-West.) on HA_2-*RVB2* and Myc_{18}-*EST2* in an *est1 yku70* null background, in which telomerase access to the telomeres is completely eliminated, under native IP-West. conditions. It is important to note that both the HA_2-Rvb2 IP-West. and Myc_{18}-Est2 IP-West. (bottom two rows) were performed on the same small volume (1/50 of total extract) in order to avoid ending up with saturating amounts of Rvb2, while, on the opposite, Est2 was barely detected in such a small volume of cell extract because it is far less abundant than Rvb2 (see main text). **(B)** Same as above in a strain harboring an *est1* null mutation (3rd lane) compared with the wild type background (2nd lane). The 1st lane is a control with no Myc_{18}-*EST2* expression to assess for HA_2-Rvb2 background.

Figure 4: Mutations in *RVB2* confer slight telomere shortening. **(A)** Genomic DNAs were prepared from individual strains containing PCR-mutagenized *RVB2* and each lane corresponds to a single strain. Southern blotting with a telomeric P^{32}-labeled probe revealed the size of the bulk of terminal $TG_{1\text{-}3}$ telomere tracts, corresponding to the DNA smear migrating at around 1.2-1.3 kb. The dashed horizontal line, representing the mean wild-type (wt) length, allowed us to obtain a better appreciation of the variations in telomere length from the wild-type value in the five indicated mutants. **(B)** Plasmids harboring mutant *rvb2* alleles were recovered from the selected original mutants, presented above, in A, transformed back into the original strains and the resulting transformants, illustrated here, further propagated in culture for 22 days at 24°C in order to make sure that the initially recorded values were maintained constant with time. Shortened telomeres of *yku70* null and *xrs2* null strains (Xrs2 is part of the Mre11-Rad50-Xrs2 complex) are shown for comparison. **(C)** Positions of the mutations in Rvb2 sequence shown aligned with its human homologue. The positions of Walker A and B motifs are shown.

Figure 1A, B
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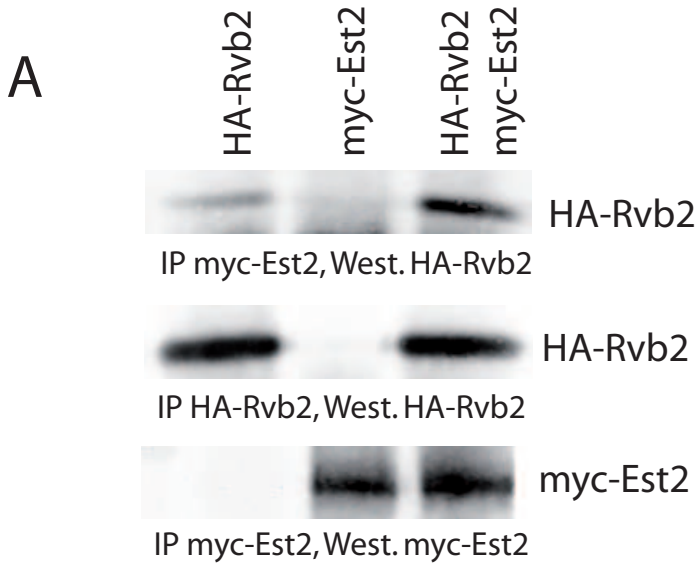
Figure 1



