TRF2 Interaction with Ku Heterotetramerization Interface Gives Insight into c-NHEJ Prevention at Human Telomeres

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SUMMARY

Telomeres are protected from nonhomologous end-joining (NHEJ) to avoid deleterious chromosome fusions, yet they associate with the Ku heterodimer that is principal in the classical NHEJ (c-NHEJ) pathway. T-loops have been proposed to inhibit Ku’s association with telomeric ends, thus inhibiting c-NHEJ; however, deficiencies in the t-loop model suggest additional mechanisms are in effect. We demonstrate that TRF2 interacts with Ku at telomeres and via residues in Ku70 helix 5 (α5), which are vital for NHEJ. We show that Ku’s interaction with a TRF2 mutant that induces telomeric fusions is significantly impaired. Additionally, we demonstrate that Ku70 α5 is required for Ku self-association in live cells, which can bridge DNA ends. Together, these findings lead us to propose a model in which telomeres are directly protected from c-NHEJ via TRF2 impeding Ku’s ability to synapse telomere ends.

INTRODUCTION

Cells continuously suffer DNA double-strand breaks (DSBs) that, if left unrepaired, threaten genomic stability. Nonhomologous end-joining (NHEJ) is the major pathway devoted to the repair of such breaks (Lieber, 2010), operating efficiently throughout the cell cycle, including G1, when homologous recombination, the other major pathway of DSB repair, is restricted (Rothkamm et al., 2003). Simultaneously, the natural ends of linear chromosomes pose potential and ever-present substrates for NHEJ. These ends, however, are protected from engagement by the telomeric nucleoprotein complex. When such protection fails, NHEJ-dependent chromosome end-to-end fusions occur, leading to cessation of cell growth, presumably due to the inability to segregate the resultant multicentric chromosomes at mitosis (Celli and de Lange, 2005).

Two NHEJ pathways have been described, referred to as the classical (or canonical) (c-NHEJ) and alternative (alt-NHEJ) pathways (Mladenov and Iliakis, 2011). One of the factors that distinguishes these pathways is Ku, a heterodimeric complex, which initiates and is required for c-NHEJ and suppresses alt-NHEJ both at DSBs and telomeres (Bombarde et al., 2010; Fattah et al., 2010; Sfeir and de Lange, 2012; Wang et al., 2006). Ku is comprised of the Ku70 and Ku80 subunits, which upon heterodimerization form a high-affinity DNA binding ring that allows Ku to thread onto DNA ends independent of sequence (Walker et al., 2001). Interestingly, Ku is associated with telomeric chromatin across species and has integral roles in telomere structure and function (Fisher and Zakian, 2005). As a result of studies in Saccharomyces cerevisiae, the “two-face” model of Ku was proposed to explain Ku’s paradoxical roles in both NHEJ and at telomeres, where the outward face, which predominantly includes Ku70 residues, is essential for DNA repair and the inward face, which predominantly includes Ku80 subunits, is responsible for telomeric functions (Ribes-Zamora et al., 2007). Ku is essential in human cells, not for its role in NHEJ but rather for its role in inhibiting catastrophic telomere loss, presumably through homology-directed repair (HDR) (Wang et al., 2009). Thus, as functional telomeres are precluded from c-NHEJ, mechanisms must exist that inhibit Ku’s c-NHEJ functions while leaving its protective properties intact.

Mammalian telomeric DNA is comprised of tandem repeats of G-rich sequence and terminates in a 3’ overhang. Telomeric ends are protected by shelterin (Palm and de Lange, 2008), a telomere-specific complex comprised of TRF1, TRF2, Rap1, TIN2, TPP1, and POT1 (Palm and de Lange, 2008). At the forefront is TRF2, which both inhibits the DNA damage response (DDR) at telomeres and plays the dominant role in preventing c-NHEJ, as evidenced by the massive Ku-dependent fusions observed upon loss of TRF2 (Celli et al., 2006; Okamoto et al., 2013). Although inhibition of the DDR is crucial to TRF2-mediated inhibition of telomeric fusions, such fusions are not completely abrogated by the absence of ATM or 53BP1 in...
The promotion of t-loop formation by TRF2 is also thought to contribute to the inhibition of c-NHEJ (Griffith et al., 1999). This telomorphic architecture, in which the telomorphic end loops back and invades the upstream duplex telomeric sequence, is proposed to prevent the loading of Ku onto the telomorphic end where it must reside to mediate c-NHEJ (de Lange, 2010). However, experimental evidence indicating that t-loops provide a protective function in cells has yet to be demonstrated, and it is unclear whether all telomeres adopt the t-loop structure (Griffith et al., 1999). In addition, t-loops must be resolved to allow for proper telomere complex to telomeres via their high affinity for the telomeric sequence (de Lange, 2012); Rap1 is specifically anchored by TRF2 (Li et al., 2000). Although the interaction of Ku with TRF2 has been established, little else has been determined about the localization of functional significance of these interactions. As Ku has many binding partners that contribute to its multitude of functions, it is highly plausible that its telomeric protein interactions negatively regulate Ku to maintain genomic stability.

