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EXTENDED EXPERIMENTAL PROCEDURES

Fluorescent PCA

PCAs for Figure S1A were performed as described in the main text with a few exceptions. Briefly, 293T cells were co-transfected with 500 ng of each indicated Venus plasmid and either 100 or 400ng DsRed-TRF2 for V[1]-TRF1/V[2]-Ku70 and V[1]-TRF2/V[2]-Rap1, respectively. The cells were fixed at 24 hr post-transfection in 4% paraformaldehyde and mounted in DAPI containing Vectashield mounting medium (Vector Laboratories). Images were taken at 100X magnification using DeltaVision (Deconvolution) Image Restoration Microscope.

Clonogenic Survival Assay

Plates containing 2.5×10^6 *Ku70*$^{-/-}$ MEF cells were co-transfected with 400 ng of a GFP containing plasmid and 4 μg of either empty plasmid or a plasmid containing Ku70 or Ku70$^{D192R/D195R}$. After 48 hr, cells were sorted by FACS and for each co-transfection, 12 plates were seeded with 3×10^3 GFP positive cells. Six hours after plating, three plates at a time received 2 Gy, 1 Gy, 0.5 Gy or no irradiation. Colonies were visualized with crystal violet and counted one week after irradiation.

Yeast Western Blots

Yeast extracts were prepared using glass beads with TMG-200 (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 10% glycerol, 0.1 mM DTT, 0.1 mM EDTA plus 200 mM NaCl). Lysates (50 μg) were resolved on 10% SDS-PAGE gels and subjected to immunoblot analysis using an anti-HA monoclonal antibody (BioVision, clone 12CA5), anti-LexA polyclonal antibody (Abcam) or anti-PGK antibody (Molecular Probes) and IRDye 800CQ conjugated secondary antibodies (Li-Cor). Fluorescence was visualized using the Li-Cor Odyssey Infrared Imaging System. The membrane was stripped using 0.1 M NaOH and reprobed.

Coimmunoprecipitation Assay

Co-immunoprecipitations were performed as described in the main text with the exception of using 600 μg WCE of 293T cells that were co-transfected with Myc-Ku70 and FLAG-Ku80 plasmids for Figure S4A. This immunoprecipitation was performed using 6 μg α-Myc antibody (Sigma) and 100 μl Protein G Plus-Agarose beads (Calbiochem). Western blots were performed as described in the main text.

