Zeng et al. "The accumulation and not the specific activity of telomerase ribonucleoprotein determines telomere maintenance deficiency in X-linked dyskeratosis congenita "

Supplementary Methods

Protein measurement by Western blot

Whole cell extracts (50 µg) were resolved in non-continuous tris-glycine SDS gels. Following PAGE, the resolved protein samples were transferred to PVDF membranes (GE HealthCare Life Sciences). PVDF membranes were incubated with the dyskerin polyclonal antibodies (Santa Cruz) and control beta-actin monoclonal antibodies (Sigma). Protein signals were detected using chemiluminescence, with the ECL Western blotting kit (GE Healthcare Life Sciences). Densitometry and the Image J software (NIH) were used to quantify protein signal.

28S and 18S rRNA quantification

Total RNA was isolated from X-DC cells with TriZol Reagents (Invitrogen) according to the manufacturer's protocol. Total RNA (1 µg) from each X-DC cell line was resolved by 1.2% denaturing agarose gel electrophoresis, in 1xMOPS buffer. Following electrophoresis, separated RNAs were visualized by staining with SybrGreen dye according to the manufacturer's protocol (Molecular Probes/Invitrogen). Typhoon Imager (GE Healthcare LifeSciences), fitted with appropriate filters and laser, captured the fluorescent images. 28S and 18S rRNA signals were quantified using ImageQuant software (GE Healthcare LifeSciences).

Stable expression of 3xFlag dyskerin in 293HEK cells

WT-3xFlag dyskerin was obtained from Kathleen Collins' laboratory. X-DC-associated dyskerins were generated with DpnI site-directed mutagenesis and sequenced to confirm cloning fidelity. A second round of DpnI site-directed mutagenesis was used to introduce silent mutations to dyskerin codon 440-450 in all recombinant 3xFlag dyskerin expression vectors.

These silent mutations render the recombinant dyskerin mRNA insensitive to the subsequent shRNA knock-down, and create a novel EaeI restriction site for identification. Fidelity of cloning was confirmed with direct sequencing of the entire coding sequence.

Forty-eight hours following calcium phosphate mediated plasmid transfection of 293HEK cells, stable integrations of plasmid vector were selected for by neomycin treatment for seven days. Expressions of recombinant 3xFlag dyskerins were confirmed by Western blot analysis (Figure S4A).

Transient knockdown of endogenous dyskerin in 293HEK cells stably expressing 3xFlag dyskerins

Transient transfection of shRNA vector against the coding sequence of dyskerin (nt 1342-1363; anti-sense 5'-TTTCACTCTCACTCTCTCGCTT-3') was mediated by the calcium phosphate method. siRNA knock-down of endogenous dyskerin was confirmed by Western blot analysis (Supplementary Figure 4A).

Immunoprecipitation of the telomerase holoenzyme complex (Supplementary Figure 4B), measurement of telomerase activity by TRAP, and normalization with TER copy number by QRT-PCR (Supplementary Figure 4A and B) were completed as described in the main text.

Supplementary Figure Legend

Supplementary Figure 1. Schematic of the dyskerin protein showing the locations of known X-DC associated mutations.

There are more than 30 known dyskerin mutations associated with X-DC and the related Hoyeraal-Hreidarsson syndrome. (Associated dyskerin mutations are shown in bold.) There are two mutational hotspots for X-DC, the N-terminal hotspot near the TruB catalytic domain, and a second hotspot located at the PUA RNA binding domain in the C-terminal.