In this study, we sought to determine the role of Ku heterotetramerization in NHEJ and shelterin’s involvement in inhibiting Ku’s NHEJ function at human telomeres. We present data to support a mechanism by which TRF2 directly protects telomere ends from engaging in c-NHEJ. We localize Ku-TRF2 and Ku-TRF1 interactions to telomeres. Ku70 had a decreased association with the TRF2AΔBΔM mutant, which is known to induce Ku heterotetramerization in vivo. Thus, these findings suggest a model of telomere end protection whereby Ku’s ability to initiate c-NHEJ via synapsing DNA ends is blocked at telomeres by its interaction with TRF2.

RESULTS

Ku70 Localizes to Telomeres when Interacting with TRF1 and TRF2

Whereas the interaction of Ku with TRF1 and TRF2 is well-established, the localization of these interactions had not been determined and warranted further assessment as Ku can be found throughout the cell (Muller et al., 2005). Additionally, TRF1 and TRF2 are found to interact with the essential NHEJ region, Ku70Δκ5, directly implicating TRF2 in regulating Ku’s ability to initiate c-NHEJ at telomeres. Additionally, Ku70 had a decreased association with the TRF2AΔBΔM mutant, which is known to induce Ku heterotetramerization in vivo. Thus, these findings suggest a model of telomere end protection whereby Ku’s ability to initiate NHEJ via synapsing DNA ends is blocked at telomeres by its interaction with TRF2.
V[2] fragments. As anticipated by the prior interaction studies (Hsu et al., 2000; Song et al., 2000), we found that coexpression of V[2]-Ku70 with either V[1]-TRF1 or V[1]-TRF2 resulted in robust fluorescence (Figure 1A), signifying an interaction. Both proteins were found to produce a weak fluorescence with Rad21, a nonspecific, nuclear protein control (Figure 1A), indicating the specificity of Ku70’s interactions with these shelterin components. Importantly, the YFP fluorescence in the PCAs was not diffuse, as might be expected if the interaction between Ku70 and the TRFs were nonspecific and simply driven by expression levels; instead, the YFP fluorescence manifested as a punctate nuclear signal, suggesting the interaction was occurring at telomeres (Figure 1B). To confirm this, we determined whether the foci colocalized with DsRed-TRF2. Following triple transfection of V[1]-TRF1 or V[1]-TRF2, V[2]-Ku70, and DsRed-TRF2 (Barrientos et al., 2008), we observed colocalization of the V[1]-TRF1/V[2]-Ku70 and the V[1]-TRF2/V[2]-Ku70 signals with the similarly punctate DsRed-TRF2 signals. TRF2-Rap1 interaction was used as a positive control, showing telomeric localization for these two known binding partners in a PCA as expected (Figure S1A).

Although TRF1 and TRF2 do not directly interact, they coexist within the shelterin complex. Therefore, it was possible that the interaction of endogenous TRF2 and the Ku70 PCA construct within shelterin may have positioned the Ku70 protein to allow for fluorescence reconstitution with the TRF1 PCA construct and vice versa with endogenous TRF1 and the Ku70 and TRF2 PCA constructs. To test these possibilities, we examined the Ku-TRF interactions in the presence of dominant negative TRF mutant proteins, TRF2ΔBΔM (van Steensel et al., 1998) and TRF1ΔAΔM (van Steensel and de Lange, 1997), which titrate the respective TRF protein off the telomere. Relative to the coexpression of the vector control, the Ku70-TRF1 fluorescence was 20% lower when TRF2ΔBΔM was coexpressed, whereas the Ku70-TRF2 fluorescence was not affected by coexpression of TRF1ΔAΔM, despite equivalent protein expression levels (Figures 1C and S1B). This could reflect...
the Ku70-TRF1 PCA fluorescence being partly due to the TRF2 interaction with Ku70. Alternatively, it could be due to a release of Ku70 from TRF1 secondary to the widespread telomere de-protection induced by TRF2.

