Supplemental Information

Figure Legends

Figure S1. Truncations within the Ring of Ku80 Impair Ku's Ability to Repair DNA Breaks and Silencing (Telomere Position Effect), Related to Figure 1. (A) Using a strain with an engineered HO endonuclease cut-site, the Ku mutants, along with Ku80WT and *ΔKu80*, were streaked onto plates containing either glucose or galactose. Galactose induces the production of HO endonuclease, triggering dsDNA break repair in cells containing functional Ku. The cells were plated at generations 40 and 200. (B) Generations 100 and 200 were analyzed for the yeast's ability to silence *URA* gene expression. (C) Generation 20 was analyzed for *ADE* gene expression. Different yeast strains were used in (A), (B) and (C), as described in Experimental Procedures.

Figure S2. Truncations within the Ring of Ku80 Do Not Impair TLC1 RNA Levels. (A) Representative Northern blot showing RNA isolated from generations 20 and 200 probed for TLC1 RNA and U1 snRNA. (B) Quantification of Northern data depicting the normalized total TLC1 RNA levels (TLC1 + TLC1-polyA) relative to WT (n = 5). (C) Quantification of rtPCR data showing the normalized TLC1 RNA levels relative to WT for generations 20 and 200 (n = 18). No substantial effect of Ku80 deletions on TLC1 RNA levels is apparent.

Figure S3. Protein and Nucleic Acid Substrates Used for Biochemical Studies, Related to Figures 3, 5, S5, S6, and S7. (A) Ku and Ku80Δ28 proteins were purified to homogeneity, analyzed by SDS-PAGE and stained with Coomassie blue. A similar amount of the Ku70 subunit co-purified with the His10-tagged Ku80Δ28 protein, indicating that the mutant subunit continued to form a Ku heterodimer. (B) dsDNA

substrates and (C) RNA substrates used in binding studies. For the TLC1-KBS RNA construct, nucleotides 286 to 332 of TLC1 (black) had additional dsRNA added to promote proper RNA folding; these sequences consisted of a region from TLC1's Ku Arm (red) and random-sequence dsRNA (blue). The 3xmutTLC1-KBS RNA mutations are highlighted in yellow.

Figure S4. Western Blot of Myc-tagged Ku80 Shows Equivalent Amounts of Protein Being Pulled Down for Both WT Ku and Ku80Δ28 in Immunoprecipitation Experiments, Related to Figure 4. Because cross-linked Ku appeared as a smear on a gel, an uncross-linked version of the experiment was performed to compare the pulled-down protein levels via western blot. First lane contains purified Ku and second lane contains molecular weight markers (MW).

Figure S5. Off-rate Determination, Related to Figure 3 and 5. (A) Set of gels used to measure the off-rate of Ku for different dsDNAs. In the left panel, the curvature of the bands is due to the gel being loaded at various time points while running, and the wedge represents increasing time from 0 to 1000 min. Similarly in the center panel, the off-set of bands between 2 and 3 hr is due to the gel continuing to run while the incubation was progressing. PM denotes pre-mix control (radiolabeled DNA and unlabeled chase DNA were pre-mixed prior to adding Ku). o/n stands for overnight time point. B represents the bound dsDNA. F denotes free dsDNA. (B) The fraction bound was quantified and fitted to an exponential dissociation equation to obtain the dissociation rate constant. (C) The off-rate for TLC1-KBS RNA was determined in a similar fashion as the dsDNA. In the sample gel shown, the wedge represents increasing time from 0 to 10 min. (D) Quantification of TLC1-KBS RNA dissociation.

Figure S6. TLC1 RNA Competes Against DNA to Bind Ku, Related to Figure 5. (A) Sample set of gels used in the competition experiment with TLC1-KBS RNA competing against dsDNA for an interaction with Ku. (B) and (C) depict similar competition experiments, but 3xmutTLC1-KBS RNA and the unrelated *Tetrahymena* P4P6 RNA, respectively, are used instead of TLC1-KBS RNA. The (PM) symbol denotes the Pre-mix control. The wedges represent increasing RNA concentrations from 0 to 1 mM. (D) Sample set of gels used in the competition experiment with TLC1-KBS RNA competing against dsDNA for an interaction with Ku. The (PM) symbol denotes the Pre-mix control. Wedge represents increasing DNA concentrations from 0 to 1 mM. (E) Sample quantification of data.

Figure S7. Separation-of-Function Ku70 Mutants Still Retain Wild-Type Affinity for DNA and RNA, Related to Figure 6. (A) Ku70 D195R and Ku70 D195A proteins were purified to homogeneity, analyzed by SDS-PAGE and stained with Coomassie blue. A similar amount of the 2MYC-10His tagged Ku80 subunit co-purified with the Ku 70 mutants protein, indicating that the mutant subunit continued to form a Ku heterodimer and migrated similarly to Ku WT. (B) Affinity binding measurements reveal that the Ku70 D195R and Ku70 D195A mutations do not affect Ku's affinity for either DNA (Telomere 3'overhang dsDNA) or for RNA (TLC1-KBS RNA). The K_d for Ku70 D195R binding to DNA is 0.17 ± 0.15 nM and 14.4 ± 4.9 nM for RNA. The K_d for Ku70 D195A for DNA is 0.07 ± 0.04 nM and 20.2 ± 0.8 nM for RNA.

Sup. Table 1. Plasmids used or generated for experiments.