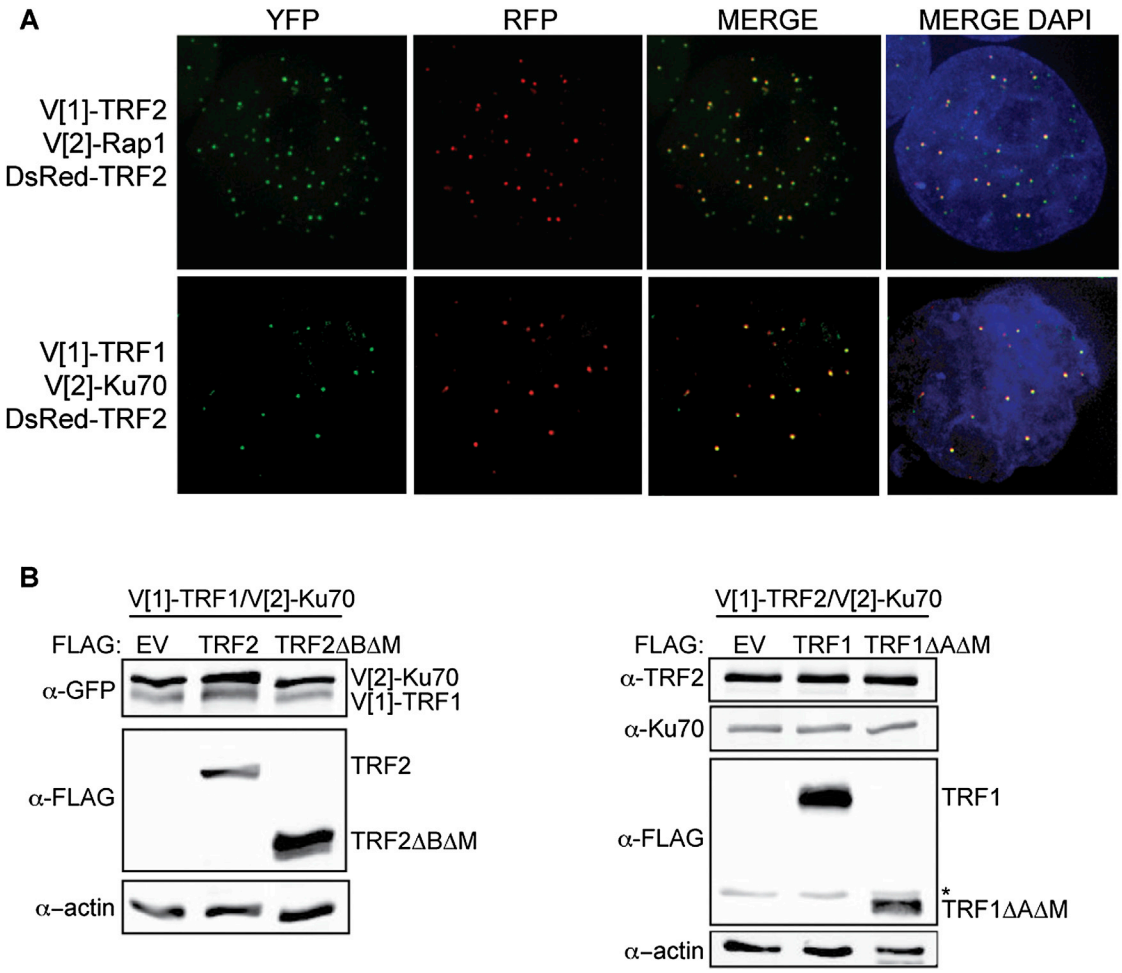

Figure S1. TRF2-Rap1 PCA Interaction Colocalizes to Telomeres, Related to Figure 1

(A) Co-localization of PCA (YFP channel) for TRF2 and Rap1 (top), and Ku70 and TRF1 (bottom) with the DsRed-TRF2 telomeric marker (merge). Cells were fixed and visualized 24 hr post-transfection at 100X magnification.

(B) Protein levels resulting from transfections in Figure 1C as detected by immunoblot analysis of WCE using the indicated antibodies. β-actin was used as a loading control. Note: primary antibodies were used for the immunoblot on the right to visualize the proteins as the V[1]-TRF2 and V[2]-Ku70 tagged proteins migrated at an equivalent position. * denotes β-actin band bleed over during secondary antibody probing.