Although, the Ku70-TRF1 fluorescence was decreased in the presence of the TRF2 mutant, the signal remained punctate (Figure 1D). Expression of the opposing full-length TRF resulted in an increase in Ku70 fluorescence with either TRF1 or TRF2, indicating a stabilizing effect when the opposing TRF is bound to the telomere. Collectively, these results are consistent with Ku70 interacting with both TRF1 and TRF2 at telomeres in vivo.

As TRF2, TRF1, and Rap1 have been implicated in the inhibition of NHEJ at telomeres, we considered the possibility that their interactions with Ku70 served as a mechanism to inhibit Ku’s NHEJ function. We therefore turned our attention to the conserved helix 5 in the Ku70 N terminus (Ku70\(_{a5}\)) (Figure 2A), which has been shown to be essential for NHEJ in budding yeast and human cells (Fell and Schild-Poulter, 2012; Ribes-Zamora et al., 2007). Consistent with previous reports, we found that, despite similar expression levels, transient transfection of Ku70\(_{a5}\)/mouse embryonic fibroblast (MEF) cells with a mutant of human Ku70 \(a5\), Ku70\(^{D192R/D195R}\) did not restore colony survival to wild-type levels after gamma irradiation (Figure 2A).

We therefore asked whether TRF1, TRF2, and/or Rap1 interacted with Ku70 via helix 5. To do this, we used three Ku70 \(a5\) mutants: Ku70\(^{D192R/D195R}\), Ku70\(^{R185D/R187D}\), and Ku70\(^{D192R/R194R/D195R}\). Overall, these mutations impaired the association of Ku70 with TRF2 up to 50% (Figure 2B). As the proteins in this assay were expressed at levels at least comparable to wild-type, the decreased fluorescence could not be attributed to decreased protein expression (Figure S2B). The impact of the

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**Figure 2.** TRF2, but Not Rap1 or TRF1, Interacts with the Ku70 NHEJ Domain

(A) Left, frontal view of Ku’s outward face. Ku70 and Ku80 are depicted in yellow and blue, respectively; Ku70 \(a5\) in orange; and DNA in black. Right, alignment of human and budding yeast Ku70 residues located in \(a5\). Residues mutated in Ku70\(^{D192R/D195R}\) are depicted in red.

(B) Fluorescence quantification of PCA for the designated Ku70 mutants or Rap21 with either TRF2 or Rap1. Shown are the averages for three independent transfections. Error bars represent SD of the mean. \(p\) values were obtained using the Student’s two-tailed unpaired t test. \(p\) values for pairs including TRF2 are as follows: \(p = 0.0072\) for Ku70 versus Ku70\(^{D192R/D195R}\), \(p = 0.0026\) for Ku70 versus Ku70\(^{D192R/R185R/D195R}\), and \(p = 0.0092\) for Ku70 versus Ku70\(^{R185D/R187D}\). \(p\) values for pairs including Rap1 are as follows: \(p = 0.3540\) for Ku70 versus Ku70\(^{D192R/D195R}\) and \(p = 0.3162\) for Ku70 versus Ku70\(^{D192R/R185R/D195R}\).

(C) Yeast two-hybrid analysis for ADH1pLexA-DBD-TRF1 or the designated ADH1pLexA-DBD-Ku70 \(1–386\) alleles (bait) with either GAL1pB42-AD-TRF2 or GAL1pB42-AD-TRF1 (prey). Serial dilutions were grown on Gal-His-Trp-Ura media without (–) or with (+) of leucine. Positive interaction is determined by growth in the absence of leucine. See also Figure S2.
Ku70 α5 mutations on Ku70-TRF2 interaction was corroborated by yeast two-hybrid analysis using a Ku70 C-terminal truncation, Ku70 1–386, encompassing the domain that includes helix 5. In this yeast two-hybrid analysis, an interaction between the TRF2 prey and Ku70 1–386 bait was demonstrated by their expression resulting in enhanced growth in the absence of leucine compared to expression of the TRF2 prey alone or the TRF2 prey in the presence of a noninteracting bait (TRF1; Figure 2C, top panel). Both Ku70 mutants exhibited a 5- to 10-fold decreased interaction with TRF2, as evidenced by a reduction in growth in the absence of leucine compared to wild-type Ku70 1–386 with TRF2 (Figures 2C, top panel, and S2C). An enhanced effect of the Ku70 mutants seen in the two-hybrid compared to the PCA are explained by the inherent irreversibility of the YFP fragments folding in the PCA, which we reason lessens the degree of disruption of the Ku70-TRF2 interaction caused by the mutations.