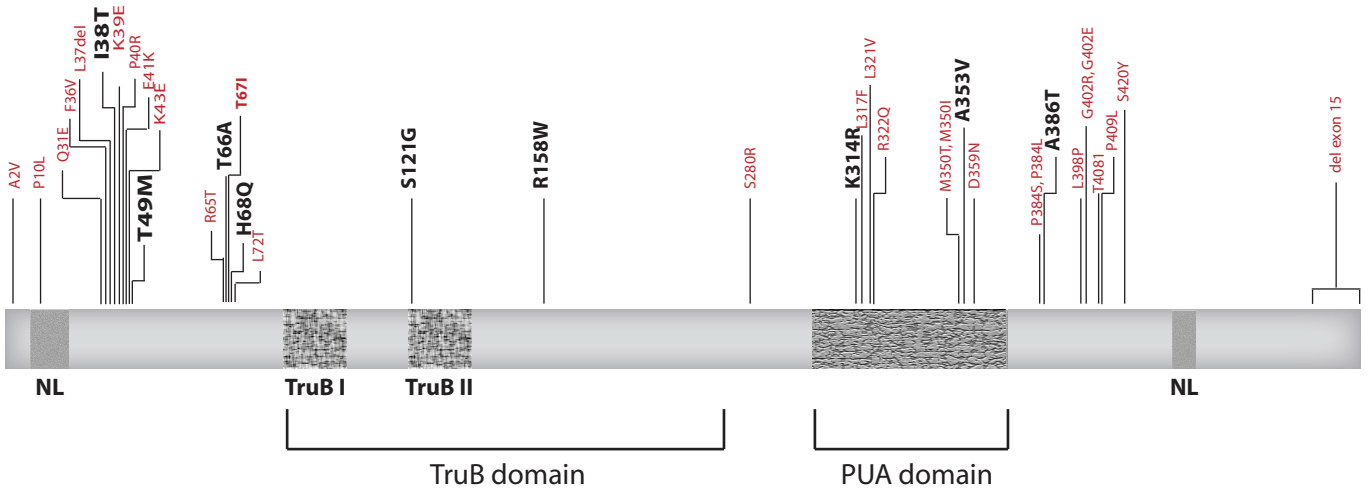
Supplementary Figure 2. Dyskerin protein levels did not differ substantially between X-DC isoforms. Expression levels of endogenous dyskerin protein were analysed by Western blotting. Dyskerin protein signals were quantified with Image J and normalized to the expression levels of beta-actin. Expression ratios of these two proteins were compared to those obtained from the wildtype sample, and this normalized value for each X-DC cell line was indicated below the respective lane. Protein quantification experiments were repeated two times with similar results (data not shown).

Supplementary Figure 3. Steady state levels of 28S and 18S ribosomal RNAs did not substantially differ between X-DC isoforms. Total RNA from each X-DC cell line was separated by denaturing agarose gel electrophoresis and stained with SybrGreen dye (A). Fluorescent signals for 28S and 18S rRNA were measured with ImageQuant software. 28S to 18S rRNA ratios were about 2 in each case, suggesting lack of degradation and generally good integrity of the isolated total RNA. There were no observable differences in rRNA quantity and quality between X-DC isoforms. Experiments were repeated three times and quantified results are shown in (B).

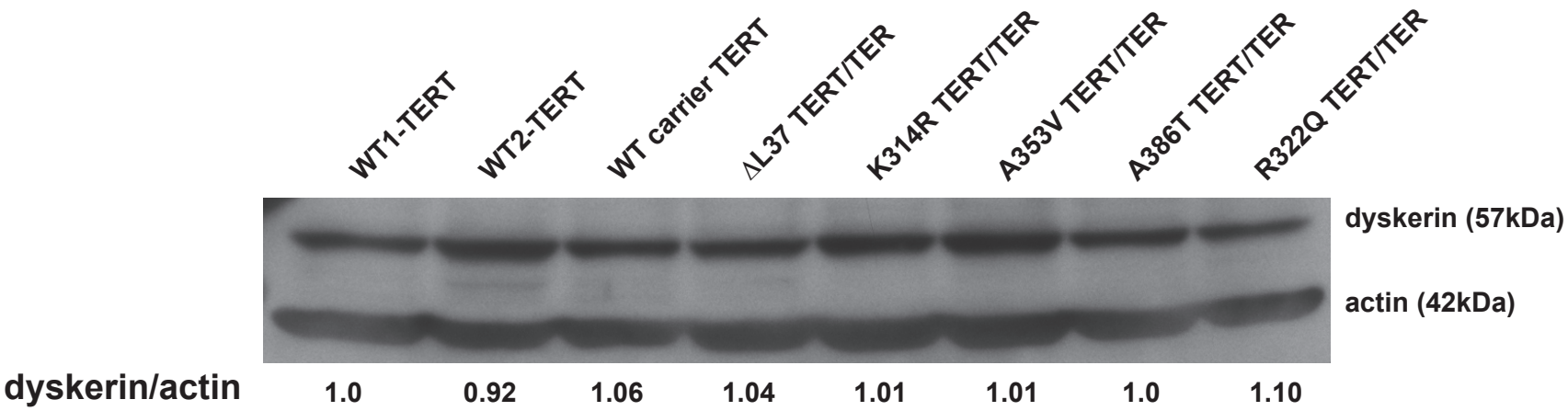
Supplementary Figure 4. WT or X-DC dyskerin-associated telomerase holoenzymes have compariable TRAP activity profiles. Generation of 293HEK cells stably expressing a dyskerin shRNA-insensitive form of recombinant WT or X-DC dyskerin (A). Western blot analysis of whole cell extracts of 293HEK cells stably expressing various 3xFlag dyskerin isoforms, before and after transient (48hr) knockdown of endogenous dyskerin expression with an shRNA vector. Corresponding steady-state TER levels by QRT-PCR following endogenous dyskerin knockdown are also shown. TRAP activity profiles of immunoprecipitated WT or X-DC dyskerin-associated telomerase holoenzyme complex. Corresponding TER copy numbers isolated from

