The Principal Role of Ku in Telomere Length Maintenance Is Promotion of Est1 Association with Telomeres

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ABSTRACT Telomere length is tightly regulated in cells that express telomerase. The *Saccharomyces cerevisiae* Ku heterodimer, a DNA end-binding complex, positively regulates telomere length in a telomerase-dependent manner. Ku associates with the telomerase RNA subunit TLC1, and this association is required for TLC1 nuclear retention. Ku–TLC1 interaction also impacts the cell-cycle-regulated association of the telomerase catalytic subunit Est2 to telomeres. The promotion of TLC1 nuclear localization and Est2 recruitment have been proposed to be the principal role of Ku in telomere length maintenance, but neither model has been directly tested. Here we study the impact of forced recruitment of Est2 to telomeres on telomere length in the absence of Ku's ability to bind TLC1 or DNA ends. We show that tethering Est2 to telomeres does not promote efficient telomere elongation in the absence of Ku–TLC1 interaction or DNA end binding. Moreover, restoration of TLC1 nuclear localization, even when combined with Est2 recruitment, does not bypass the role of Ku. In contrast, forced recruitment of Est1, which has roles in telomerase recruitment and activation, to telomeres promotes efficient and progressive telomere elongation in the absence of Ku–TLC1 interaction, Ku DNA end binding, or Ku altogether. Ku associates with Est1 and Est2 in a TLC1-dependent manner and enhances Est1 recruitment to telomeres independently of Est2. Together, our results unexpectedly demonstrate that the principal role of Ku in telomere length maintenance is to promote the association of Est1 with telomeres, which may in turn allow for efficient recruitment and activation of the telomerase holoenzyme.

TELOMERES are the specialized nucleoprotein structures at the ends of linear chromosomes, which protect the natural chromosome termini from degradation, recombination, and fusion. Telomere length is tightly regulated in cells expressing the telomere replication enzyme telomerase, and proper telomere maintenance is important for the ability of telomeres to promote genome stability. In *Saccharomyces cerevisiae*, telomeres are maintained at 250–350 bp of double-stranded $TG_{(1\text{-}3)}$ DNA repeats with a terminal 3′ single-stranded G-rich overhang (G-tail) and a repertoire of proteins, which associate either directly or indirectly with the duplex telomeric repeats or G-tails (Wellinger and Zakian 2012). Due to the inability of the semiconservative DNA replication machinery to fully replicate DNA ends of linear chromosomes, terminal chromosomal DNA is lost with each cell division, which, in the absence of a mechanism to restore telomere length, eventually leads to critically short telomeres and cellular senescence or apoptosis.

Most eukaryotes studied utilize telomerase to circumvent this loss of telomeric DNA. In *S. cerevisiae*, telomerase is composed of an RNA component, TLC1 (Singer and Gottschling 1994), and three protein subunits, Est1, Est2, and Est3 (Lin and Zakian 1995; Lingner *et al.* 1997b; Hughes *et al.* 2000). Absence of any one of these components results in a telomerase deficiency phenotype *in vivo*, with progressive telomere shortening and eventual cellular senescence, referred to as an *est* phenotype (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay *et al.* 1996). In contrast, only the absence of TLC1, which provides the telomeric repeat sequence template, or Est2, the catalytic subunit, results in telomerase deficiency in cell extracts, indicating roles for Est1 and Est3 in the activity of telomerase in the context

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of the cell and telomeric chromatin (Cohn and Blackburn 1995; Lingner *et al.* 1997a).

The Est1 protein has been proposed to mediate two functions in telomere replication. First, it is thought to mediate the recruitment of telomerase to telomeres via its interaction with Cdc13 (Evans and Lundblad 1999; Qi and Zakian 2000; Pennock *et al.* 2001; Bianchi *et al.* 2004), which binds single-stranded $TG_{1\text{-}3}$ DNA with high affinity (Lin and Zakian 1996; Nugent *et al.* 1996). This essential role of Est1 in telomere elongation, however, can be bypassed by expression of a Cdc13–Est2 fusion protein, which enables telomeres to be stably maintained by telomerase and remain viable in the absence of Est1 (Evans and Lundblad 1999). Second, Est1 is proposed to play a role in telomerase activation. This was first suggested by the failure of the Cdc13–Est2 fusion protein to extensively elongate telomeres in the absence of Est1 (Evans and Lundblad 1999). Further support for this role has come from the identification of separation-of-function alleles of *EST1* that retain telomerase association but are defective in promoting extensive telomere lengthening (Evans and Lundblad 2002). An Est1 activation function has also been demonstrated *in vitro*, as addition of purified Est1 protein stimulates telomerase activity in cell extracts (Dezwaan and Freeman 2009; Talley *et al.* 2011). Furthermore, Est1 interacts directly with Est3 and is required for telomere association of Est3 (Tuzon *et al.* 2011). Although the function of Est3 is not known, Est3 is required for *in vivo* telomerase activity (Lendvay *et al.* 1996); therefore, part of Est1's activation function may be to recruit Est3 to telomeres (Tuzon *et al.* 2011).

The evolutionarily conserved Ku heterodimer also contributes to telomere length maintenance. In contrast to the telomerase subunits, the absence of Ku does not result in an *est* phenotype (Boulton and Jackson 1996; Porter *et al.* 1996). Composed of Yku70 and Yku80 subunits in budding yeast, Ku binds DNA ends with high affinity via a preformed DNA-binding channel in a sequence-independent manner (Walker *et al.* 2001). Ku associates with telomeric ends throughout the cell cycle (Fisher *et al.* 2004) and interacts directly with a 48-nucleotide stem loop structure of TLC1 (Peterson *et al.* 2001). How Ku impacts telomere length has been a major area of investigation.

Budding yeast strains lacking Ku have short but stable telomeres (Boulton and Jackson 1996; Porter *et al.* 1996). Several models have been proposed for this effect. One model proposes that Ku's major contribution to telomere length is to aid in the recruitment of Est2 to telomeres (Fisher *et al.* 2004; Chan *et al.* 2008). Chromatin immunoprecipitation (ChIP) assays have demonstrated that, although Est2 is telomere-associated throughout the cell cycle, it exhibits two peaks, one in G1 and the other in late S/G2 (Fisher *et al.* 2004). The G1 association of Est2 is strictly dependent on the interaction between Ku and TLC1 leading to the hypothesis that a principal role of Ku in telomere length maintenance is the recruitment of Est2 to telomeres. However, telomerase is not active in G1 (Diede and Gottschling 1999; Marcand *et al.* 2000). Moreover, visualization of TLC1's association with telomeres in G1 demonstrated that its association is transient, in contrast to in late S/G2 cells, when it is stably associated (Gallardo *et al.* 2011), suggesting that the Est2 association in G1 observed in ChIP assays is not indicative of a stable association of telomerase with telomeres. Together, these findings question the significance of the G1 Ku-dependent telomere association of Est2.

Ku also impacts the late S/G2 phase association of Est2 when telomerase is active. The impact, however, is only partial, as strains deficient in Ku–TLC1 interaction (*tlc1Δ48* and *yku80-135i* strains) have an ~50% reduction in the late S/G2 telomere association (Fisher *et al.* 2004; Chan *et al.* 2008). Est1 association is similarly reduced. Therefore, Ku's impact on telomere length may be secondary to the reduction in telomere association of one or both of these telomerase components. Notably, disruption of telomerase recruitment by Cdc13 via the *cdc13-2* allele also results in a 50% reduction of Est1 and Est2 in late S/G2 (Chan *et al.* 2008). In contrast to *tlc1Δ48* and *yku80-135i* strains, however, a *cdc13-2* strain exhibits an *est* phenotype (Lendvay *et al.* 1996), indicating an inherent difference between the Ku and Cdc13 pathways of telomerase recruitment.

Ku–TLC1 association is also necessary for nuclear localization of TLC1 RNA (Gallardo *et al.* 2008). When the ability of Ku to bind telomerase is lost, TLC1 is no longer retained in the nucleus in G1. Thus, an alternative model proposes that it is the failed retention of TLC1 in the nucleus that underlies the telomere length defect in *yku80-135i* or *yku80Δ* strains. However, telomeres are maintained via telomerase in these strains; therefore, enough TLC1 must be in the nucleus as part of the telomerase holoenzyme at least at some point in time.

In addition, Ku must bind to DNA ends to perform its telomeric functions (Lopez *et al.* 2011). A mutant allele of *YKU70* (*yku70-R456E*) that has severely reduced DNA end-binding activity but retains the ability to heterodimerize and associate with TLC1 was found to be defective for telomere length maintenance (Lopez *et al.* 2011). As Ku is unable to simultaneously bind RNA and DNA (Pfingsten *et al.* 2012), this argues against a model in which Ku is bound to telomerase via interaction with TLC1 and delivers telomerase to the telomere via binding the chromosome end. Therefore, a complete understanding of how Ku promotes elongation remains unclear.

In this study, we aimed to define the primary role of Ku in telomere length maintenance. To determine whether reduced telomere association of Est2 is the main reason that telomeres are short when Ku cannot interact with TLC1, we tethered Est2 to telomeres using a Cdc13–Est2 fusion protein (Evans and Lundblad 1999). Surprisingly, the Cdc13–Est2 fusion was unable to elongate telomeres as efficiently in the absence of Ku–TLC1 interaction. Furthermore, we found that expression of the Cdc13–Est2 fusion also resulted in partial retention of TLC1 in the nucleus, indicating an impact of Ku on telomere length that is independent not