Although Ku70 showed a robust interaction with Rap1, in contrast to the effect on TRF2 interaction, mutations in Ku70 α5 did not impact the Ku70-Rap1 PCA, further arguing for the specificity of the influence of Ku70 α5 on the Ku70-TRF2 interaction (Figures 2B and S2D). Because the results in Figure 1C show that TRF2 may influence the Ku70-TRF1 PCA interaction, we used yeast two-hybrid to analyze the effect of the Ku70 mutants on the TRF1 interaction. The Ku70 D192R/D195R mutant did not show a decrease in interaction with TRF1, despite a robust interaction and comparable protein expression levels (Figures 2C, bottom panel, and S2E). Taken together, these results demonstrate that TRF2, but not Rap1 or TRF1, interacts with a region of Ku70 required for NHEJ.

As the binding of TRF2 to Ku70 involved α5, it could provide a mechanism by which c-NHEJ is directly inhibited at telomeres. We next determined whether TRF2ΔBM mutant, which leads to c-NHEJ-dependent telomeric fusions (Smogorzewska et al., 2002; van Steensel et al., 1998), is impaired for the interaction. Consistent with this, we found that TRF2ΔBM had a 45% lower fluorescence in the PCA with Ku70 as compared to wild-type TRF2 (Figure 3). This opens the possibility that loss of Ku-TRF2 interaction may contribute to the telomere fusion phenotype observed in cells expressing TRF2ΔBM.

Ku70 α5 Is Not Required for Ku70’s Association with XRCC4 or XLF

Having established that TRF2 interacted with Ku via a region required for NHEJ, we next sought to determine the role of Ku70 α5 in NHEJ. One possibility was that Ku70 α5 is required for Ku’s ability to interact with one of its NHEJ binding partners. Whereas Ku’s interaction with DNA-PKcs has been localized to the Ku80 C-terminal domain (Singleton et al., 1999), the region(s) required for its interactions with key NHEJ factors XRCC4 (Mari et al., 2006) and XLF (Yano et al., 2008) have not been defined. Whereas PCA demonstrated an interaction between Ku70 and XRCC4 and XLF, we found that V[1]-Ku70D192R/D195R was able to restore fluorescence with V[2]-XRCC4 and V[2]-XLF to the same extent as wild-type V[1]-Ku70 (Figure 4); therefore, the radiation sensitivity of Ku70D192R/D195R was not due to the loss of its ability to interact with XRCC4 or XLF. Additionally, the lack of impact of V[1]-Ku70D192R/D195R on fluorescence with V[2]-XRCC4 and V[2]-XLF as compared to wild-type further supports the specificity of the reduction in fluorescence observed when the mutant was expressed with V[2]-TRF2 (Figure 2B).

Ku Self-Associates Independently of DNA-PKcs

Given the localization of Ku70 α5 to the outward face of Ku as opposed to the face oriented inward along the DNA continuum (Figure 2A), we next considered the possibility that this region contributed to the Ku-Ku interactions that had been previously observed in vitro with recombinant proteins (Cary et al., 1997; Ramsden and Gellert, 1998). First, we wanted to determine...
whether Ku-Ku interactions could be detected in vivo; therefore, we performed coimmunoprecipitation (coIP) assays using whole-cell extracts (WCEs) prepared from cells cotransfected with differentially tagged versions of Ku70. Consistent with prior studies indicating Ku self-association, we detected Myc-Ku70 in FLAG-Ku70 immunoprecipitates (Figure 5A) and, conversely, FLAG-Ku70 in Myc-Ku70 immunoprecipitates (Figure 6A, middle panel). Similar results were obtained when using differentially tagged versions of Ku80 (Figure S3A). Because Ku bound to DNA forms a complex with DNA-PKcs, which has been implicated in bridging DNA ends (DeFazio et al., 2002), we tested the possibility that the coIP of differentially tagged Ku subunits was the result of DNA-PKcs interactions and not direct Ku interactions. To do this, we repeated the coIP assays in HCT116 DNA-PKcs−/− cells (Ruis et al., 2008). We found that differentially tagged Ku70 subunits associated in the absence

Figure 4. Ku70 α5 Is Not Required for Ku’s Association with XRCC4 or XLF
(A) Visualization of PCA using Ku70 or Ku70D192R/D195R with XLF or XRCC4. Images are at 4x magnification.
(B) Fluorescence quantification of PCA shown in (A). The error bars represent SD of the mean.
(C) Protein levels resulting from transfections in (A) detected by immunoblot analysis of WCE using a GFP antibody. β-actin was used as a loading control.
of DNA-PKcs (Figure 5B). We also found that the coIP of the differentially tagged Ku70 or Ku80 subunits was not the result of different heterodimers being bound to opposing ends of the same DNA fragment because the interaction was not eliminated by treatment of the WCEs with the combination of DNaseI and Benzonase nuclease prior to immunoprecipitation (Figures 5A, 5B, and S3A). As neither Ku70 nor Ku80 homodimerize, and both are unstable when not heterodimerized (Errami et al., 1996; Gu et al., 1997), these results are consistent with the differentially tagged Ku heterodimers forming multimers.