each immunoprecipitation eluate are indicated below the gel image (B). The whole experiment was repeated once with similar results.

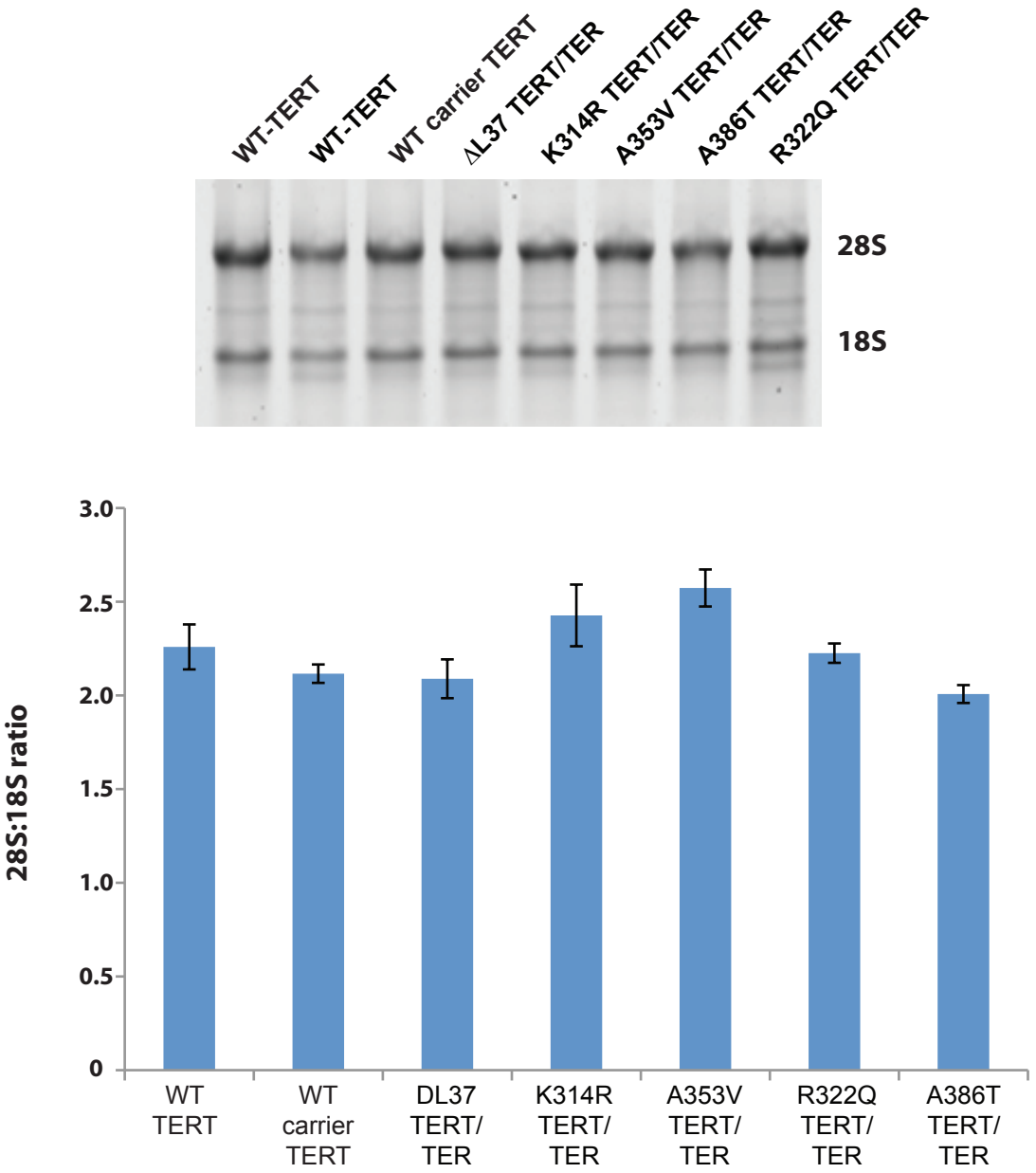
Supplementary Figure 1