Table 1 Yeast strains

Strains	Genotype
yAB289	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*
yAB621	*MAT***a** *yku80-135i ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*
yAB766	*MAT***a** *yku80Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*
yAB471	*MAT***a** *cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB718	*MAT***a** *yku80Δ::HPH^R cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB719	*MAT***a** *yku80-135i cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB620	*MAT***a***yku70-R456E cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB285	*MAT***a** *yku70-R456E YKU80-G8$_{myc18}$::TRP1 TEL VII-L::URA3 bar1-Δ::KAN^R ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* (YTSF79 derivative)
yAB470	*MAT***a** *yku70Δ::HPH^R cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB472	*MAT***a** *cdc13Δ::NAT^R tlc1Δ48 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB725	*MAT***a** *yku70Δ::HPH^R exo1Δ::KAN^R cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB761	*MAT***a/α** *yku80Δ::KAN^R/yku80-135i est1Δ::HIS3/EST1 cdc13Δ::NAT^R/CDC13 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*
yAB800	*MAT***a** *EST1-(MYC)$_{13}$::HIS3 cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB801	*MAT***a** *EST1-(MYC)$_{13}$::HIS3 yku80Δ::HPH^R cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
yAB802	*MAT***a** *EST1-(MYC)$_{13}$::HIS3 yku80-135i cdc13Δ::NAT^R ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1* pVL438
YVL3803	*MAT***a** *EST1-G6-(MYC)$_{12}$ (MYC)$_{12}$-G6-EST2 bar1Δ::NAT^R leu2 trp1 ura3-52 prb prc pep4-3* (Lubin *et al.* 2012)
YVL3906	*MAT***a** *EST1-G6-(MYC)$_{12}$ (MYC)$_{12}$-G6-EST2 YKU80-(FLAG)$_3$* ::TRP1 *bar1Δ::NAT^R leu2 trp1 ura3-52 prb prc pep4-3* (V. Lundblad)
YVL3493	*MAT***a** *EST1-G6-(MYC)$_{12}$ (FLAG)$_3$-(MYC)$_{12}$-G6-EST2 leu2 trp1 ura3-52 prb prc pep4-3* (Lubin *et al.* 2012)

only of Est2 recruitment but also of TLC1 localization. In contrast, expression of a Cdc13–Est1 fusion resulted in progressive telomere elongation in the absence of Ku–TLC1 interaction or DNA end binding to the same extent as in wild-type (WT) strains. Moreover, we found that Ku associates with both Est1 and Est2 in a TLC1-dependent manner and that Est1-telomere association was affected by Ku–TLC1 interaction independently of Est2 recruitment. These results reveal a previously unidentified interaction between Est1 and Ku that is dependent on Ku's association with TLC1 and is important for telomere length maintenance.

Materials and Methods

Strains and plasmids

Strains and plasmids used in this work are listed in Table 1 and Table 2, respectively. *S. cerevisiae* strains used are isogenic derivatives of YPH499 except for yVL3803, yVL3906, and yVL3493, which are isogenic derivatives of the protease-deficient strain *MAT***a** *leu2$^-$ trp1$^-$ ura3-52 prb$^-$ prc$^-$ pep4-3*. Gene deletions were created using one-step allele replacement using the indicated markers.

Genetic methods

For experiments involving a *CDC13-EST2* or *CDC13-EST1* fusion plasmid, indicated strains containing pVL438 (*CDC13 URA3 CEN*) were transformed with pVL1107 or pVL1091, respectively (Evans and Lundblad 1999). Transformants were struck out immediately (1× streakout) on −leu 5-fluoroorotic acid media to select for loss of the *CDC13*-covering plasmid. Strains were subsequently struck out (2×, 3×, etc.) on −leu media every 2 days. For *est1Δ yku80-135i* epistasis analysis, diploid strain yAB761 was transformed with plasmids pVL1107 and pVL1037 (*YKU80 TRP1 CEN*), sporulated, and dissected to obtain the haploid spores *yku80Δ::HPH^R cdc13Δ::NAT^R* pVL1037 pVL1107 (WT), *est1Δ::HIS3 yku80Δ::HPH^R cdc13Δ::NAT^R* pVL1037 pVL1107 (*est1Δ*), *est1Δ::HIS3 yku80-135i cdc13Δ::NAT^R* pVL1107 (*est1Δ yku80-135i*), and *yku80Δ::HPH^R cdc13Δ::NAT^R* pVL1107 (*yku80Δ*).

Telomere length analysis

Strains were grown in 8 ml of appropriate minimal media at 28° overnight. Genomic DNA was prepared by zirconia/silica bead disruption using a standard phenol extraction. DNA was digested with *Xho*I and separated on a 0.8% agarose gel. Gels were transferred to a Hybond XL membrane (Amersham) using an alkaline transfer and probed with a radiolabeled telomeric DNA fragment excised from plasmid Ap135. Telomere length analysis was performed at least three times for each strain.

Fluorescence in situ hybridization

Yeast fixation and fluorescence *in situ* hybridization (FISH) to detect endogenous *TLC1* RNA was performed as described (Pfingsten *et al.* 2012).

Image acquisition, deconvolution, and processing

All images were acquired using an Axio Imager 2-Carl Zeiss epifluorescence upright microscope equipped with a 100× DIC H (1.4 N.A.) objective and with a Photometrics CoolSNAP fx CCD camera. Images were acquired with Zeiss software and processed with Image J. One hundred

Table 2 Plasmids

Plasmid	Description
pVL999	*ADH1-EST2 2μ LEU2* (Nugent *et al.* 1998)
pAB753	*ADH1-Est1 2μ TRP1*
pAB830	*ADH1-TLC1 2μ URA3*
pVL1107	*CDC13-EST2 CEN LEU2* (Evans and Lundblad 1999)
pVL1091	*CDC13-EST1 CEN LEU2* (Evans and Lundblad 1999)
pVL438	*CDC13 CEN URA3* (Evans and Lundblad 1999)
pAB548	*YKU80-3XFLAG CEN LEU2*
pAB922	*yku80-135i-3XFLAG CEN LEU2*
pVL1037	*YKU80 CEN TRP1* (Bertuch and Lundblad 2004)
Ap135	$TG_{(1-3)}$ probe
pAB126	TyB probe

fields of yeast cells were acquired as *z* stacks of 12 planes minimum, with 0.5 μm between planes in the *z* axis. Maximal projection of *z* stacks was performed, deconvolved by Image J or Autoquant X3 software using a theoretical point spread function algorithm, and merged with a DAPI signal for quantification of localization. For each yeast strain, a total of 200 unbudded cells were randomly scored in three independent experiments, and numbers are expressed as a percentage of cells with TLC1 located mainly in the nucleus, cytoplasm, or distributed between both.

Co-immunoprecipitation assays

Fifty milliliter cultures (OD_{600} = 1.0) of yAB718 pAB548, yAB801 pAB548, yAB801 pAB922, yAB801 pVL1037, YVL3803, YVL3906, and YVL3493 were lysed in 400 μl of TMG (10 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, 10% (v/v) glycerol, 0.1 mM DTT, 0.1 mM EDTA)–50 mM NaCl with silica beads. Fifty microliters of α-FLAG M2 agarose beads (Sigma) were added to 4 mg of total protein in 500 μl TMG-50 + 0.5% Tween-20 and rotated at 4° for 1 hr. For RNase A and DNase I treatments, 20 units of RNase A (Affymetrics) or 10 μl of DNase I (NEB) plus 2.5 mM $MgCl_2$ were added to extracts and incubated at 37° for 15 min prior to addition of α-FLAG M2 beads. Beads were washed once with TMG-50 + 0.5% Tween-20, three times with TMG-300 mM NaCl + 0.5% Tween-20, once with TMG-500 mM NaCl + 0.5% Tween-20, and once with TMG-50. A total of 130 μg of "input" and total immunoprecipitation ("IP") samples were run on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with α-myc (Sigma) α-flag (Sigma), and α-PGK (Abcam) primary antibodies and IRDye 800CW-conjugated goat α-rabbit or goat α-mouse secondary antibodies (LiCor).

Cell cycle arrest

For cell cycle arrest experiments, 50 ng/ml α-factor, 0.2 M hydroxyurea, or 15 μg/ml nocodazole were added to 50 ml YPD cultures (OD_{600} = 0.5) and grown for 2.5 hr. Cells were then harvested and used in co-immunoprecipitation experiments.

ChIP assays

One hundred milliliter −leu cultures of 2× streakouts of yAB800, yAB801, and yAB802 transformed with pVL1107 were grown at 28° to an OD_{600} = 1.0. Cultures were washed once with YPD and transferred to 100 ml YPD and allowed to grow for 15 min before cross-linking. Formaldehyde cross-linking and cell lysis using a multi-tube vortexer were carried out as described previously (Aparicio *et al.* 2005) with minor modifications. Lysates were sonicated using a Misonex Sonicator 3000 (power level 2: 2 min, six cycles). Total protein levels were equilibrated across samples (typically 13 mg of protein per sample). IPs were performed in 800 μl lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodiun deoxycholate and 0.1% SDS) incubated with 6 μl of 9E10 (Sigma, 2.1 mg/ml) antibody overnight at 4°. Protein G-plus agarose beads (Calbiochem) were added, and samples were incubated an additional 1.5 hr. IP washes, reversal of cross-links, and DNA precipitation were done according to Aparicio *et al.* (2005). DNA was dot-blotted on a Hybond XL membrane (Amersham) according to the manufacturer's protocol. IP samples were probed with a randomly labeled telomeric DNA fragment excised from plasmid Ap135 and inputs with a randomly labeled TyB DNA fragment excised from plasmid pAB126. Membranes were then exposed to a phosphorimager screen and quantified using ImageQuant software. To monitor IP efficiency, 100 μg of input and 40 μl of IP sample were run on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with α-myc (9E10 Sigma) antibody.

Results

Overexpression of telomerase subunits or tethering Est2 to telomeres cannot bypass the role of Ku or Ku–TLC1 interaction in telomere elongation

We first sought to determine whether reduced amounts of Est2 at telomeres played a major role in the telomere shortening observed in the absence of Ku or Ku–TLC1 interaction. To do this, we overexpressed Est2 and Est1 subunits in an attempt to drive more telomerase to telomeres. We found that Est2 overexpression had no impact on telomere length in *yku80-135i* or, as previously reported, in *yku80*Δ strains (Figure 1A and Supporting Information, Figure S1) (Teo and Jackson 2001). As previously reported, a slight increase in telomere length was observed when Est1 was overexpressed in WT strains (Virta-Pearlman *et al.* 1996; Zhang *et al.* 2010), and a similar effect was observed in *yku80-135i* and *yku80*Δ strains. However, in contrast to WT strains, where simultaneous overexpression of Est1 and Est2 resulted in synergistic telomere elongation, Est1/Est2 co-overexpression had minimal impact on telomere length in the *yku80-135i* mutant or in the absence of Ku, as telomeres were no longer than when Est1 was overexpressed alone (Figure 1A and Figure S1). Since telomere elongation was minimal when Est2 was overexpressed alone or in combination with Est1, these results suggest that reduced amounts of Est2 at telomeres in the absence of Ku–TLC1 interaction may not be the main reason that telomeres are short.