To investigate Ku-Ku interactions further in living cells, we performed PCA. We found that transiently transfected V[1]-Ku70 reconstituted fluorescence robustly when coexpressed with either V[2]-Ku80 or V[2]-Ku70 but only weakly with V[2]-Rad21, a nonspecific control (Figures 5C and S3B). In contrast, V[1]-Ku80 did not reconstitute fluorescence with V[2]-Ku80 (Figure 5C). V[1]-Ku80 did, however, strongly reconstitute fluorescence with V[2]-Ku70 (Figure S3B), indicating the functionality of this fusion protein. Reversing V[1] and V[2] fragments in each protein gave comparable results (Figures 5C and S3B). Although there was some variation in steady-state protein levels, each of the proteins was readily detected and the differences in fluorescence intensities could not be attributed to differences in protein levels (Figure 5D). The N terminus of Ku70 and Ku80 map to opposite faces of the Ku heterodimer, and Ku binds DNA ends with a polarity that places the Ku70 N terminus facing outward (Walker et al., 2001). Thus, the findings that coexpressed N-terminally tagged Ku70 proteins were able to reconstitute fluorescence in the PCA but coexpressed N-terminally tagged Ku80 could not are consistent with Ku heterodimers interacting with each other in an orientation bringing the outward faces together.

**Ku70 α5 Is Required for Heterotetramerization**

Because interactions involving the outward faces of two Ku heterodimers might contribute to the synopsis of the two ends of a DSB during NHEJ, we then asked whether Ku70 α5 might contribute to Ku heterotetramerization (i.e., the association of
two Ku heterodimers) and thereby facilitate NHEJ. Expression of differentially tagged Ku70\(^{D192R/D195R}\) mutants resulted in an approximately 50% reduction in the amount of FLAG-Ku70\(^{D192R/D195R}\) recovered in the Myc-Ku70\(^{D192R/D195R}\) immunoprecipitates when compared to wild-type (Figure 6A). Similar reductions were observed in the amount of Myc-Ku70\(^{D192R/D195R}\) detected in FLAG-Ku70\(^{D192R/D195R}\) immunoprecipitates with respect to wild-type. Importantly, expression of Myc-Ku70\(^{D192R/D195R}\) pulled down wild-type levels of FLAG-Ku80, indicating the effect of the mutation was not due to destabilization of the heterodimer (Figure S4A).

Similar to the coIP results, V[1]-Ku70\(^{D192R/D195R}\) showed a significant reduction in its ability to reconstitute fluorescence in the PCA with V[2]-Ku70\(^{D192R/D195R}\) when compared with wild-type V[1]-Ku70 and V[2]-Ku70 (Figures 6B and 6C) despite comparable steady-state protein levels (Figure 6D). The Ku70\(^{D192R/R194D/D195R}\) and Ku70\(^{R185D/R187D}\) mutants exhibited a decreased self-interaction in the PCA as well (Figure S4B). Thus, Ku70 x5 is utilized for both heterotetramerization, where the two outward faces in each heterodimer interact with each other, and NHEJ, suggesting NHEJ requires Ku heterotetramerization.

**DISCUSSION**

A fundamental question regarding telomeres is how they are protected from engagement in c-NHEJ. The t-loop model is simple and elegant; however, it is likely that additional mechanisms exist to confer this protection. Our findings provide evidence to support the hypothesis that TRF2, the primary inhibitor of c-NHEJ at telomeres, directly inhibits c-NHEJ by negatively regulating Ku. Here, we show that TRF2 interacts with Ku via Ku70 x5, a region that maps to Ku’s outward face and is necessary for NHEJ. The PCA method, which requires the split Venus fragments to come into such close proximity to fold together, allowed us to determine that TRF2 and Ku70 interact at telomeres. Furthermore, we show that Ku70 x5 contributes to Ku-Ku association, which has been shown to bridge DNA ends.
Figure 7. Models for Ku Heterotetramerization and Its Inhibition at Telomeres by TRF2

(A) Top view of a hypothetical Ku tetramer obtained via protein docking using HADDOCK web server. Yellow: Ku70. Blue: Ku80. Orange: Ku70 α5. Residues R185, D192, and D195 from Ku70 are shown in stick representation. The vWA domain from Ku70 would have to move to accommodate this model of heterotetramerization. Its original position with respect to its native heterodimer is shown in “ghost” (semitransparent) representation.