Plasmid name	Description	Source
pVL1037	*CEN TRP1 KU80*	Gift from A. Bertuch
pRS414	*CEN TRP1*	Gift from A. Bertuch
pJP1	*CEN TRP1 ku80Δ4*	pVL1037
pJP2	*CEN TRP1 ku80Δ12*	pVL1037
pJP3	*CEN TRP1 ku80Δ20*	pVL1037
pJP4	*CEN TRP1 ku80Δ28*	pVL1037
pJP5	*CEN TRP1 ku80Δ36*	pVL1037
pJP6	*CEN TRP1 ku80Δ40*	pVL1037
pJP7	*CEN TRP1 KU80*-4Gly-2Myc-10His	pVL1037
pJP8	*CEN TRP1 ku80Δ4*-4Gly-2Myc-10His	pJP1
pJP9	*CEN TRP1 ku80Δ12*-4Gly-2Myc-10His	pJP2
pJP10	*CEN TRP1 ku80Δ20*-4Gly-2Myc-10His	pJP3
pJP11	*CEN TRP1 ku80Δ28*-4Gly-2Myc-10His	pJP4
pJP12	*CEN TRP1 ku80Δ36*-4Gly-2Myc-10His	pJP5
pJP13	*CEN TRP1 ku80Δ40*-4Gly-2Myc-10His	pJP6
pRS423TEF-*YKU80*-TAP	*TRP1* TEF-*KU80*-TAP	Gift from D. Gottschling
pRS425TEF-*YKU70*	*LEU* TEF-*KU70*	Gift from D. Gottschling[1]
pJP14	*TRP1* TEF-ku80-4Gly-2Myc-10His	pRS423TEF-*YKU80*-TAP
pJP15	*TRP1* TEF- *ku80Δ28*-4Gly-2Myc-10His	pRS423TEF-*YKU80*-TAP
pJP16	*LEU* TEF-*KU70*	pRS425TEF-*YKU70*[1]
pJP17	T7-TLC1-KBS RNA	
pJP18	T7-3xmutTLC1-KBS RNA	pJP17
pJP19	*LEU* TEF-*KU70 D195R*	pJP16
pJP20	*LEU* TEF-*KU70 D195A*	pJP16

1. pRS425TEF-*YKU70* contained the point mutation or polymorphism D473G. This point mutation was tested in all assays conducted with purified Ku and performed similarly to the corrected Ku. Figure 4 and S6 panel C contains data that was acquired with the Ku70 from the pRS425TEF-*YKU70* plasmid.

Sup. Table 2. Strains used in experiments.

Strain	Genotype	Source
YVL885	*MAT***a** *yku80Δ::kanr ura3-52 lys2-801 trp1Δ63 his3Δ200 leu2Δ1 ppr1::HIS3 adh4::URA3*-(*URA3* at TEL VIIL) DIA5-1 (*ADE2* at TEL VR)	Gift from A. Bertuch
YVL2236	*MAT*α *yku80Δ::kanr tlc1Δ::LEU2 hmlΔ::ADE1 hmrΔ::ADE1 ADE1 lys5 leu2 ura3-52 trp1Δ::hisG ADE3::GAL::HO* pSD120(*CEN URA3 TLC1*)	Gift from A. Bertuch
BJ2168	*MAT***a** *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407gal2*	Gift from D. Gottschling

Extended Experimental Procedures

Western Blots

Liquid cultures grew overnight at 30°C in the appropriate drop-out liquid media. An OD_{600} was taken for each culture and the cell concentration was calculated. Appropriate amounts of the liquid cultures were taken to ensure that at all the samples contained equal numbers of cells in the volume of 800 µl of liquid culture. The protocol followed that of (Knop et al., 1999) except that the cells were in media, not water; volumes were adjusted for 800 µl; TCA was 50% and made fresh; incubations were for 10 min and samples were pelleted for 3 min, washed with 1 ml of cold acetone and pelleted again.

After the lysis of the cells, the resulting pellets were resuspended in 4x NuPAGE® LDS Sample Buffer (Invitrogen NP0008) with 4% BME. After heating at 85°C for a few minutes, the samples were then loaded onto a NuPAGE® 4-12% Bis Tris Gel. After electrophoresis, the proteins were transferred to Amersham Hybond™ ECL™(GE RPN78D) membrane using western transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol) for 45 min at 0.5 Amps in a Bio-Rad Mini Trans-Blot® apparatus.

Real-time PCR

Using the total RNA samples prepared for Northern blots, the levels of TLC1 and ACT1 were measured via real-time RT-PCR. Preparation of the standard RNAs was performed as described in (Mozdy and Cech, 2006). The primers used were based on those from (Mozdy and Cech, 2006); TLC1 template: 38&39 ccatgggaagcctaccat, ttagcaaagtttgcacgagttc (IDT) and ACT1 exon: 32&33 ctggtatgtgtaaagccggt, tgggtcaaaaagactcctacgt (IDT). cDNAs were synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems , #4368813), following the manufacturer's protocol and using 1µg of total RNA in 10 µl of water. The reactions incubated for 2 hours at 37 °C, and were then heat inactivated at 75 °C for 10 min. The samples were then diluted 2 fold and used in real-time PCR reactions using iQ™ SYBR® Green Supermix (Bio-Rad#170-8882). Reaction set up was: 10 µl of the Supermix, 1 µl of 2 µM primer stock containing both forward and reverse primers, 2 µl of the cDNA and 7 µl of water for a total of 20 µl. In the same 96 well plate, duplicates were performed for each sample. Three replicates were performed for each biological sample for a n = 18. A LightCycler 480 (Roche) was used to perform the rt-PCR. The run primer efficiencies were calculated as described in (Pfaffl, 2001) using the standard

RNAs. The primer efficiencies were used to calculate the ratio of TLC1 to ACT1. The ratios were normalized to WT.