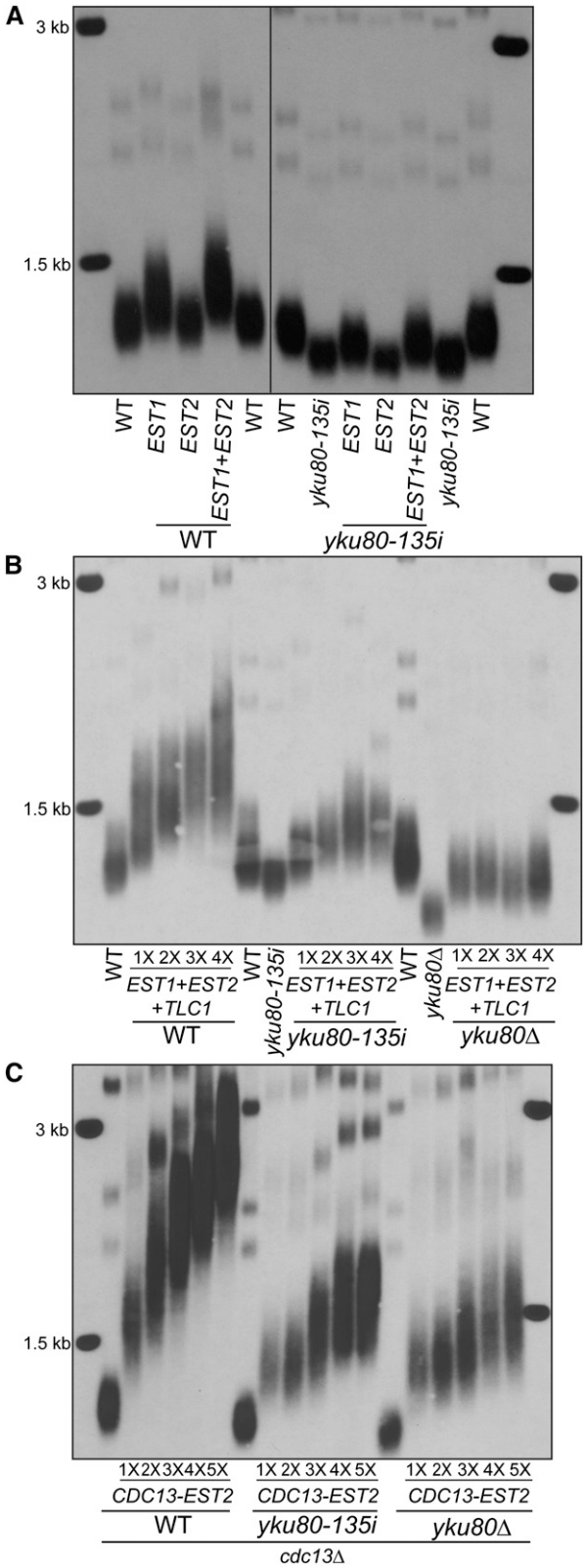

Figure 1 Telomerase overexpression or Cdc13-Est2 expression has differential effects in WT, *yku80-135i*, and *yku80*Δ strains. (A) Telomere length analysis by Southern blot of *Xho*I-digested DNA isolated from WT, *yku80-135i*, and *yku80*Δ strains transformed with Est1 and Est2 overexpression plasmids, singly and in combination. (B) Telomere length analysis by Southern blotting of 1×–4× serial single-colony streakouts of WT, *yku80-135i*, and *yku80*Δ strains simultaneously overexpressing Est1, Est2, and TLC1. (C) Telomere length analysis of 1×–5× serial single-colony streakouts of *cdc13*Δ (WT), *cdc13*Δ *yku80-135i* (*yku80-135i*), and *cdc13*Δ *yku80*Δ (*yku80*Δ) strains expressing a Cdc13–Est2 fusion.

The loss of Ku or Ku–TLC1 interaction results in a slight reduction in total TLC1 RNA (Zappulla *et al.* 2011), and TLC1 is detected mainly in the cytoplasm (Gallardo *et al.* 2008). Therefore, it was possible that TLC1 was the limiting factor in telomere elongation when Est1 and Est2 were overexpressed. To address this possibility, we also overexpressed TLC1. Examination of telomere length revealed no effect of TLC1 overexpression alone or in combination with Est1 or Est2 in WT, *yku80-135i*, or *yku80*Δ strains (Table 3 and Figure S1). However, triple overexpression of TLC1, Est1, and Est2 had a synergistic effect on telomere lengthening in WT and *yku80-135i* strains but not in *yku80*Δ strains (again, the additional effect in the WT strain was indicative that TLC1 was overexpressed) (Figure 1B and Table 3). Telomere length analysis of single-colony serial streakouts resulted in progressive telomere elongation in WT strains, which continued for four successive streakouts (indicated by 1×, 2×, etc.), whereas elongation plateaued in *yku80-135i* strains after three streakouts (Figure 1B; Table 3; Figure S1D). These results indicate that Ku, and specifically Ku–TLC1 interaction, has a role in promoting telomere elongation that cannot be completely bypassed by increasing the amount of telomerase. However, the greater telomere elongation observed in the *yku80-135i* strain, when Ku can bind DNA but not RNA, compared to the Ku null strain suggests that Ku may have a role in promoting telomere elongation independently of its interaction with TLC1.

Although in the previous experiment telomerase subunits were overexpressed, we did not know if that led to increased telomerase associated with telomeres. To directly test whether recruitment of the catalytic subunit, Est2, plays a major role in Ku-mediated telomere length maintenance, we made use of a Cdc13–Est2 fusion protein to tether Est2 to telomeres (Evans and Lundblad 1999). Expression of the Cdc13–Est2 fusion results in extensive telomere elongation in WT strains likely due to increased recruitment of Est2 to telomeres (Evans and Lundblad 1999). Furthermore, Cdc13 recruitment to telomeres is not dependent on Ku–TLC1 interaction, as the *tlc1*Δ*48* mutation has no impact on Cdc13 telomere association and the amount of Cdc13 at telomeres increases in the absence of Ku, presumably due to the increase in G-tail DNA (Fisher *et al.* 2004). Thus, expression of a Cdc13–Est2 fusion should lead to efficient recruitment of Est2 to telomeres in the absence of Ku–TLC1 interaction or Ku. Additionally, Cdc13 is associated with telomeres throughout the cell cycle in the absence of Ku as opposed to WT and strains lacking Ku–TLC1 interaction where Cdc13 telomere association is restricted to S phase (Fisher *et al.* 2004). For this reason, expression of a Cdc13–Est2 fusion would result in recruitment

Table 3 Summary of the effects of telomerase subunit overexpression on telomere length in WT and *yku80* mutant strains

	WT	*yku80-135i*	*yku80*Δ
Est1	Slight, stable increase	Slight, stable increase	Slight, stable increase
Est2	Slight, progressive increase	No effect	No effect
TLC1	No effect	No effect	No effect
Est1+Est2	Progressive increase	Slight, stable increase	Slight, stable increase
Est1+TLC1	Slight, stable increase	Slight, stable increase	Slight, stable increase
Est2+TLC1	No effect	No effect	No effect
Est1+Est2 +TLC1	Progressive increase, additive effect of TLC1	Stable increase, additive effect of TLC1	Slight, stable increase, no additive effect of TLC1

of Est2 to telomeres in G1 in *yku80*Δ strains, allowing us to specifically test the role of Est2 recruitment to telomeres in G1 in telomere length regulation.

For experiments involving a Cdc13 fusion protein, *cdc13*Δ, *cdc13*Δ *yku80-135i*, and *cdc13*Δ *yku80*Δ strains were constructed to eliminate competition between endogenous Cdc13 and the fusion. We will refer to these strains subsequently as simply WT, *yku80-135i*, and *yku80*Δ, respectively, indicating the mutation status of Ku. As previously demonstrated, expression of a Cdc13–Est2 fusion resulted in extensive, progressive telomere elongation over successive colony streakouts in WT strains (Figure 1C). Interestingly, the effect was greatly attenuated in *yku80-135i* and *yku80*Δ strains, in which Ku does not interact with TLC1 or is absent (Figure 1C), with telomeres elongating to a much lesser extent. A similar effect was observed in *tlc1Δ48* and *yku70*Δ strains expressing a Cdc13–Est2 fusion (Figure S2), confirming that the defect in telomere elongation was due to loss of Ku–TLC1 interaction or Ku function. Furthermore, the failure of the expression of the Cdc13–Est2 fusion to extensively elongate telomeres in the *yku80*Δ strain, in particular, argues that Ku-dependent Est2 recruitment to telomeres in G1 does not contribute significantly to telomere length regulation.

Ku's role in promoting telomere elongation requires both DNA and RNA binding but is independent of end protection

After finding that Ku was unable to promote extensive telomere elongation in the presence of a Cdc13–Est2 fusion in the absence of TLC1 binding, we wanted to know if Ku's interaction with TLC1 in the absence of DNA binding was sufficient to promote telomere elongation. The ability of Ku–TLC1 interaction to promote telomere elongation in the absence of DNA binding was plausible because Ku cannot bind TLC1 and DNA ends simultaneously (Pfingsten *et al.* 2012). To test the requirement of DNA binding to promote extensive telomere elongation in the presence of a Cdc13–Est2 fusion, we made use of a previously characterized DNA-binding-defective allele of Yku70, *yku70-R456E* (Lopez *et al.* 2011). The single amino acid substitution in the DNA-binding channel of Yku70 results in a Ku heterodimer that can no longer efficiently bind DNA ends but still associates with TLC1 *in vivo* as determined by co-immunoprecipitation (Lopez *et al.* 2011). However, much like the *yku80-135i* and *yku80*Δ strains, telomeres did not extensively elongate when a Cdc13–Est2 fusion was expressed in the absence of DNA binding by Ku (Figure 2A, *yku70-R456E* strain). The reduced telomere elongation was unlikely due to TLC1 mislocalization in the cytoplasm as TLC1 was mainly nuclear in a *yku70-R456E* strain (Figure 2B). These data suggest that, although Ku does not associate with RNA and DNA simultaneously, its role in telomere length maintenance requires it to retain the ability to bind both TLC1 and DNA ends.

DNA end binding by Ku is known to inhibit nucleolytic processing by Exo1. In the absence of Ku, telomeres are deprotected, resulting in extensive single-stranded telomeric DNA (Maringele and Lydall 2002; Bertuch and Lundblad 2004). Therefore, it was possible that the failure of telomeres to progressively elongate in Ku null strains in the presence of a Cdc13–Est2 fusion was a secondary effect of loss of end protection. To address this possibility, *EXO1* was deleted in the *yku70*Δ strain to reduce the amount of single-stranded telomeric DNA (Maringele and Lydall 2002; Bertuch and Lundblad 2004). However, expression of a Cdc13–Est2 fusion in an *yku70*Δ *exo1*Δ strain did not allow for progressive telomere elongation (Figure 2C), just an initial increase that stabilized, indicating that Ku's role in promoting telomere elongation is independent of its end protection function. These results further demonstrate that the Ku heterodimer has a previously unidentified role in telomere elongation.