Supplementary Figure 2

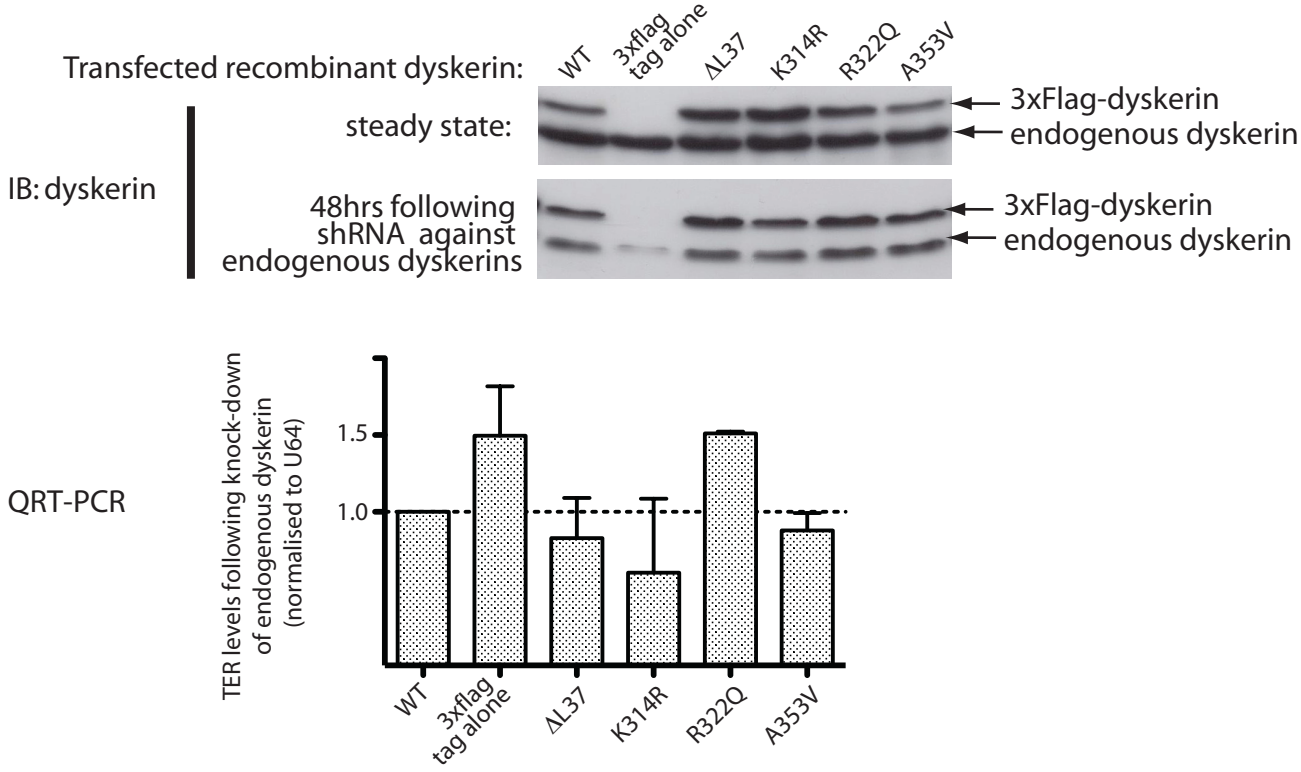


Supplementary Figure 3



Supplementary Figure 4

A. 293HEK+3xFlag-dyskerin cell models



B. Immunoprecipitation of Telomerase Holoenzyme Complex