Determination of Off-rates

To determine the off-rate for dsDNA, 2 nM Ku was incubated with 1 nM end-labeled dsDNA in the buffer [21 mM HEPES pH 7.5, 150 mM NaCl, 11% glycerol, 5 mM $MgCl_2$, 25 □g/mL tRNA, 0.1 mg/ml BSA, 1 mM DTT] for about 1 hour. Then cold dsDNA in 10 mM Tris pH 8, 50 mM NaCl, 0.1 mM EDTA was added to the final concentration of 1 µM and samples of the reaction were loaded onto a running native 5% acrylamide gel and onto a running native 4-20 % acrylamide gel (Invitrogen) at varying time points. A premix control was included in which the Ku had equal opportunity to interact with the cold and labeled dsDNA. The gels were dried and exposed to phosphorimager screens. After the scanning, the bands were measured using Imagequant TL. The fraction bound was fitted to an exponential dissociation equation to yield the k_{off}.
For the determination of the RNA off-rate, a protocol similar to that described above was used, but with a few adjustments. 143 nM Ku was incubated with 20 nM TLC1-KBS RNA, which contained trace amounts of radiolabeled RNA. To fold the RNA, the RNA was diluted into storage buffer [25 mM Tris pH 8, 200 mM NaCl, 20% glycerol, 2 mM DTT and 1 mM EDTA], heated to 85 ºC for 60 s, and snap cooled on ice for 15 min. After the RNA and Ku incubated for an hour, the cold TLC1-KBS RNA was added. From this point, the samples were treated the same as the dsDNA samples.

Mixing Assay

To assay for Ku's ability to bind both dsDNA and RNA concurrently, a mixing assay was employed. 144 nM Ku had equal opportunity to interact with 50 nM dsDNA and 50 nM

TLC1-KBS RNA in the reaction buffer [21 mM Hepes pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 16% glycerol, 7 mM Tris pH 8, 0.77 mM EDTA, 25 µg/ml tRNA, 0.1 mg/ml BSA, 1.4 mM DTT]. The RNA was folded as previously mentioned. The RNA and dsDNA samples were spiked with end-labeled versions. Samples of just dsDNA, RNA, dsDNA plus Ku, and RNA and Ku were included to compare migrations and amount of substrate bound by Ku. The samples were allowed to incubate for a period of time that was at least five times greater than the slowest half-life of the DNA-Ku interaction, which would be enough time for the samples to come to equilibrium. In this case, the samples incubated for 15-16 hours at room temperature. The samples were run on a native 5% acrylamide TBE gel, which ran at 12 watts at 4 ^{o}C. The gel was dried and processed as described above.

Competition Assay

To assay the TLC1-KBS RNA's ability to compete off dsDNA from Ku, 1 nM end-labeled dsDNA was incubated with 2 nM Ku in the buffer [21 mM HEPES pH 7.5, 6.5 mM TRIS pH 8, 150 mM NaCl, 16% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 25 µg/ml tRNA, 0.1 mg/ml BSA, 1 mM DTT]. Increasing amounts of TLC1-KBS RNA, 3xmutTLC1-KBS RNA, or P4P6 were added to the dsDNA plus Ku samples. To fold the RNA, the RNA was diluted into storage buffer [25 mM Tris pH8, 200 mM NaCl, 20% glycerol, 2 mM DTT and 1 mM EDTA], heated to 85 ºC for 60 s, and snap cooled on ice for 15 min. The RNA was folded at a 1 µM concentration and concentrated to ~10 µM using a 10, 000 MWCO Amicon Ultra. The samples were incubated at room temperature for 15-16 h, which was enough time for the samples to come to equilibrium. This statement is based on comparisons with samples containing the maximum amount of RNA and the pre-mix controls, which contained both DNA and the maximum amount of RNA prior to

the addition of Ku. The fraction bound was similar to one another in these samples, which indicates equilibrium being reached. The samples were run on a native 4-20% acrylamide gel (Invitrogen). The gels were processed as described above. The calculated fraction bound was fitted to the Linn and Riggs equation to ascertain the K_I for the RNA.

To assay the ability of dsDNA to compete off TLC1-KBS RNA, 143 µM Ku was combined with 20 µM TLC1-KBS RNA, which contained trace amount of end-labeled RNA. The RNA was folded as previously described. Increasing amounts of dsDNA were added to the samples. The samples incubated for approximately 15 to 16 h at room temperature in the following buffer [21 mM HEPES pH 7.5, 2.5 mM TRIS pH 8, 150 mM NaCl, 11% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 25 µg/ml tRNA, 0.1 mg/ml BSA, 1 mM DTT]. The samples were processed and analyzed as previously described.