Recruitment of Est1 to telomeres via a Cdc13–Est1 fusion is sufficient to bypass the role of Ku in promoting telomere elongation

The above results indicate that the recruitment of Est2 to telomeres is not the main role of Ku in telomere elongation. As Est1 recruitment to telomeres in the absence of Ku–TLC1 interaction or Ku is also reduced (Fisher *et al.* 2004), we next wanted to determine whether expression of a Cdc13–Est1 fusion could bypass the role of Ku. It was previously demonstrated that, after ~100 generations of growth, telomere lengths in WT and *yku70*Δ strains expressing a Cdc13–Est1 fusion were similarly elongated (Grandin *et al.* 2000). These analyses, however, were conducted at high temperature to reveal effects conferred by the *cdc13-1* allele also present in the strains, and it is known that telomere and

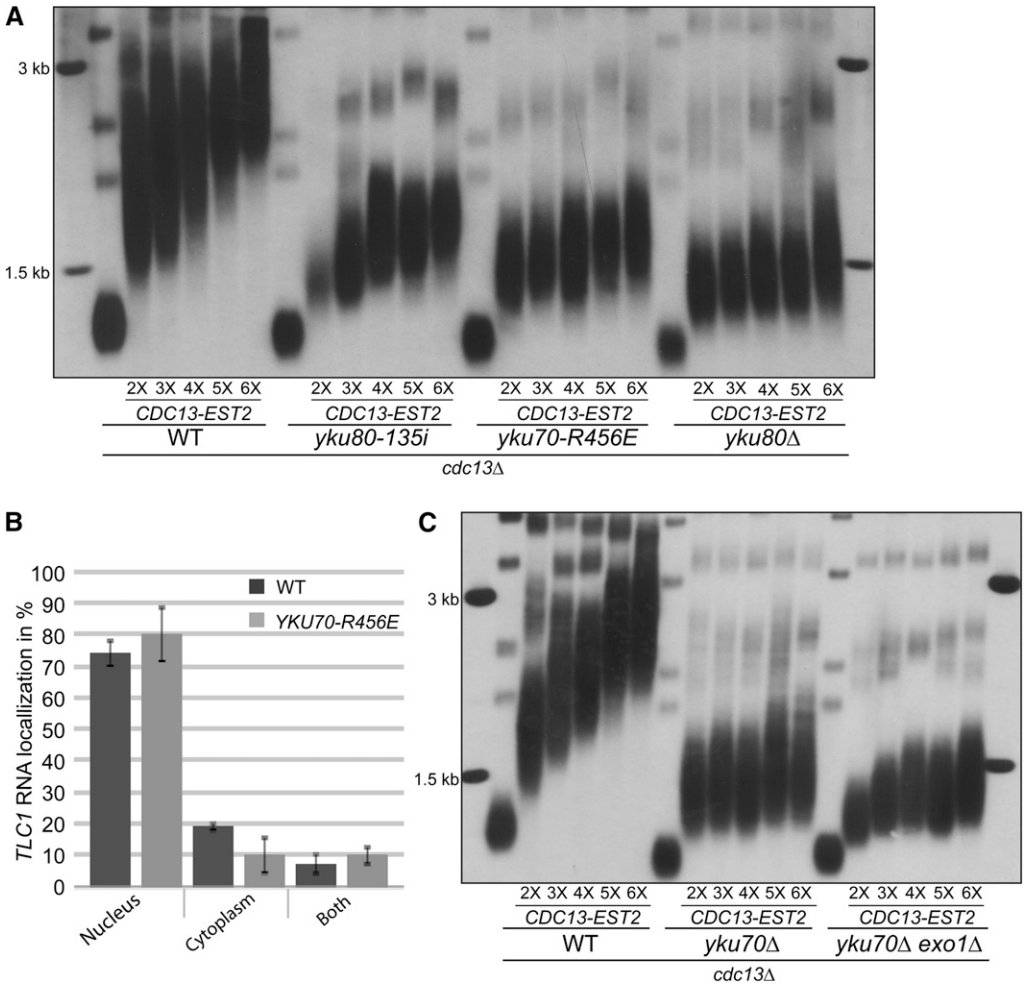

Figure 2 TLC1 nuclear retention in addition to Est2 recruitment cannot bypass the role of Ku. (A) Telomere length analysis of 2×–6× serial single-colony streakouts of *cdc13*Δ (WT), *cdc13*Δ *yku70-R456E* (*yku70-R456E*), and *cdc13*Δ *yku70*Δ (*yku70*Δ) strains expressing a Cdc13–Est2 fusion. (B) Quantification of TLC1 localization by FISH in a *yku70-R456E* strain. Error bars represent ± 1 SD. Unbudded cells from asynchronous cultures were analyzed. However, TLC1 localization was similar in all cells (data not shown). (C) Telomere length analysis of 2×–6× serial single-colony streakouts of *cdc13*Δ (WT), *cdc13*Δ *yku70*Δ (*yku70*Δ), and *cdc13*Δ *yku70*Δ *exo1*Δ (*yku70*Δ *exo1*Δ) strains expressing a Cdc13–Est2 fusion.

subtelomeric structure is altered in Ku-deficient strains at high temperature (Gravel *et al.* 1998; Fellerhoff *et al.* 2000; Maringele and Lydall 2002) due to an apparently naturally thermolabile telomere-specific activity (Paschini *et al.* 2012). Additionally, the presence of extensive G-tails in the *yku70*Δ strain would have resulted in a marked increase in binding sites of the Cdc13–Est1 fusion. Therefore, we wanted to test whether defects in Ku–TLC1 interaction alone affects the ability of a Cdc13–Est1 fusion to promote progressive telomere elongation. To do this, we examined telomere length in WT, *yku80-135i*, and *yku80*Δ strains expressing a Cdc13–Est1 fusion over successive colony streakouts. In contrast to what was seen in the presence of a Cdc13–Est2 fusion, expression of a Cdc13–Est1 fusion resulted in progressive telomere elongation to approximately the same extent in all strains (Figure 3A and Figure S2). Because a *yku80-135i* strain does not exhibit extensive G-tails (Stellwagen *et al.* 2003), this indicates that the extensive elongation observed in the *yku80-135i* strain was not a consequence of additional Cdc13-binding sites. These results suggest that Ku influences telomere length mainly via Est1.

The reason telomeres are short in a *yku70-R456E* mutant is not clear. As TLC1 is mainly nuclear in this mutant (Figure 2C) yet telomeres are still short, one possibility is that Ku bound to TLC1 in the absence of DNA binding sequesters TLC1 from associating with active telomerase. We therefore sought to determine the impact of Ku–TLC1 interaction in the absence of DNA end binding by Ku on the ability of a Cdc13–Est1 fusion to promote telomere elongation. Expression of a Cdc13–Est1 fusion in a *yku70-R456E* strain resulted in efficient and progressive telomere elongation (Figure 3B), refuting the hypothesis that end-binding-deficient Ku sequesters TLC1. These results demonstrate that both Ku's DNA binding-dependent and RNA binding-dependent roles in telomere elongation can be bypassed by tethering Est1 to telomeres.

Expression of a Cdc13–Est2 or Cdc13–Est1 fusion partially rescues TLC1 nuclear localization in the absence of Ku–TLC1 interaction or Ku

In the context of a fusion, Cdc13-Est1 and Cdc13-Est2 might differentially promote TLC1 nuclear localization in the absence of Ku–TLC1 interaction and, thereby, result in differences in telomere elongation. To examine this possibility, TLC1 localization was determined by FISH in WT, *yku80-135i*, and *yku80*Δ strains alone or expressing a Cdc13–Est1 or Cdc13–Est2 fusion. As previously reported, TLC1 was mainly cytoplasmic in *yku80-135i* and *yku80*Δ strains not expressing a fusion protein

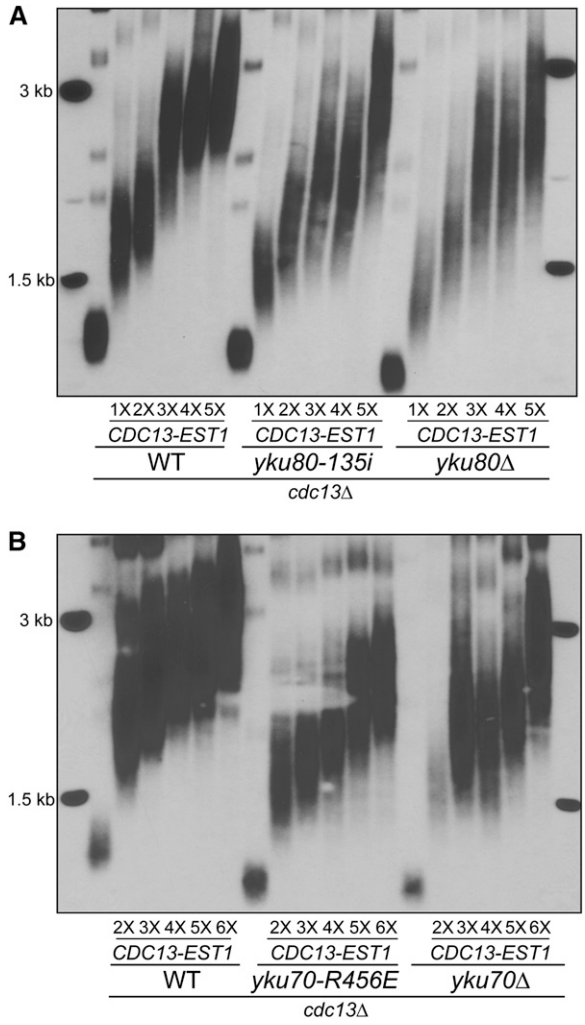

Figure 3 Tethering Est1 to telomeres promotes efficient telomere elongation in Ku mutant strains. (A) Telomere length analysis by Southern blot of *Xho*I-digested DNA isolated from 1×–5× serial single-colony streakouts of *cdc13*Δ (WT), *cdc13*Δ *yku80-135i* (*yku80-135i*), and *cdc13*Δ *yku80*Δ (*yku80*Δ) strains expressing a Cdc13–Est1 fusion. (B) Telomere length analysis of 2×–6× serial single-colony streakouts of *cdc13*Δ (WT), *cdc13*Δ *yku70-R456E* (*yku70-R456E*), and *cdc13*Δ *yku70*Δ (*yku70*Δ) strains expressing a Cdc13–Est1 fusion.