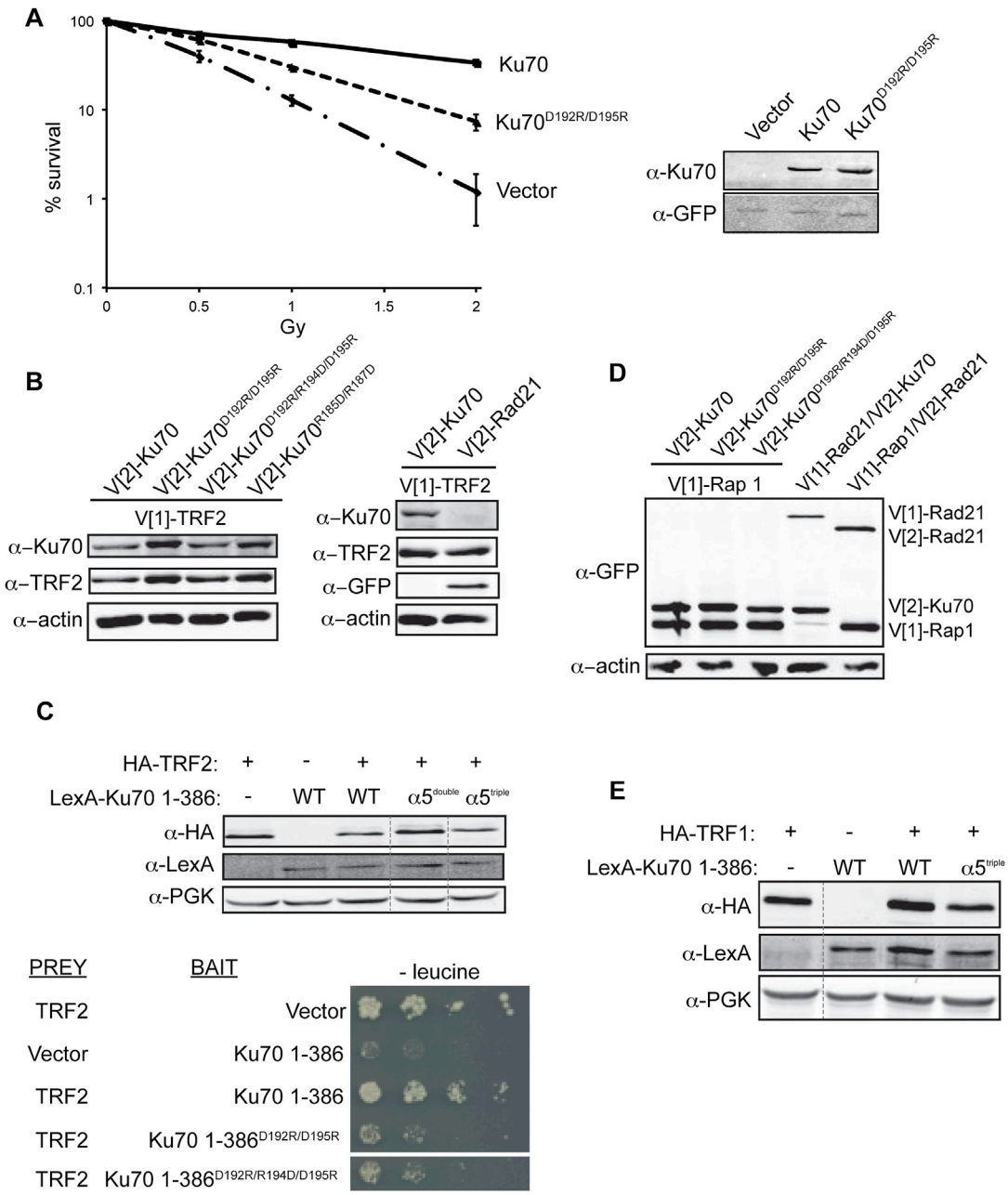

Figure S2. Ku70 α5 Is Required for NHEJ, Related to Figure 2

(A) (left) Clonogenic survival assays of Ku70−/− MEFs co-transfected in a 1:10 ratio with a GFP plasmid and either Ku70, Ku70$^{D192R/D195R}$, or vector. For each co-transfection, equal amounts of GFP positive cells were sorted by flow cytometry and exposed to increasing amounts of irradiation. Colonies were counted one week after irradiation. (right) Protein levels were detected 48 hr after co-transfection and before FACS and irradiation using a Ku70 antibody. GFP was used as a loading control.

(B) Protein levels resulting from transfections in Figure 2B for TRF2 as detected by immunoblot analysis of WCE using anti-GFP to detect Rad21 and primary antibodies to detect V[1]-TRF2 and V[2]-Ku70 as the tagged proteins migrated at an equivalent position. β-actin was used as a loading control. The Rad21 control blot is on the right.

(C) Protein levels (top) resulting from yeast two-hybrid of TRF2 with comparable results as in Figure 2C (bottom) as detected by immunoblot analysis of WCE using anti-HA and anti-LexA. PGK was used as a loading control. Double, D192R/D195R; triple, D192R/R194D/D195R.

(D) Protein levels resulting from transfections in Figure 2B for Rap1 as detected by immunoblot analysis of WCE using anti-GFP. β-actin was used as a loading control.

(E) Protein levels resulting from yeast two-hybrid of TRF1 in Figure 2C as detected the same as in (C). Triple, D192R/R194D/D195R.