(B) Mechanistic model for the role of Ku’s heterotetramerization during NHEJ. We propose that Ku heterotetramerization is required in the initial NHEJ steps to synapse the DNA ends of a DSB prior to the DNA-PKcs recruitment. Formation of the DNA-PK complex displaces Ku away from the end, which effectively

(legend continued on next page)
in vitro (Ramsden and Gellert, 1998). These data lead us to propose a model in which TRF2 directly inhibits NHEJ at telomeres by blocking Ku from bridging two telomere ends in preparation for ligation (Figure 7). Whereas Ku may be excluded from the telomeric end via mechanisms promoted by TRF2 (e.g., via t-loop formation), the ability of TRF2 to bind a region of Ku required for Ku-Ku interaction and NHEJ may add an additional layer of protection, particularly should Ku gain access to the end.

We additionally found that the TRF2ΔBΔM dominant-negative mutant has a diminished association with Ku70. As engagement in NHEJ presumably requires single Ku heterodimers bound to each DNA end, then even a moderate release of Ku’s inhibition has the potential to lead to telomere fusions when the ends are accessible. Thus, the catastrophic telomere fusion phenotype seen in this TRF2 mutant may be promoted by the liberation of Ku70’s NHEJ ability. Even though functional telomeres are recognized as DNA damage in G2 (Verdun et al., 2005), TRF2 is still telomeric and presumed capable of inhibiting Ku, which may explain why fusions do not occur at this time. As the Ku70 α5 mutant is defective for NHEJ but presumably proficient for DNA end-binding (Pfingsten et al., 2012), it would be expected to significantly suppress the TRF2ΔBΔM fusion phenotype without allowing alt-NHEJ fusions to occur. The identification of Ku mutants that are impaired for TRF2 binding, but not c-NHEJ, or TRF2 mutants that are still able to localize to telomeres but are defective for Ku70 binding would be valuable for this determination. Such mutants would provide important, direct evidence in support of our proposed model. However, if Ku70 interacts with multiple domains in TRF2, then we anticipate it will be difficult, if not impossible, to identify a separation-of-function mutation that would eliminate Ku70 binding yet preserve TRF2’s interaction with both telomeric DNA and its telomeric binding partners (e.g., Rap1, TIN2, and Apollo). Additionally, our model argues that it might not be possible to identify a separation-of-function allele for Ku where NHEJ is fully functional and TRF2 binding is eliminated if the residues required for TRF2 binding completely overlap with those required for NHEJ.

There are conflicting data as to whether TRF2 itself or its binding partner Rap1 leads to the inhibition of NHEJ (Bae and Baumann, 2007; Martinez et al., 2010; Sarthy et al., 2009; Sfeir et al., 2010). Although the artificial tethering of Rap1 to the telomeric tract in HeLa cells bypassed the requirement of TRF2 for the prevention of telomere fusions (Sarthy et al., 2009), MEFs lacking Rap1 or expressing an allele of TRF2 that cannot bind Rap1 did not result in a DNA damage response or exhibit a telomere fusion phenotype (Martinez et al., 2010; Okamoto et al., 2013; Sfeir et al., 2010). Therefore, Rap1 is dispensable for the protection of telomeres from NHEJ, at the very least, in the mouse model system. Whereas our data suggest a direct role for TRF2 in c-NHEJ inhibition in human cells, we were not able to implicate Rap1, as it did not associate with the NHEJ region of Ku70.

There are also conflicting data for the involvement of TRF1 in the inhibition of NHEJ at telomeres. Whereas a TRF1 knockout mouse model showed notable levels of telomere fusions (Martinez et al., 2009), a conditional TRF1 knockout in MEFs resulted in telomere fusions in only 2% of cells (Sfeir et al., 2009). These two studies also differ in whether the deletion of TRF1 resulted in solely ATR activation or combined ATM/ATR activation, which could further explain the disparities seen in telomere fusions. Our results are consistent with TRF1’s interaction with Ku70 not playing a major role in inhibiting telomeric c-NHEJ in human cells.