Determination of Equilibrium Constants and Type of Inhibition

For the determination of the equilibrium dissociation constant, trace levels of end-labeled TLC1-KBS RNA (pre-folded as described above) or dsDNA were incubated with increasing amounts of Ku at room temperature for 15-16 h. The buffer used for binding was [21 mM HEPES pH 7.5, 6 mM TRIS pH 8, 150 mM NaCl, 16% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 25 µg/ml tRNA, 0.1 mg/ml BSA, 1 mM DTT]. The samples were run on a native 4-20% acrylamide TBE gel (Invitrogen). The gels were processed as described above. The calculated fraction bound was fitted to the Langmuir isotherm to acquire K_d.

The preceding protocol was also used for determining the type of inhibition, except either 100 nM TLC1-KBS RNA or 3xmutTLC1-KBS RNA was included in the samples. The radiolabeled telomeric 3'overhang dsDNA served as the substrate.

Immunoprecipitation of TLC1-Ku Complexes

Protocol incorporated methods from Fisher et al. (2004) and Lopez et al. (2011). Yeast expressing myc-tagged Ku (100 ml) were treated with formaldehyde (1% final). Cells were washed, treated with zymolyase, and protease inhibitors (Roche, Complete EDTA free) added. After sonication (Bioruptor UCD-200, Diagenode), insoluble debris was removed by centrifugation. After adding Rnasin (Promega), SuperRnasin (Ambion) and Tween-20 to 0.5%, anti-myc antibody (Sigma M4439) was added to half of each sample. The other half comprised the "minus antibody" control. Following nutation at 4^{o}C overnight, complexes were pulled-down on Protein G Plus/Protein A Agarose (Calbiochem) beads, then treated with proteinase K, chloroform extraction, and RNeasy (Qiagen) RNA purification followed by real-time RT-PCR.

REFERENCES

Fisher, T.S., Taggart, A.K., and Zakian, V.A. (2004). Cell cycle-dependent regulation of yeast telomerase by Ku. Nat Struct Mol Biol *11*, 1198-1205.

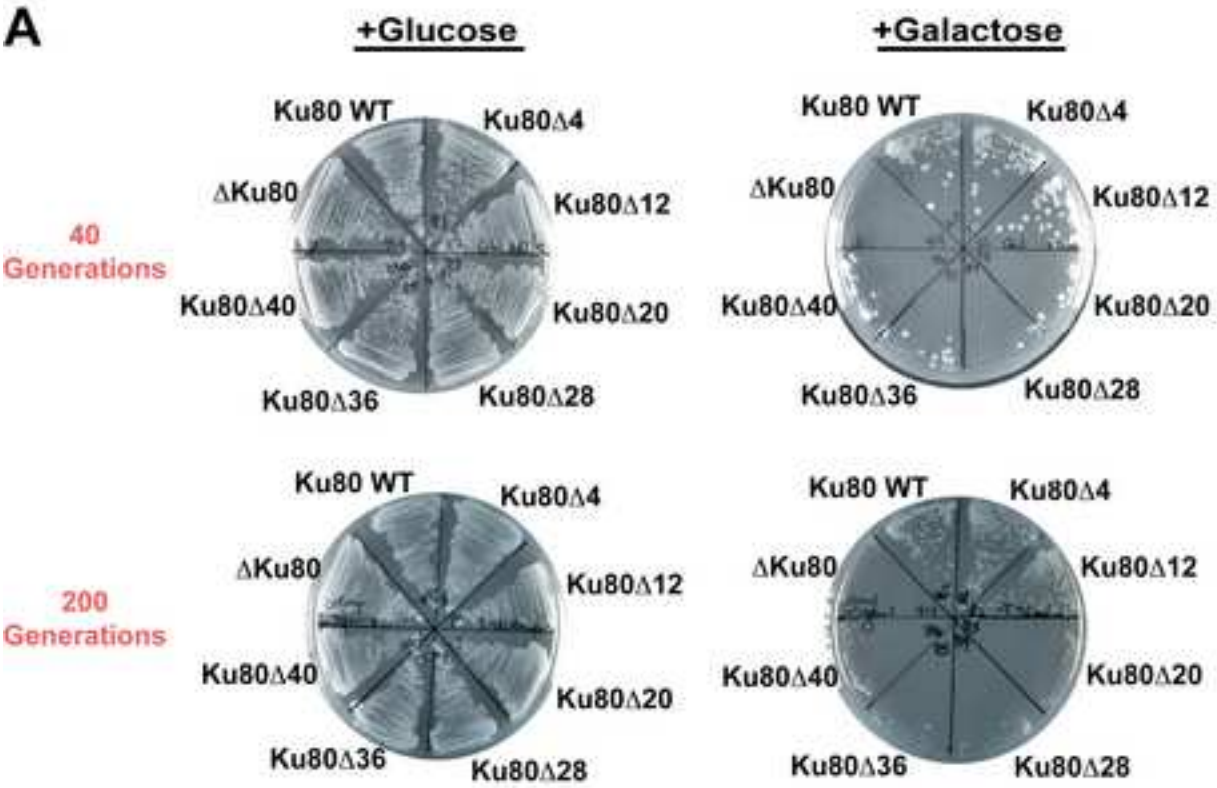
Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963-972.