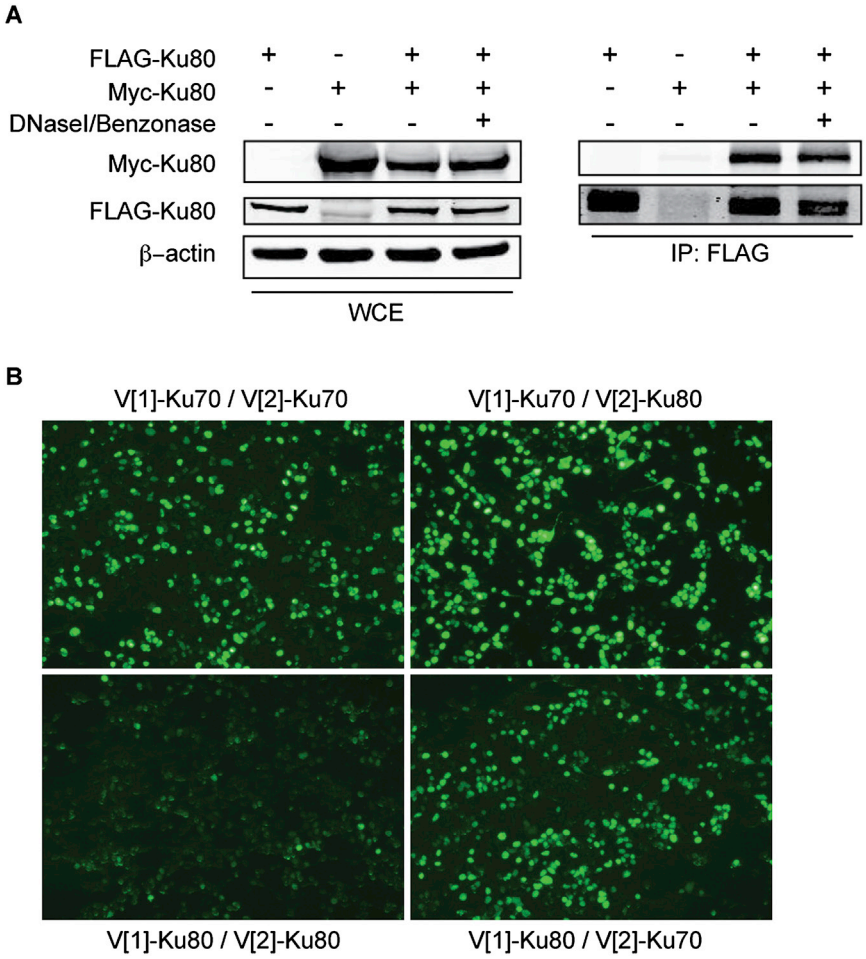

Figure S3. Ku80 Subunits Coimmunoprecipitate Together and Ku70 Associates with Both Ku70 and Ku80, Whereas Ku80 Only Associates with Ku70 in PCA, Related to Figure 5

(A) Co-immunoprecipitation of Myc-Ku80 with FLAG-Ku80. Immunoprecipitations with anti-FLAG antibody were performed using WCEs, untreated (-) or treated (+) with DNaseI/Benzonase. FLAG and Myc immunoblots were performed on the WCEs (left) and immunoprecipitates (right, IP:FLAG). β-actin represents a loading control.

(B) Protein-fragment complementation assays (PCA) in 293T cells transiently transfected with the indicated combinations of V[1]-Ku70, V[2]-Ku70, V[1]-Ku80 and V[2]-Ku80. Images were obtained 48 hr after transfection using 10X magnification.