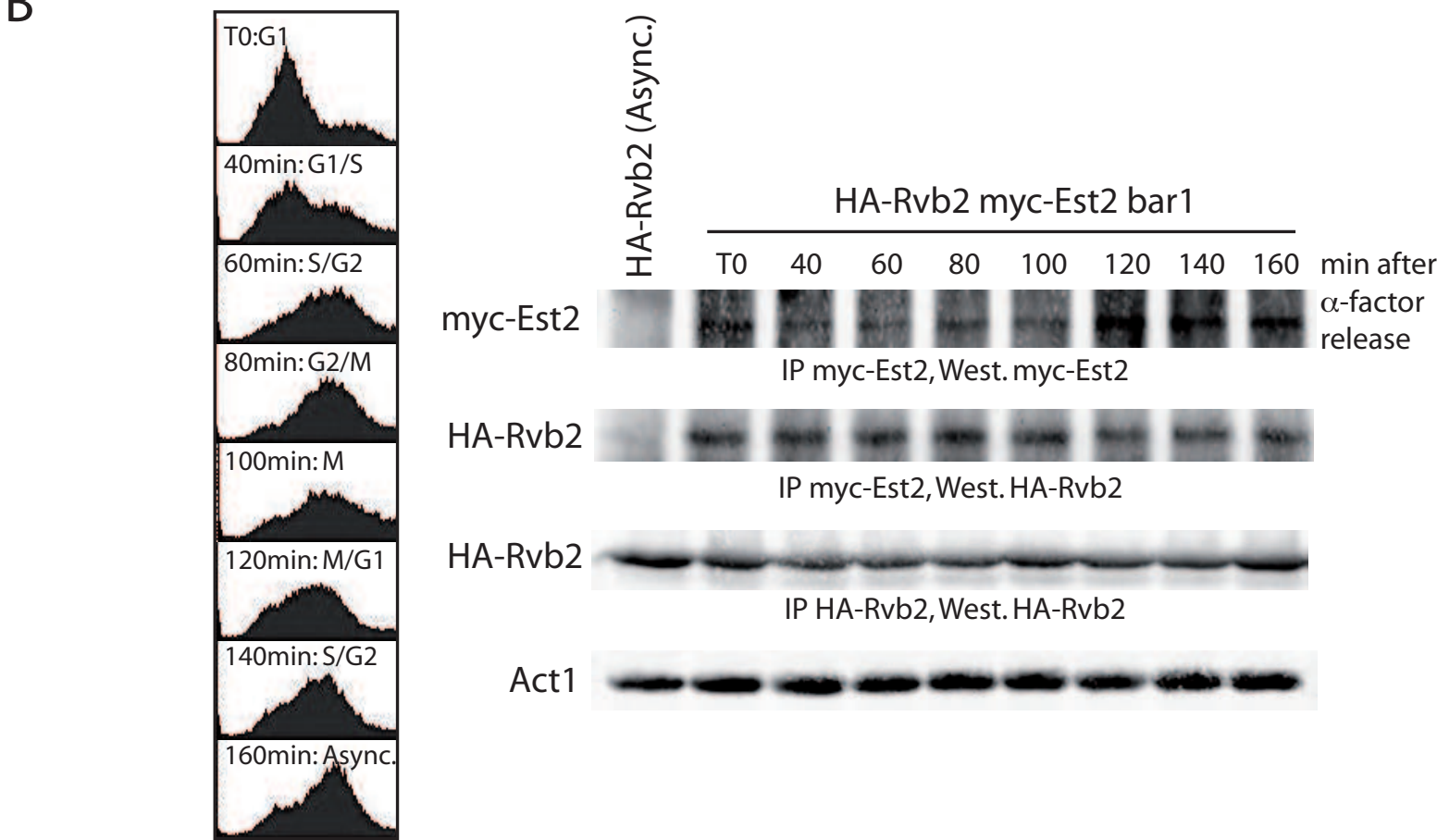


Figure 1C, D, E

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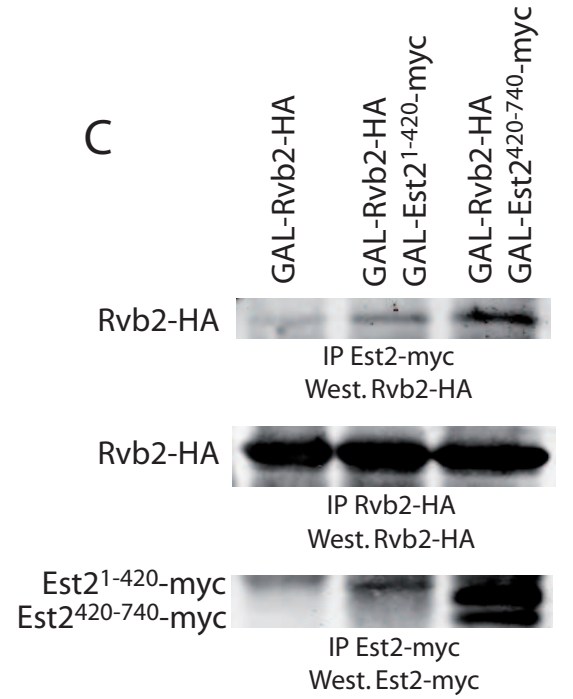