In this study, we also address the mechanism of Ku that is specifically blocked at telomeres. We show here that interactions between Ku heterodimers can be detected in living cells. We found that Ku70 α5 mutations disrupted heterotetramer formation and DNA repair, suggesting that Ku heterotetramerization is required for c-NHEJ. Together, our data lead us to propose a model in which Ku heterotetramerization occurs via an interaction of the two outward faces of each Ku heterodimer with Ku70 α5 at the interface (Figure 7A). In the context of a DSB, Ku heterotetramerization would mediate the synopsis of the broken ends (Figures 7A and 7B). Whereas the current mechanistic model for c-NHEJ places DNA-PKcs as the protein responsible for bridging two DNA ends of a DSB (Dobbs et al., 2010; Líorca, 2007), we propose a role for Ku via heterotetramerization, which would occur right after Ku loads onto each end and prior to the recruitment of DNA-PKcs (Figure 7B). Thus, Ku heterotetramerization would play the initial role in restraining the two broken ends. Subsequently, DNA-PKcs recruitment to the Ku-bound DSB would lead to the separation of the heterotetramers and displacement of Ku internally along the DNA. DNA-PKcs would then replace Ku in the task of synapsing the two ends of the DSB. Further c-NHEJ functions of Ku beyond this point, namely recruitment of other c-NHEJ factors, should occur, therefore, in the absence of heterotetramers. This is consistent with our finding that Ku mutations that disrupt heterotetramerization do not disrupt binding with either XRCC4 or XLF.

Although our PCA data do not exclude the possibility that the restored fluorescence for Ku70-Ku70 was caused by one or more proteins bridging the differentially tagged heterodimers, this is unlikely. First, Ku-Ku interactions have been detected in vitro in the absence of other proteins (Cary et al., 1997; Ramsden and Gellert, 1998). Second, our differentially tagged coIP associations were detected in the absence of DNA or DNA-PKcs, two factors known to bridge Ku heterodimers. Third, although the maximal distance between Venus YFP fragments that allows them to come together and fold has not been experimentally determined, the relatively short flexible peptide linkers (ten amino acids [aa] in length) between Ku70 and Venus fragments would indicate that the two Ku70 N termini must allow the Venus fragment to come in very close proximity. For example, the same peptide linker robustly supported dihydrofolate reductase fragments coming together in a PCA if they were separated by

dissociates the Ku-Ku interaction and allows DNA-PKcs to replace Ku in synapsing the two ends. Later roles of Ku in recruiting NHEJ factors to DSBs would not require heterotetramer formation.

(C) Mechanistic model for the inhibition of Ku’s heterotetramerization by TRF2 at telomeres. We propose that TRF2’s interaction with the Ku70 α5 helix effectively inhibits Ku heterotetramerization at telomeres and the synopsis of telomeric ends, thereby blocking telomeric NHEJ.
39 Å (~10 aa in length) but only weakly if they were separated by 73 Å (Remy et al., 1999). Therefore, we strongly favor the conclusion that restored fluorescence observed in the Ku70-Ku70 PCA was the result of direct interaction between Ku heterodimers.

If TRF2 interaction with Ku70 can inhibit Ku’s ability to initiate NHEJ, why then would TRF2 inhibit Ku at telomeres and not at DSBs? TRF2 has a high affinity for telomeric repeats and is found to be fully chromatin-bound (Takai et al., 2010), the vast majority being telomeric with additional detection at interstitial sequences (Simonet et al., 2011; Yang et al., 2011). Consistent with a role in telomeric function, we localized Ku70’s interaction with TRF2 to telomeres. Because the TRF2-Ku70 interaction we observed in the PCA was punctate and robust, we speculate that it may be facilitated by TRF2 binding to telomeric DNA. This could also explain why the growth for the yeast two-hybrid TRF2-Ku70 interaction, although present, was not as robust, as it would not recapitulate TRF2 bound to telomeric DNA.

In vitro binding experiments with purified proteins in the presence of DNA, however, showed that the TRF2-Ku70 interaction, although present, was not as robust, as it would not recapitulate TRF2 bound to telomeric DNA. This could also explain why the growth for the yeast two-hybrid TRF2-Ku70 interaction, although present, was not as robust, as it would not recapitulate TRF2 bound to telomeric DNA.

Cutting-edge technologies and tools that allow us to gain insights into the complex interplay between telomere function and Ku's role in protecting telomeres have been developed. These include next-generation sequencing and single-molecule imaging, which allow us to visualize and analyze telomere dynamics in real-time. This has led to the discovery of new mechanisms by which telomeres are protected from fusions via TRF2.