(Figure 4A). Interestingly, both the Cdc13-Est1 and the Cdc13-Est2 fusion restored TLC1 nuclear localization to about the same extent in *yku80-135i* and *yku80*Δ strains with ~40–60% of cells having mainly nuclear TLC1 (Figure 4A). Expression of a Cdc13–Est1 fusion had a slightly greater effect on TLC1 nuclear retention in *yku80-135i* and *yku80*Δ strains compared to expression of a Cdc13–Est2 fusion. However, the *yku80-135i/CDC13-EST2* and *yku80Δ/CDC13-EST1* strains had a comparable number of cells with TLC1 mainly in the nucleus (51 and 49.5%, respectively), yet telomere lengths in these strains were vastly different (Figure 4B). Therefore, differences in the ability of the Cdc13–Est1 and Cdc13–Est2 fusion to retain TLC1 in the nucleus could not fully account for differences in their ability to promote telomere elongation in Ku mutant strains. These findings suggest that, although TLC1 nuclear localization is important for telomere length maintenance, Ku makes additional contributions to telomere elongation.

Ku, Est1, and Est2 are present in a complex, which is dependent on TLC1

The ability of the Cdc13–Est1 fusion to bypass the role of Ku suggests that Ku's primary contribution to telomere length maintenance is via Est1. In further support of an interaction between Ku and Est1, we found that myc-tagged Est1 co-immunoprecipitated with flag-tagged Yku80 in asynchronous cells (Figure 5A). This interaction was dependent on the ability of Ku to bind TLC1 as the Ku–Est1 interaction was reduced when a *yku80-135i* allele was expressed (Figure 5A). Using a strain harboring identically myc-tagged Est1 and Est2, we found that Est2, in addition to Est1, co-immunoprecipitated with flag-tagged Yku80 (Figure 5, B–D). Consistent with the interaction between Ku and Est1 being dependent upon Ku's ability to bind TLC1, treatment of lysates with RNase A abolished the co-immunoprecipitation of Est1 and Est2 with Yku80 (Figure 5B). Furthermore, the interaction between Ku and Est1 and Est2 was not mediated by DNA as treatment of lysates with DNase I had no impact on the co-immunoprecipitation efficiency (Figure 5C and Figure S3A). Although no genomic DNA was detected in the lysates used in this experiment, samples were treated under conditions sufficient to degrade 3 μg of a 1.2-kb PCR fragment (Figure S3B). Therefore, any endogenous DNA in the extracts should have been degraded.

Next, to determine if Ku's interaction with telomerase subunits is cell-cycle-regulated, co-immunoprecipitation experiments were performed in G1-, S-, and G2/M-arrested cells by treatment with α-factor, hydroxyurea, and nocodazole, respectively. Interaction between Ku and Est2 was detected at all three points in the cell cycle (Figure 5D). As expression of Est1 is low in G1, no interaction between Ku and Est1 was detected in α-factor-arrested cells (Figure 5D). However, in both hydroxyurea- and nocodazole-treated cells, when Est1 was equivalently expressed, Est1 co-immunoprecipitated with Ku with similar efficiency (Figure 5D). These findings indicate that Est1 interacts with Ku in a TLC1-dependent manner in S phase and that the interaction persists through G2/M. Given that Est1 and Est2 shared the identical myc epitope tag, we could compare their relative association with Ku in these assays. We found that 3.5 and 1.8 times more Est1 than Est2 was associated with Ku in hydroxyurea- and nocodazole-arrested cells, respectively. Together with the ability of Est2 to interact with Ku in G1 in the absence of Est1, these results suggest that Ku can form separate complexes involving Est1 and Est2. However, compared to the association of Est1 with Est2, the association between Est1 and Ku was much less robust (Figure 5E, *EST1-MYC FLAG-MYC-EST2 vs. EST1-MYC MYC-EST2 YKU80-FLAG* strain). As equivalent amounts of protein were used in Est2 and Ku immunoprecipitations, only a small portion of Est1 and Est2 are associated with Ku. Therefore, although Ku associates with telomerase subunits throughout the cell

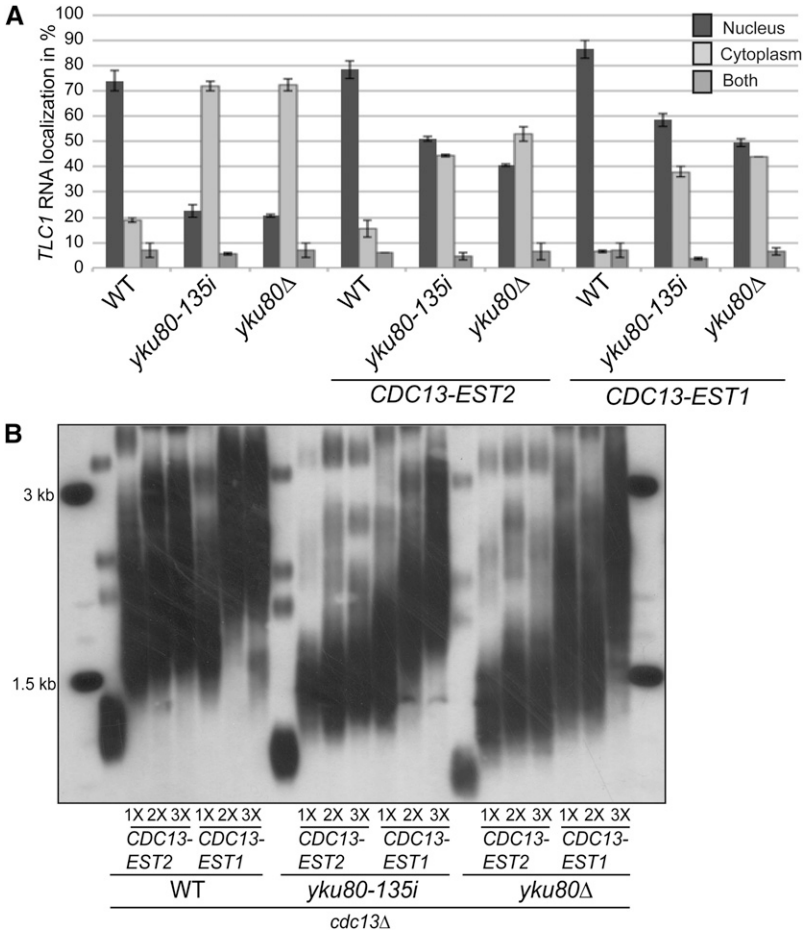

Figure 4 Cdc13-Est2 or Cdc13-Est1 expression partially rescues TLC1 nuclear localization in *yku80-135i* and *yku80*Δ strains. (A) Quantification of TLC1 localization by FISH in *cdc13*Δ (WT), *cdc13*Δ *yku80-135i* (*yku80-135i*), and *cdc13*Δ *yku80*Δ strains expressing either a Cdc13–Est2 or Cdc13–Est1 fusion protein. Error bars represent ± 1 SD. Unbudded cells from asynchronous cultures were analyzed. However, TLC1 localization was similar in all cells (data not shown). (B) Telomere length analysis of 1×–3× serial single-colony streakouts of *cdc13*Δ (WT), *cdc13*Δ *yku80-135i* (*yku80-135i*), and *cdc13*Δ *yku80*Δ (*yku80*Δ) strains expressing a Cdc13–Est2 or Cdc13–Est1 fusion.

cycle, the associations are transient or unable to be sustained under the conditions of these experiments.

Ku–TLC1 interaction promotes efficient Est1 association with telomeres independently of Est2

To explore the role that Ku's interaction with Est1 may play in telomere length maintenance, we examined the impact of Ku on Est1's association with telomeres. It was previously shown that both Est1 and Est2 telomere association is reduced in the absence of Ku–TLC1 interaction (Fisher *et al.* 2004; Chan *et al.* 2008). However, robust association of Est1 and Est2 are mutually dependent, with the absence of one subunit resulting in reduced telomere association of the other (Chan *et al.* 2008). Therefore, it is difficult to determine whether Ku is important for recruitment of one or both of the subunits. As telomere association of Est2 in G1 is completely dependent on Ku (Fisher *et al.* 2004), one hypothesis is that Ku is required for efficient Est2 recruitment and the reduction of Est1 at telomeres is a secondary effect of less Est2 present. The results with the Cdc13 fusion proteins, however, do not support this hypothesis as tethering Est2 to telomeres in the absence of Ku–TLC1 interaction or Ku could not bypass the role of Ku in promoting telomere elongation (Figure 1C). Furthermore, tethering Est1 to telomeres is sufficient for extensive telomere elongation in the absence of Ku–TLC1 interaction or Ku (Figure 3A), suggesting that a major role of Ku may be to promote Est1 recruitment to telomeres. To determine if Ku influences Est1's telomere association independently of promoting Est2 recruitment, ChIP experiments were performed in WT, *yku80*Δ, and *yku80-135i* strains expressing a Cdc13–Est2 fusion. To facilitate immunoprecipitation, endogenous Est1 was C-terminally tagged with a 13×-myc epitope. We found that both *yku80*Δ and *yku80-135i* mutants had reduced telomere association of Est1 compared to WT strains when a Cdc13–Est2 fusion was expressed (Figure 6, A and B). The decrease in Est1 telomere association could not be explained by differences in Est1 protein level or immunoprecipitation efficiency in Ku mutant strains as Est1 protein levels were equivalent in input and IP samples (Figure 6C).