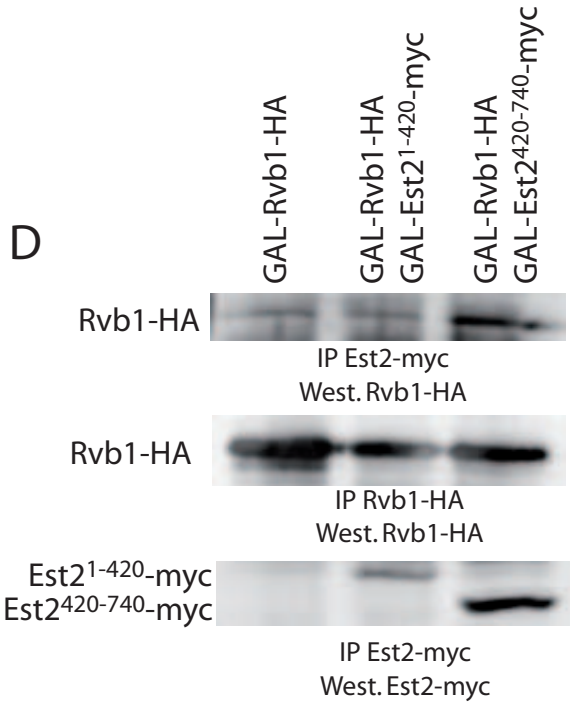

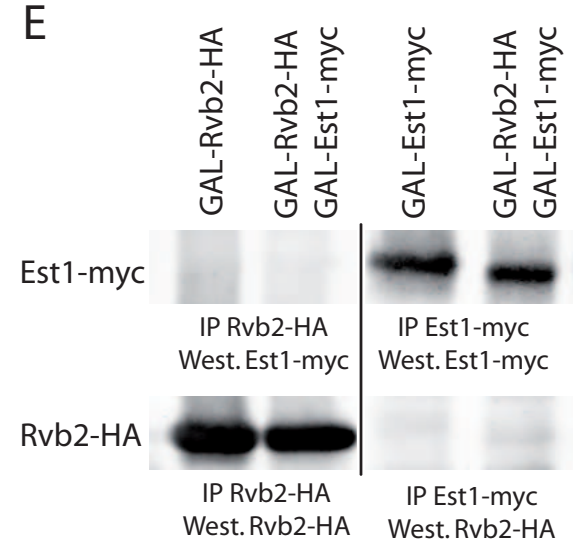

Figure 2

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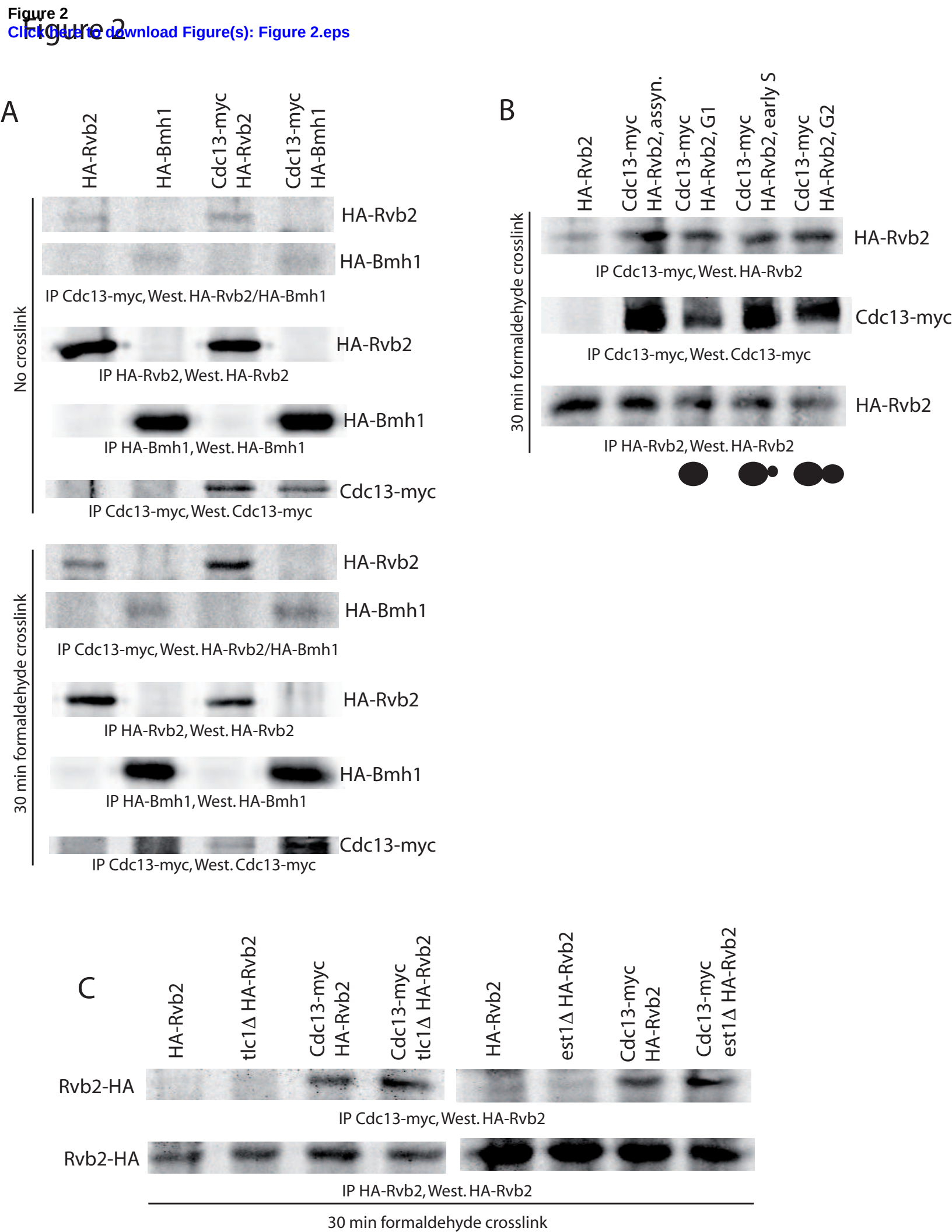