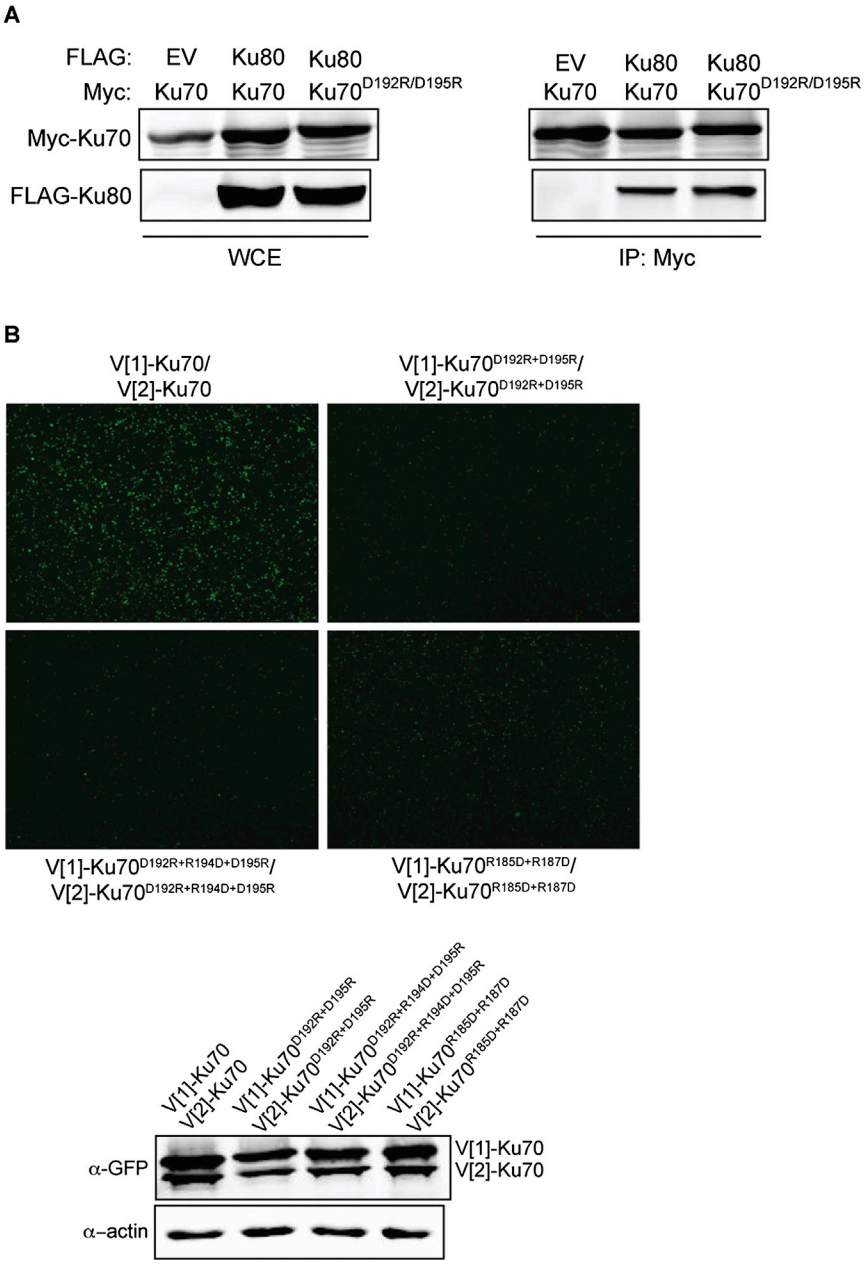

Figure S4. Ku70 α5 Heterodimerizes with Ku80 and Several Mutations in Ku70 α5 Impair Heterotetramerization, Related to Figure 6

(A) Co-immunoprecipitation of Myc-Ku70$^{D192R/D195R}$ with FLAG-Ku80. Immunoprecipitations with anti-Myc antibody were performed using cells co-transfected with Myc-Ku70 or Myc-Ku70$^{D192R/D195R}$ and FLAG-Ku80. FLAG empty vector (EV) was used as a negative control. FLAG and Myc immunoblots were performed on the WCEs (left) and the Myc immunoprecipitates.

(B) (left) Visualization of PCA using V[1]-Ku70 and V[2]-Ku70 bearing the indicated α5 mutations. Images were obtained 48 hr after transfection using 4X magnification. (right) Protein levels resulting from transfection on left detected by immunoblot analysis of WCE using anti-GFP antibody. β-actin was used as a loading control.