In the absence of a Cdc13 fusion, Est1 and Est2 telomere association is dependent on TLC1 (Chan *et al.* 2008); therefore, it is possible that the reduced amount of Est1 at telomeres in *yku80-135i* and *yku80*Δ strains expressing a Cdc13–Est2 fusion was due to decreased TLC1 nuclear localization. If the failure of telomeres to elongate was completely due to limiting amounts of nuclear TLC1, similar results would be expected when a Cdc13–Est1 fusion was expressed as less Est2 would be associated with telomeres. However, as telomeres elongated equivalently in WT, *yku80-135i*, and *yku80*Δ strains in the presence of a

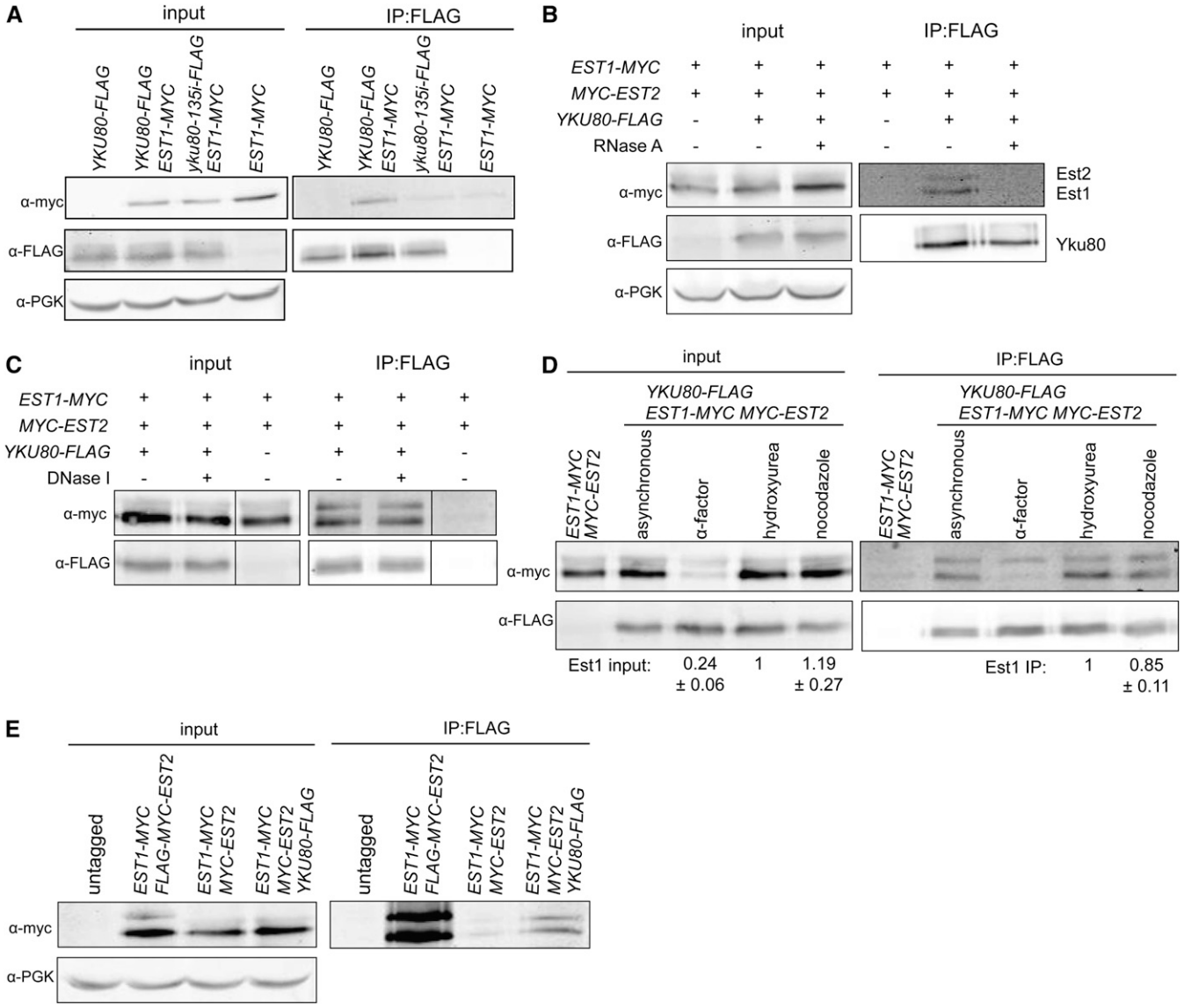

Figure 5 Ku associates with Est1 and Est2 in a TLC1-dependent manner. (A) Co-immunoprecipitation of Est1-myc with Yku80-FLAG and Yku80-135i-FLAG. Anti-FLAG immunoprecipitations were performed with whole-cell extracts of indicated strains. Inputs and IPs were analyzed by Western blotting with α-myc to detect Est1 and with α-FLAG to detect Yku80 or Yku80-135i. Inputs were also probed with α-PGK as a loading control. (B) Co-immunoprecipitation of Est1-myc and myc-Est2 with Yku80-FLAG in RNase A-treated and untreated extracts. Western blots were probed with α-myc to detect Est1 (bottom band) and Est2 (top band). (C) Co-immunoprecipitation of Est1-myc and myc-Est2 with Yku80-FLAG in DNase I-treated and untreated extracts. (D) Co-immunoprecipitation of Est1-myc and myc-Est2 with Yku80-FLAG in asynchronous, α-factor-, hydroxyurea-, and nocodazole-arrested cells. Quantification of relative amount of Est1 in inputs and immunoprecipitates represents the average and standard deviation of four independent experiments. (E) Co-immunoprecipitation of Est1 with Est2 (*EST1-MYC FLAG-MYC-EST2* strain) or Yku80 (*EST1-MYC MYC-EST2 YKU80-FLAG* strain).

Cdc13–Est1 fusion (Figure 3A and Figure S2), this suggests that Est2 was efficiently recruited in all three strains.

Ku has a minor role in telomere length maintenance that is independent of Est1

It has been previously demonstrated that, in an *est1*Δ strain, expression of a Cdc13–Est2 fusion can bypass Est1's telomerase recruitment function, resulting in approximately WT length telomeres (Evans and Lundblad 1999). Furthermore, the Cdc13–Est2 fusion was unable to promote extensive, progressive elongation in an *est1*Δ strain, leading to the conclusion that Est1 has a telomerase activation function (Evans and Lundblad 1999). The inability of the Cdc13–Est2 fusion to promote telomere elongation to the same extent as in a WT strain in *yku80-135i* or *yku80*Δ mutants suggests that Ku may also have a role in telomerase activation. Additionally, the ability of the Cdc13–Est1 fusion to promote progressive telomere elongation in the absence of Ku indicates that Ku's role in telomere length maintenance may be primarily through an Est1-dependent pathway. To test this, we performed epistasis

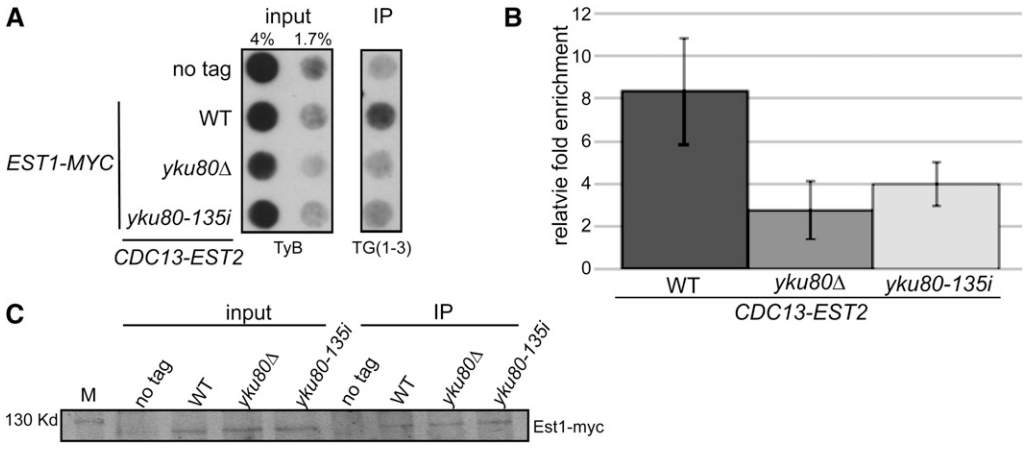

Figure 6 The association of Est1 with telomeres is dependent on Ku–TLC1 interaction even when Est2 is tethered to the telomere. (A) Myc-tagged Est1 was immunoprecipitated from formaldehyde cross-linked *cdc13*Δ (WT), *cdc13*Δ *yku80*Δ (*yku80*Δ), and *cdc13*Δ *yku80-135i* (*yku80-135i*) strains expressing a Cdc13–Est2 fusion. Isolated DNA was dot-blotted onto a membrane and probed with a radiolabeled TyB (inputs) or telomere-specific $T\text{-}G_{(1\text{-}3)}$ (IPs) probe. (B) Graphical representation of the average IP/input signal relative to the no-tag control strain based on four independent experiments. Error bars represent ± 1 SD. (C) Western blot showing equivalent amounts of Est1 protein immunoprecipitated from *cdc13*Δ (WT), *cdc13*Δ *yku80*Δ (*yku80*Δ), and *cdc13*Δ *yku80-135i* (*yku80-135i*) strains expressing a Cdc13–Est2 fusion. A total of 100 μg of whole-cell extract prior to immunoprecipitation (input) was loaded, demonstrating that Est1 protein level is equal in all three strains.

analysis of *EST1* and the *yku80-135i* mutation in the presence of a Cdc13–Est2 fusion. To enable recovery of all genotypes of interest from a single parental stain, *EST1/est1*Δ *yku80*Δ/*yku80-135i* *CDC13/cdc13*Δ diploid strains harboring both *YKU80* and *CDC13-EST2* plasmids were sporulated and dissected, ensuring similar initial telomere lengths. After dissection of 20 tetrads, however, no *est1*Δ *yku80*Δ double mutants were recovered even in the presence of a Cdc13–Est2 fusion. This was presumably due to the previously reported synthetic lethality of *est1*Δ *yku80*Δ mutations (Nugent *et al.* 1998) and increased single-stranded DNA that cannot be rescued by a Cdc13–Est2 fusion (Tong *et al.* 2011). Telomere length analysis of recovered haploid genotypes revealed, as expected, extensive telomere elongation in the WT strain expressing a Cdc13–Est2 fusion whereas telomeres were stably maintained but not elongated in the absence of Est1 (Figure 7). Interestingly, combining *est1*Δ and *yku80-135i* mutations had a slightly additive negative effect, with telomeres shorter in the *est1*Δ *yku80-135i* strain than in the *est1*Δ strain in the presence of a Cdc13–Est2 fusion (Figure 7). Although tethering Est1 to telomeres is sufficient to bypass the role of Ku in promoting telomere elongation, the *est1*Δ *yku80-135i* epistasis results demonstrate a specific contribution of Ku–TLC1 interaction that is independent of Est1. However, deletion of Est1 has a much greater impact on telomere lengthening in the presence of a Cdc13–Est2 fusion than deletion of *YKU80* (Figure 7, *est1*Δ strain compared to *yku80*Δ strain), suggesting that any contribution that Ku may have in telomerase activation is minor compared to that of Est1.