Figure 3

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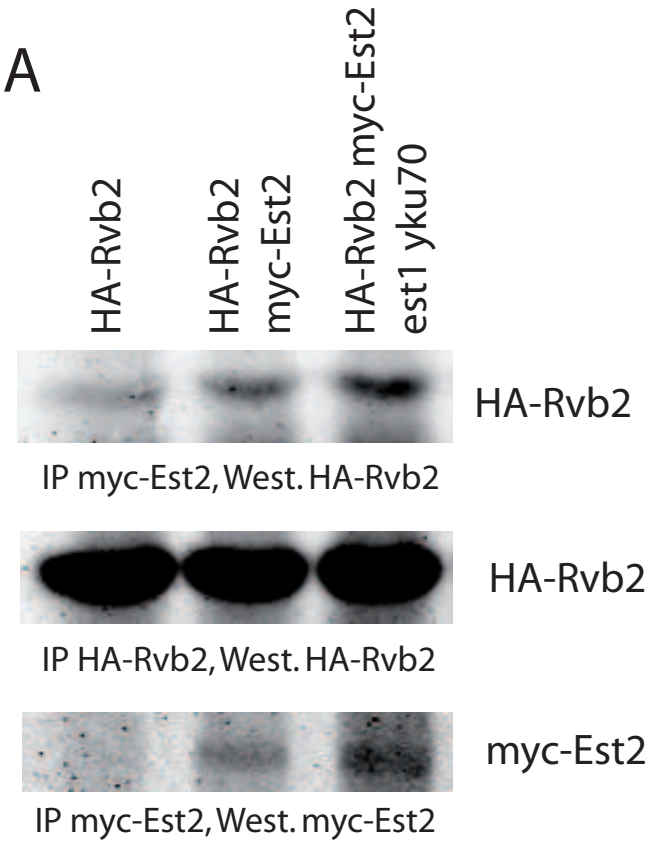
Figure 3