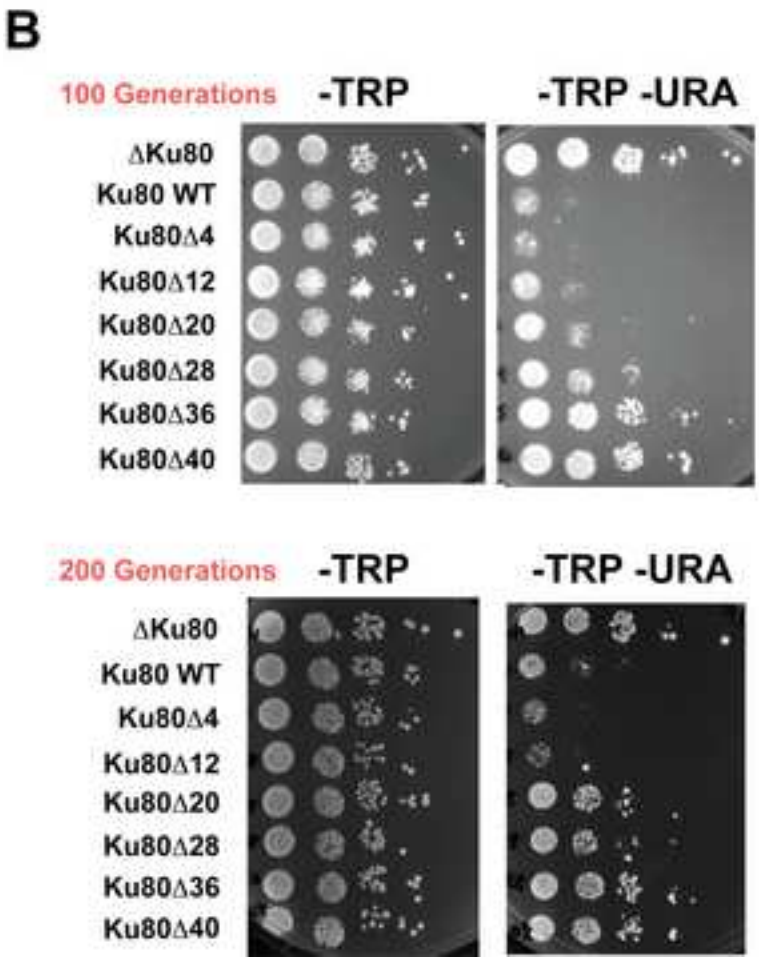
Lopez, C.R., Ribes-Zamora, A., Indiviglio, S.M., Williams, C.L., Haricharan, S., and Bertuch, A.A. (2011). Ku must load directly onto the chromosome end in order to mediate its telomeric functions. PLoS Genet. *7*, e1002233.

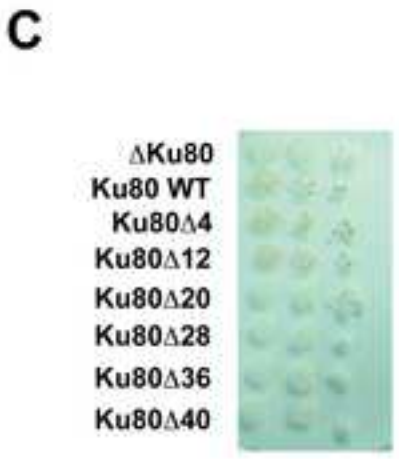
Mozdy, A.D., and Cech, T.R. (2006). Low abundance of telomerase in yeast: implications for telomerase haploinsufficiency. Rna *12*, 1721-1737.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29*, e45.