Discussion

The role of Ku at telomeres is complex, making Ku's contribution specifically to telomere length maintenance difficult to examine. Prior to this study, the two predominant hypotheses for the major contribution of Ku to telomere length were to promote the recruitment of Est2, and therefore telomerase, to telomeres and to enforce the nuclear localization of TLC1. Although both of these functions are important, our results demonstrate that neither tethering Est2 to telomeres nor promoting TLC1 nuclear accumulation is sufficient to bypass the role of Ku in telomere elongation. Instead, we have uncovered an interaction between Est1 and Ku that appears to be the main determinant of Ku's impact on telomere elongation.

Previous work supports a genetic interaction between Ku and Est1. A study by Evans and Lundblad (2002) discovered a class of Est1 mutants (*est1-50* and *est1-51*) that they proposed functioned in the same pathways as Ku. This was in part due to the lack of synthetic lethality when combined with a *yku70* deletion. These mutants had short telomeres but did not senesce and, although telomeres elongated in the presence of a Cdc13–Est2 fusion, they did not to the same extent as in a WT strain (Evans and Lundblad 2002). The phenotype of the *est1-50* and *est1-51* mutants is reminiscent of a *yku80-135i* strain and supports a role of Ku in telomere length maintenance that involves Est1. In further support of an interaction between Ku and Est1, tethering of chromosomes to the nuclear periphery by Ku is dependent on TLC1 interaction and Est1 (Schober *et al.* 2009). However, disruption of tethering had no effect on telomere length, which argues against tethering as a mechanism of telomere length maintenance by Ku.

Our data support a model in which Ku's main contribution to telomere length maintenance is to promote the stable association of Est1 with the telomere, where Est1 performs both its telomerase activation and its recruitment functions. Although Ku has other functions at the telomere, the defect in telomere elongation can be completely bypassed by tethering Est1, but not Est2, to telomeres. The different effects of these fusions are consistent with this model, with the

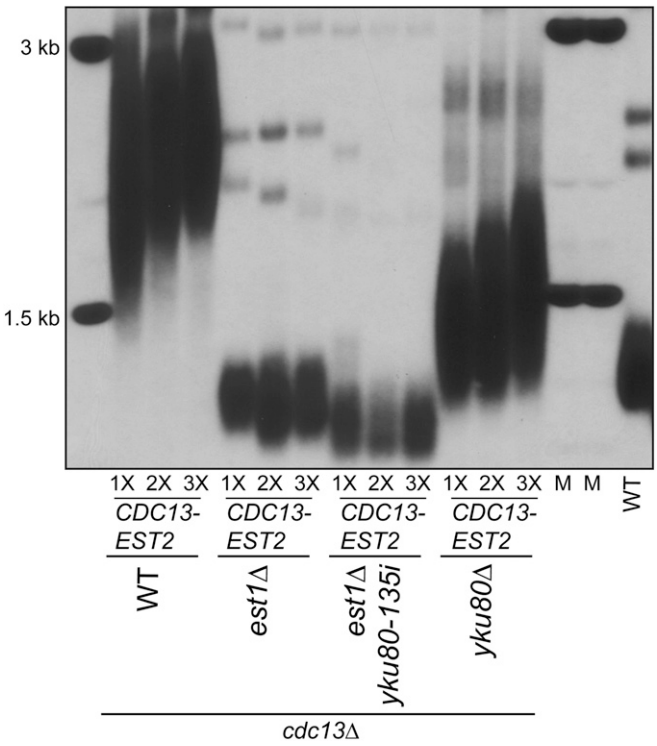

Figure 7 Ku has a minor role in telomere length maintenance that is independent of Est1. Telomere length analysis by Southern blot of 1×–3× serial single-colony streakouts of haploid strains *cdc13*Δ (WT), *cdc13*Δ *est1*Δ (*est1*Δ), *cdc13*Δ *est1*Δ *yku80-135i* (*est1*Δ *yku80-135i*), and *cdc13*Δ *yku80*Δ (*yku80*Δ) strains expressing a Cdc13–Est2 fusion dissected from a *EST1/est1*Δ *yku80*Δ*/yku80-135i CDC13/cdc13*Δ diploid strain harboring both *YKU80* and *CDC13-EST2* plasmids.

caveat that the promotion of telomere elongation in the context of the fusions may be fundamentally different from what occurs under physiologic conditions.

As Est1 recruitment to telomeres was not significantly different between *yku80-135i* and *yku80*Δ strains in the presence of a Cdc13–Est2 fusion (Figure 6B), Ku–TLC1 interaction is necessary for efficient or stable Est1 recruitment. We observed a physical association between Est1 and Ku by co-immunoprecipitation that is dependent on Ku's ability to bind TLC1 (Figure 5, A and B), demonstrating the presence of a complex containing Est1, TLC1, and Ku. We also detected an RNA-dependent association of Ku with Est2 (Figure 5B). From these experiments we are unable to determine if Ku, Est1, and Est2 are part of a single complex. However, we found that Est2 associated with Ku in G1 in the absence of Est1 (Figure 5D). In other phases of the cell cycle, immunoprecipitation of Yku80 resulted in more Est1 than Est2 co-immunoprecipitated. These results suggest that Ku can form independent complexes containing Est1 and Est2.

The influence of the Ku–Est1 interaction on telomere length maintenance and the mechanism by which Ku impacts Est1's association with telomeres remain to be elucidated. The small fraction of Est1 that co-immunoprecipitates with Ku may indicate that the interaction is transient. One possibility is that Ku binding to TLC1 promotes a conformational change or otherwise facilitates Est1 binding to TLC1. As association of Est1 with telomeres is dependent on TLC1 and Est2 (Chan *et al.* 2008), an influence of Ku on telomerase holoenzyme formation could promote Est1's recruitment to telomeres. Additionally, it has previously been proposed that the higher affinity of Ku for DNA than RNA may result in the release of TLC1 from Ku, thereby allowing telomerase recruitment by Cdc13 and telomere elongation (Pfingsten *et al.* 2012). Therefore, Ku may promote interaction between Cdc13 and Est1, leading to stable association of Est1 with the telomere. However, the binding of Ku to the telomeric end must occur prior to Cdc13 binding, as *in vitro* studies have shown that Ku cannot bind to an end prebound by Cdc13 (Wu *et al.* 2009). This suggests that Cdc13 would not yet be present at the telomere to receive telomerase handed off by Ku. Thus, the timing of these interactions is critical and remains unclear.

Similar to our findings with Ku, replication protein A (RPA) has been shown to be required for telomeric binding of Est1 *in vivo* (Schramke *et al.* 2004). RPA, which associates with telomeres and acts in the telomerase pathway (Schramke *et al.* 2004), also interacts with Ku, Cdc13, Est2, and TLC1 *in vivo* and weakly binds Est1 *in vitro* (Wu and Zakian 2011; Luciano *et al.* 2012). Its interaction with Ku occurs independently of RNA, whereas its association with TLC1 is dependent on both Ku and Est1 (Luciano *et al.* 2012). These results have led to a model whereby the two telomerase recruitment pathways contribute to RPA's interaction with telomerase at telomeres, which then facilitates telomere elongation. While these results raise the possibility that RPA may be the protein that receives telomerase handed off by Ku, the same concerns regarding the timing of RPA *vs.* Ku association with telomeric DNA theoretically pertain.

Furthermore, the cell-cycle-regulated recruitment of Est3 to telomeres is dependent upon Est1 and has been proposed to be part of Est1's telomerase activation function (Tuzon *et al.* 2011). This raises the possibility that reduced telomere elongation in Ku mutant strains is partly due to less Est3 recruitment. However, we found reduced Est1 associated with telomeres in Ku mutant strains expressing a Cdc13–Est2 fusion (Figure 6). Efficient Est1 association with telomeres is not dependent on Est3 (Tuzon *et al.* 2011). Therefore, an impact of Ku on Est3 recruitment would most likely be an indirect effect of less Est1 at telomeres.

Additionally, we found that the *yku70-R456E* mutant strain, which has markedly reduced association of Ku at telomeres (Lopez *et al.* 2011), maintained WT levels of TLC1 nuclear localization (Figure 2B). This result is consistent with the finding that Ku's association with TLC1 and DNA ends is mutually exclusive (Pfingsten *et al.* 2012) and, importantly, establishes that Ku docks TLC1 in the nucleus via a mechanism independent of its association with DNA ends. However, Ku's interaction with TLC1 alone is not sufficient for telomere length maintenance as telomeres are

short in the *yku70-R456E* mutant (Lopez *et al.* 2011) and do not extensively elongate when a Cdc13–Est2 fusion is expressed (Figure 2A). In this case, Est2 recruitment and TLC1 nuclear localization are achieved but telomere elongation is still reduced in the absence of telomere-bound Ku. Furthermore, expression of a Cdc13–Est2 fusion in a *yku80-135i* strain partially rescued nuclear localization of TLC1 (Figure 4A). In this strain, Ku is at the telomere, Est2 is recruited to telomeres via the Cdc13–Est2 fusion, and TLC1 is in the nucleus yet the telomere elongation defect is not overcome. Taken together, these data suggest that the Ku heterodimer must have the ability to bind both DNA and RNA for proper telomere maintenance. As DNA and RNA binding is mutually exclusive (Pfingsten *et al.* 2012), it precludes the possibility that Ku binds to the telomere and tethers telomerase via an interaction with TLC1. Therefore, the mechanism by which Ku's interaction with TLC1 and DNA ends influences telomere length is unclear. One possibility is that separate pools of Ku exist—one that is bound to the DNA and another that associates with TLC1—and both must be present for proper telomere elongation. This is probable given that the amount of Ku molecules per cell greatly exceeds the amount of TLC1. Alternatively, one Ku heterodimer may need to interact with both RNA and DNA but these interactions occur at different times in the cell cycle.