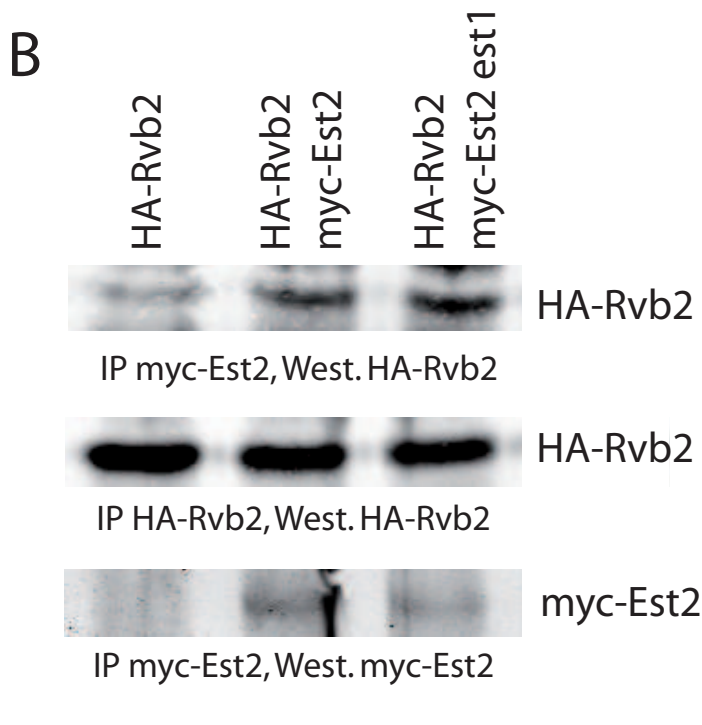


Figure 4A, B

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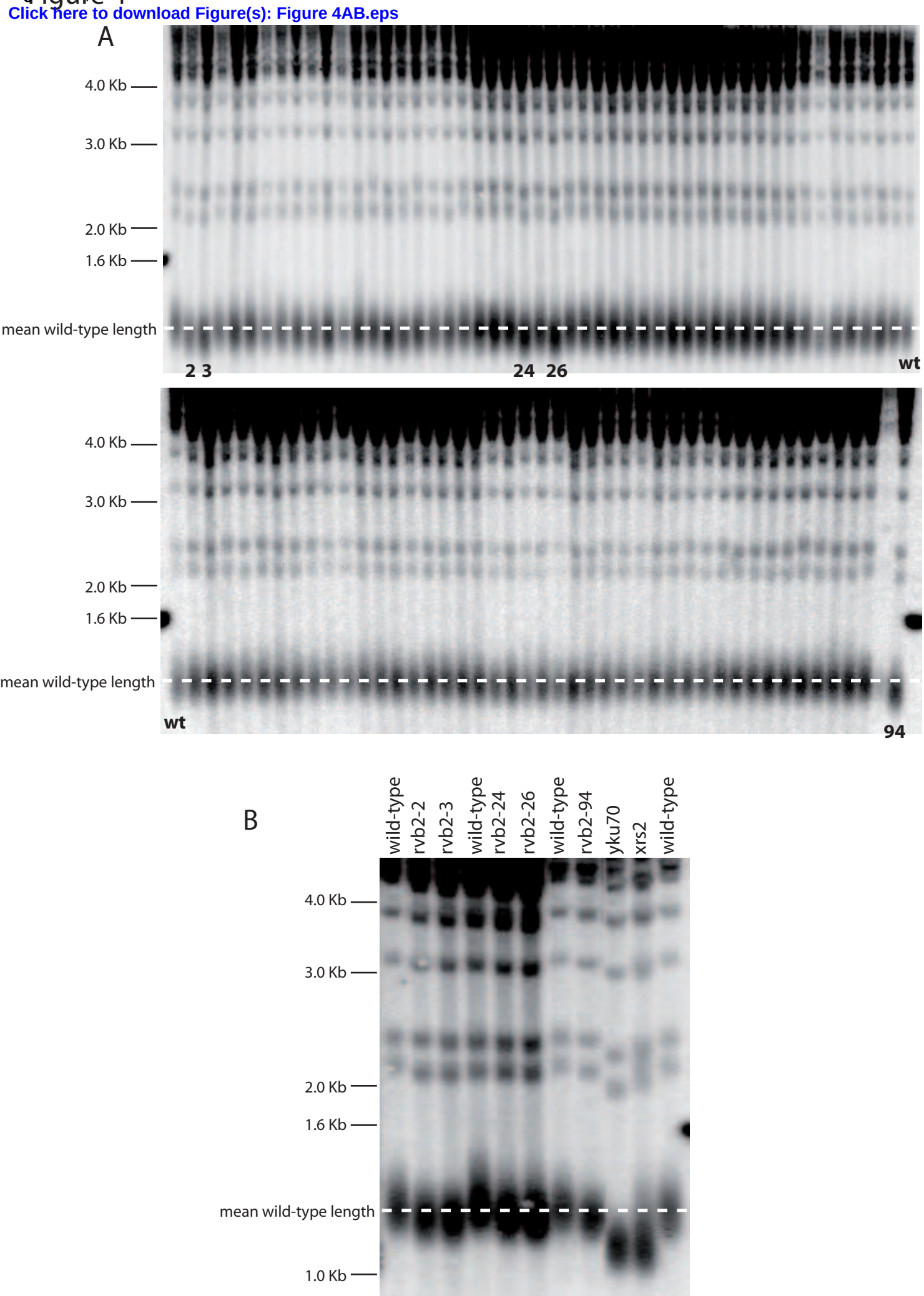

Figure 4C

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Figure 4 C