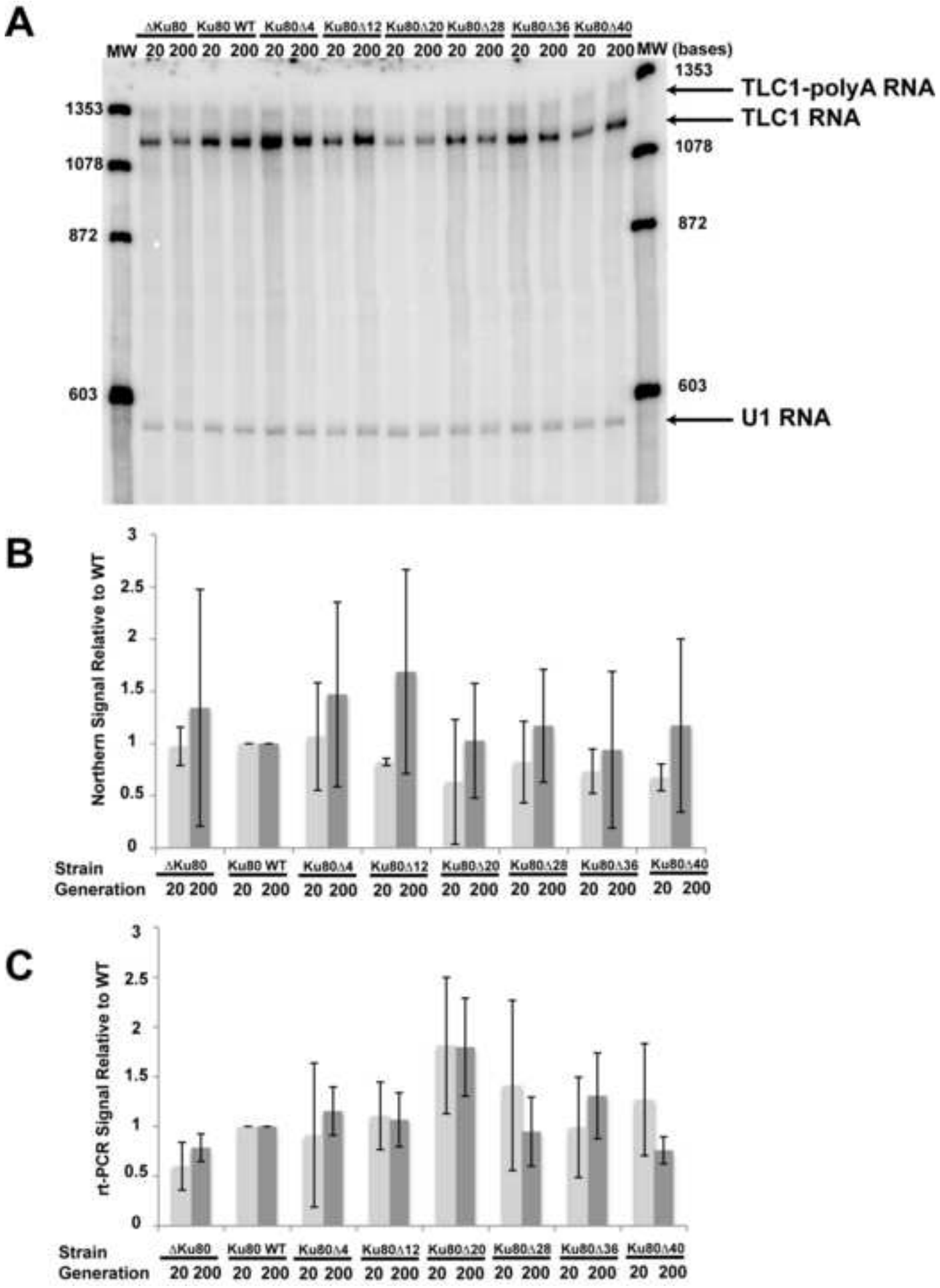
Supplemental Figure 1



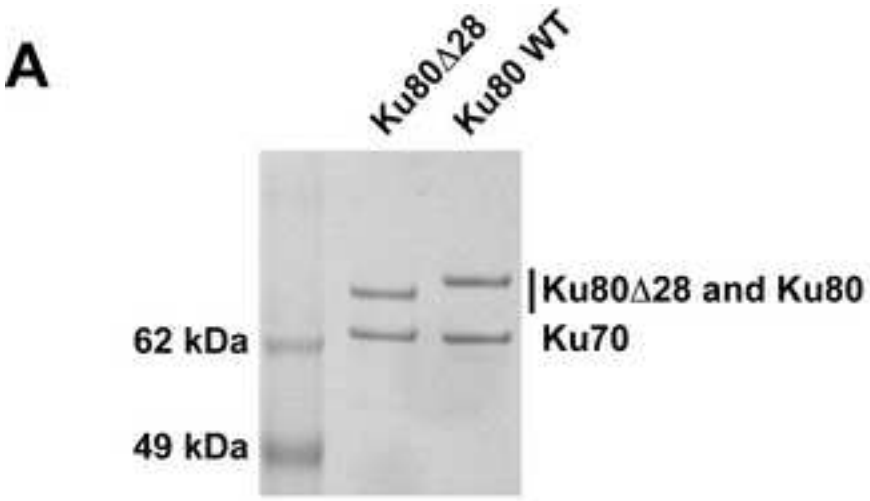




Supplemental Figure 2



Supplemental Figure 3

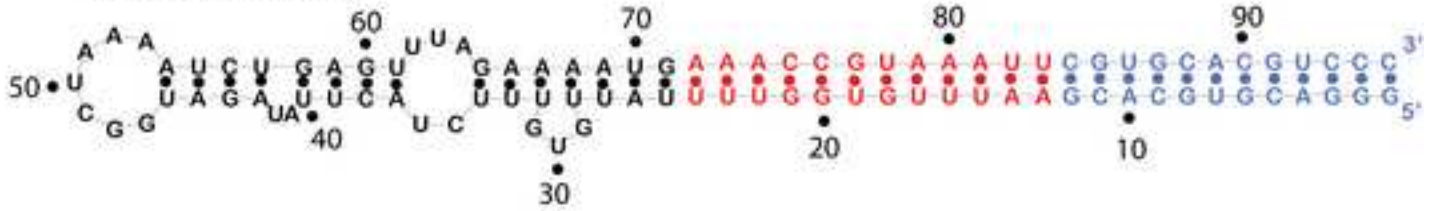


B

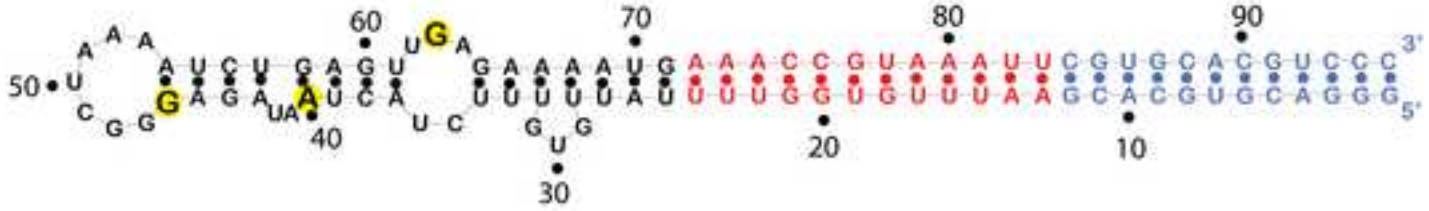
Random 3' overhang	5'	ggacaaaagactatcgacatttgatagaaatatcaatgg 3'
	3'	cctgttttctgatagctgtaaa 5'
Blunt	5'	ggacaaaagactatcgacattt 3'
	3'	cctgttttctgatagctgtaaa 5'
Telomeric 3'overhang	5'	ggacaaaagactatcgacatttgggtgtggggtgtg 3'
	3'	cctgttttctgatagctgtaaa 5'