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The Principal Role of Ku in Telomere Length Maintenance Is Promotion of Est1 Association with Telomeres

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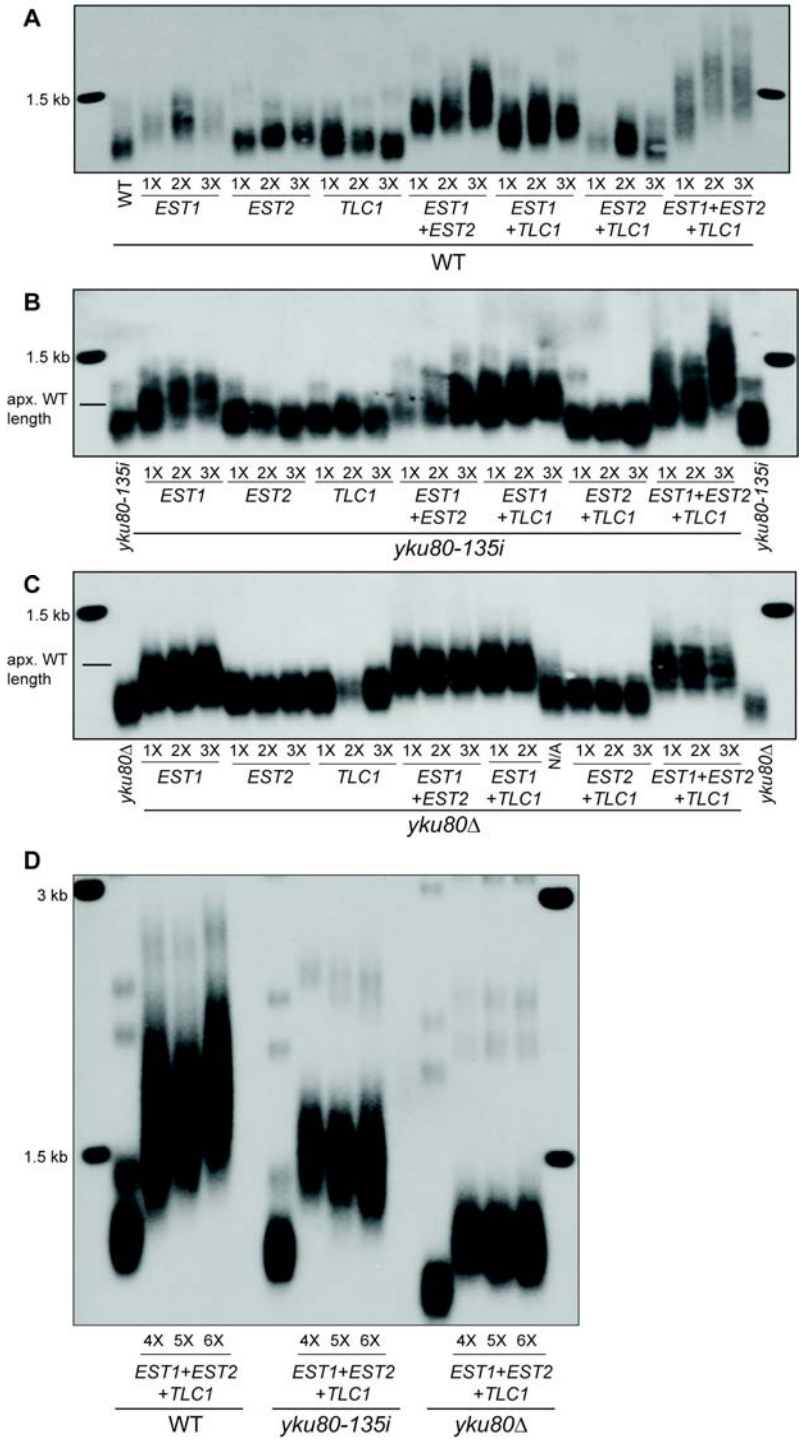

Figure S1 Overexpression of telomerase subunits has differential effects in WT, *yku80-135i* and *yku80Δ* strains. Telomere length analysis by Southern blot of XhoI digested DNA isolated from 1X-3X serial single colony streakouts of (A) WT, (B) *yku80-135i* and (C) *yku80Δ* strains transformed with *EST1*, *EST2* and *TLC1* overexpression plasmids singly and in combination. Approximate WT length depicted in B and C were based on migration in A as all gels were run under the same conditions. (D) Telomere length analysis of 4X-6X streakout of WT, yku80-135i and *yku80Δ* strains simultaneously overexpressing Est1, Est2 and TLC1.

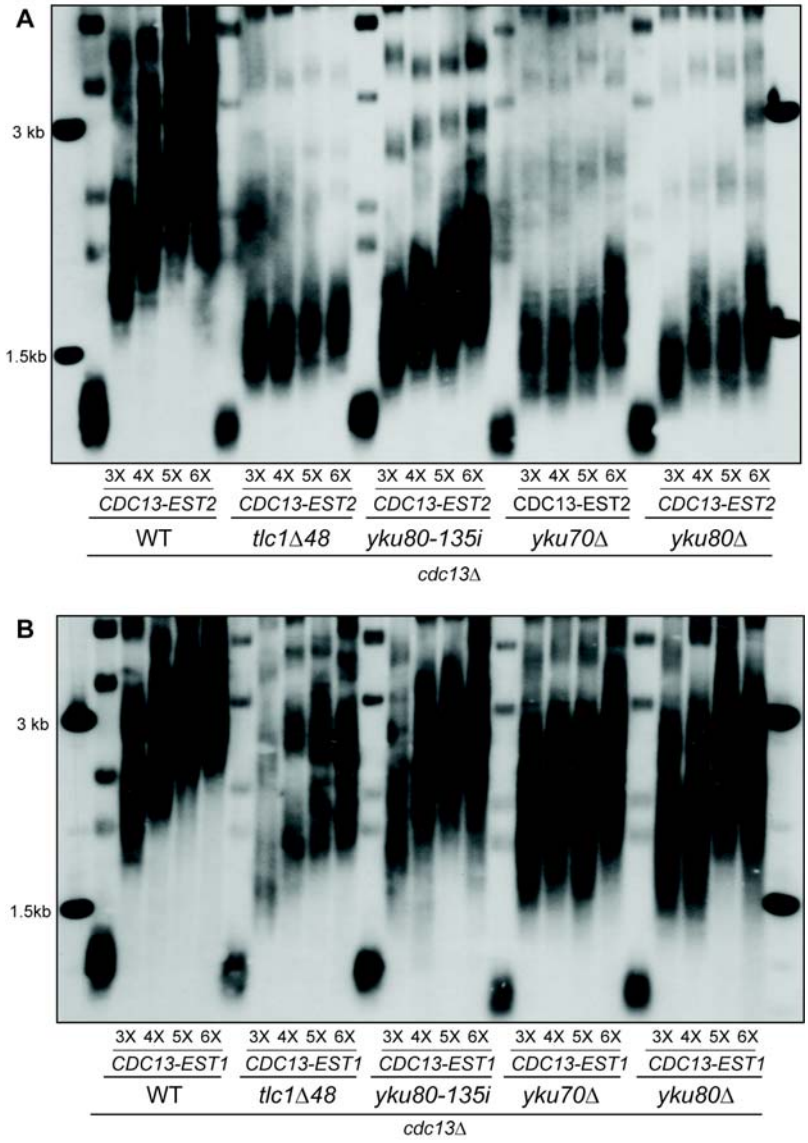

Figure S2 Effects of expression of a Cdc13-Est2 or Cdc13-Est1 fusion in *tlc1Δ48* or *yku70Δ* are similar to those observed in *yku80-135i* and *yku80Δ* strains, respectively. Telomere length analysis by Southern blot of XhoI digested DNA isolated from 3X-6X serial single colony streakouts of *cdc13Δ* (WT), *cdc13Δ tlcΔ48* (*tlcΔ48*), *cdc13Δ yku80-135i* (*yku80-135i*), *cdc13Δ yku70Δ* (*yku70Δ*), and *cdc13Δ yku80Δ* (*yku80Δ*) strains expressing a (A) Cdc13-Est2 or (B) Cdc13-Est1 fusion.

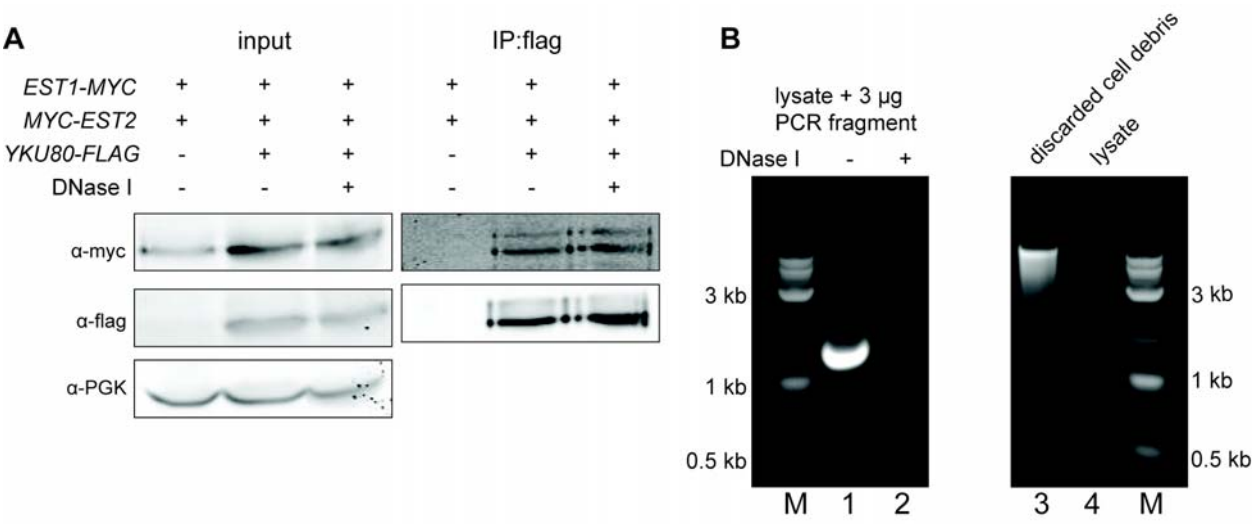

Figure S3 Treatment of lysates with DNase I does not affect the association of Est1 and Ku. (A) Independent co-immunoprecipitation experiment of Est1 and Est2 with Ku in the presence of DNase I confirming the results in Figure 5C. (B) A 1.2 kb PCR fragment was added to lysates and treated with DNase I under the same conditions as Figure 5C and S3A. DNA isolation and analysis by ethidium bromide stained agarose gel demonstrates conditions were sufficient to degrade 3 µg of DNA (lanes 1 and 2). The protein preparation used in these experiments resulted in no detectable genomic DNA shown in the lane marked 'lysate' (lane 4). Instead genomic DNA was present in the cell debris that was discarded during preparation of lysates (lane 3).