In vitro experiments with purified proteins in the presence of DNA, however, showed that the TRF2-Ku70 interaction, although present, was not as robust, as it would not recapitulate TRF2 bound to telomeric DNA. This could also explain why the growth for the yeast two-hybrid TRF2-Ku70 interaction, although present, was not as robust, as it would not recapitulate TRF2 bound to telomeric DNA.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mutagenesis**

N-terminal Myc- and/or FLAG-tagged versions of Ku70, Ku80, TRF1, TRF1Δ4A3M, TRF2, and TRF2Δ4BΔM were produced by cloning each gene in either either CS2-MT or pFLAG-CMV2 vectors. Ku70, Ku80, Rad21, XRCC4, XLF, TRF1, TRF2, and Rap1 were fused at their N terminus with either Venus [1] (V1) or Venus [2] (V2) fragments by replacing the leucine zipper in either Venus [1]-GCN4-leucine zipper or Venus [2]-GCN4-leucine zipper constructs (provided by S. Michnick). List of plasmids, cloning details, and oligo sequences can be provided upon request. Mutations in Ku70 Δ5 were generated using oligonucleotide single-stranded mutagenesis. Rap1 was subcloned from pLP/Lrap1-FL, a gift from Titia de Lange (Addgene no. 12542). V2]-TRF2Δ4BΔM was generated by PCR of TRF2 and encodes aa 45–455 and contains three additional aa, WRE, at the N terminus.

**Colp Assays**

For Myc-Ku70 or Myc-Ku80 immunoprecipitations, 5 × 10⁶ human embryonic kidney 293T cells (HEK293T) or HCT116 DNA-PKcs−/− cells (donated by E. Hendrickson) were cotransfected with plasmids containing FLAG-Ku70 or FLAG-Ku80 using Lipofectamine and Plus Reagents (Invitrogen) according to manufacturer’s protocol. WCEs were prepared with radio immunoprecipitation assay (RIPA) buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, and Protease Inhibitor Cocktail III (Calbiochem)) 48 hr after transfection. For each cotransfection, 1.5 mg of WCE was diluted in 500 μl and, where indicated, DNA was removed by adding 10 μl of DnaseI (New England Biolabs), 5 μl of Benzonase nuclease (Novagen), and 2.5 μl of MgCl₂ for 1 hr on ice. Extracts were precleared with 150 μl of Protein G Plus-Agarose beads (Calbiochem) for 1 hr at 4°C. After centrifugation and removal of beads, extracts were mixed with 10 μg of α-Myc antibody (Sigma), rotated for 1 hr at 4°C, and then added to 150 μl of Protein G Plus-Agarose beads followed by overnight rotation at 4°C. Beads were pelleted and washed four times with RIPA buffer, and proteins were eluted by heat. The same procedure was followed for FLAG-Ku70 or FLAG-Ku80 immunoprecipitations but adding 150 μl of FLAG-conjugated agarose beads slurry (Sigma) instead of α-Myc antibody followed by Protein G beads. WCEs and immunoprecipitates were subjected to SDS-PAGE on 10% gels and analyzed by western blotting with either Myc, FLAG, or p-actin (Sigma) primary antibodies and IRDye 800CW-conjugated goat anti-mouse secondary antibody (Li-Cor). Fluorescence was visualized using the Li-Cor Odyssey Infrared Imaging System, and quantitation was performed using ImageQuant software (Molecular Dynamics).

**Fluorescent PCA**

Plates containing 5 × 10⁶ HEK293T cells were cotransfected with 500 ng of each indicated plasmid, and 48 hr after transfection, cells were washed with PBS and pictures were taken using a magnification of 4x on an Olympus IX71 fluorescence microscope with 500 ms exposure and ISO200. Deconvolution images were obtained using the same procedure. An emission filter was applied to remove any background fluorescence. Cells were then fixed with 3% PFA and nuclei were stained with DAPI. For each transfection, three images were acquired, and the fluorescence activity averaged. Remaining cells from each transfaction were used for immunoblot analysis using a GFP antibody (Abcam), anti-Ku70 (Lab Vision), anti-TRF2 (Santa Cruz), anti-FLAG (Sigma), or anti-p-actin (Sigma).

**Yeast Two-Hybrid Assays**

EGY48 was transformed with a prey plasmid containing TRF1 (pAB884) or TRF2 (pAB879) and then a bait plasmid containing either TRF1 (pAB 650), Ku70 1–386 truncation (pAB668), Ku70 1–386D192R/D194R/D195R (pAB877), or Ku70 1–386D192R/D194R/D195R (pAB869). Interaction between the bait and prey constructs was determined by growth on Gal-Leu-His-Trp-Ura plates.

**Protein Docking**

To construct the model shown in Figure 7A, protein docking was performed using HADDOCK web server (Dominguez et al., 2003). All simulations were done in the absence of DNA, which was later modeled into the final complex. The Ku70-Ku80 dimer configuration was taken from the complex deposited in Protein Data Bank as 1JEY (Walker et al., 2001). In the first production run, the
Supplemental Information

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.040.

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