C

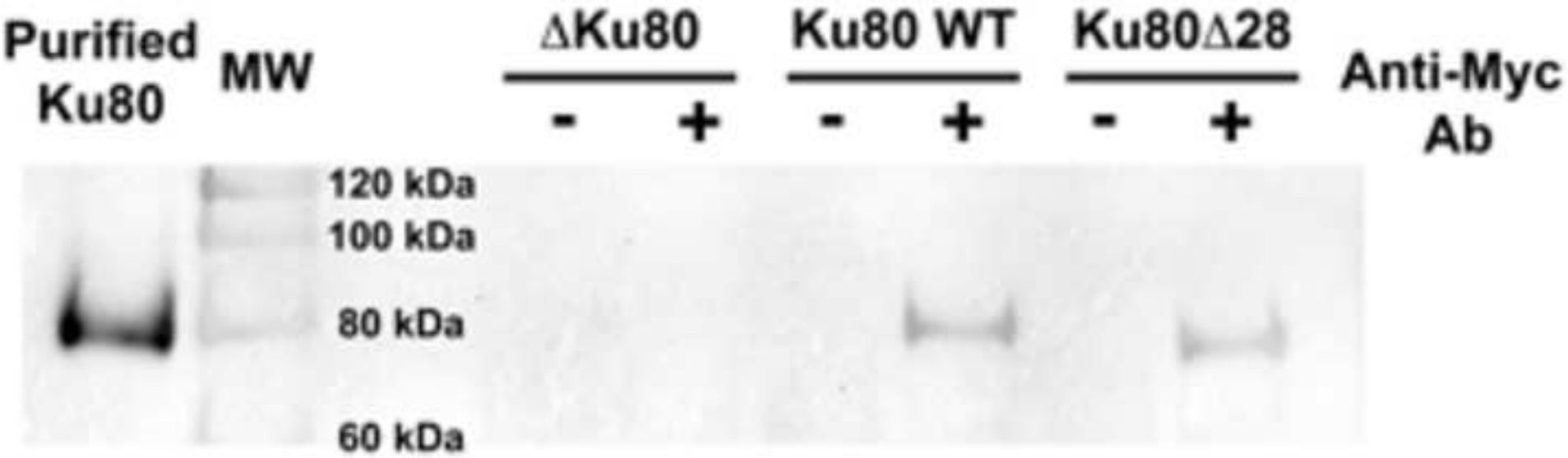
TLC1-KBS RNA



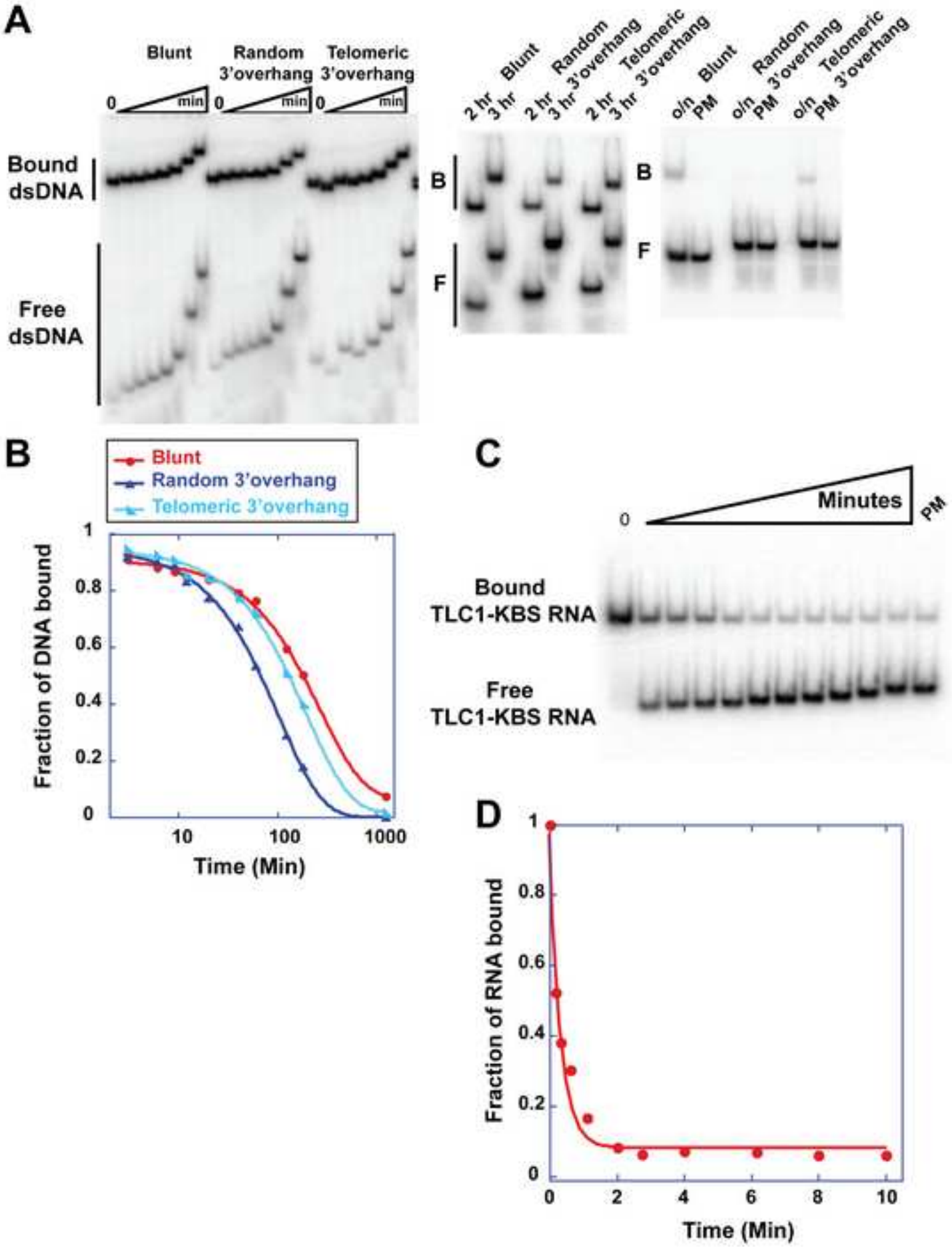
3xmutTLC1-KBS RNA



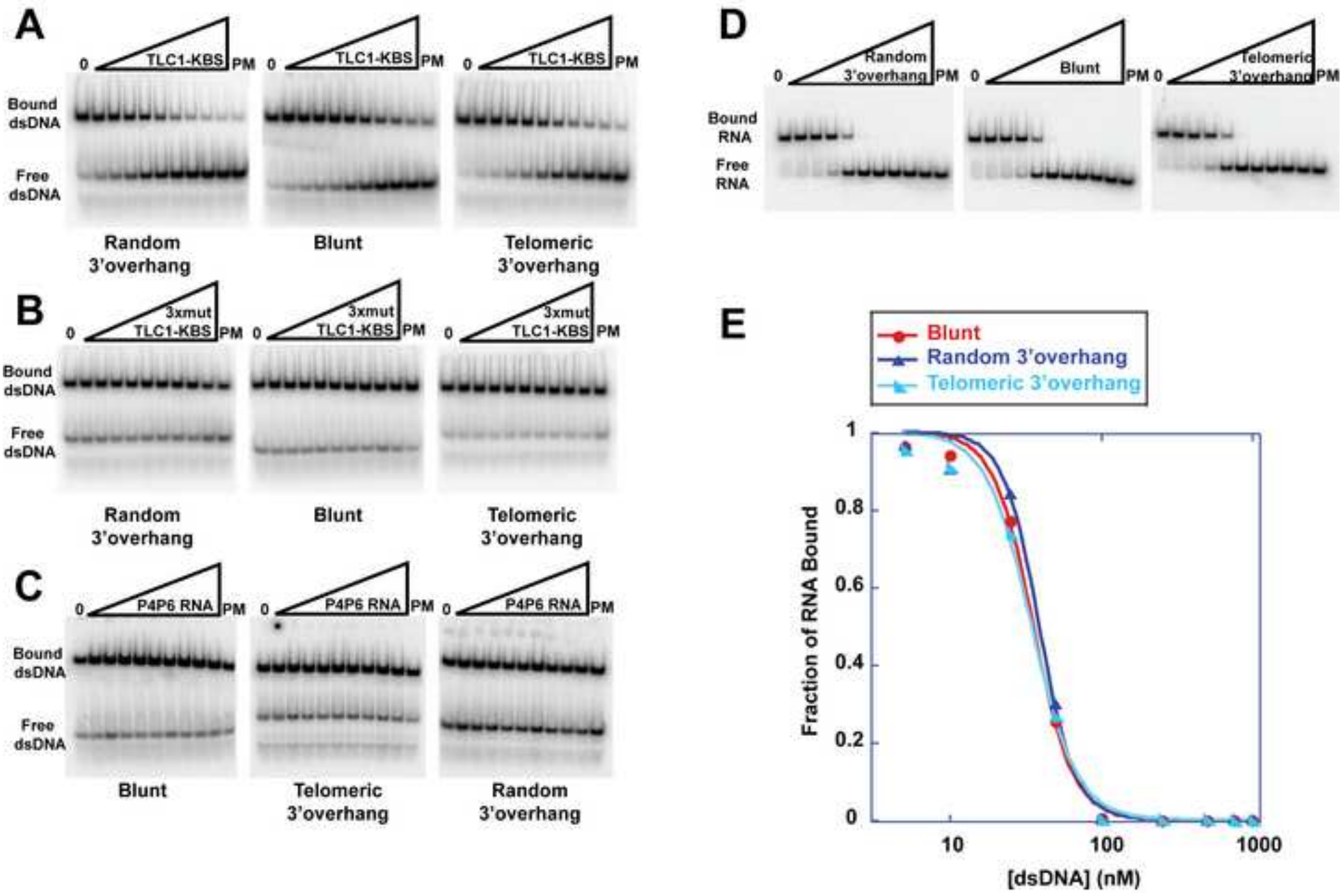
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7