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Effects of DNA nonhomologous end-joining factors on telomere length and chromosomal stability in mammalian cells

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DNA repair by nonhomologous end-joining (NHEJ) relies on the Ku70:Ku80 heterodimer in species ranging from yeast to man. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, Ku also controls telomere functions. Here, we show that Ku70, Ku80, and DNA-PKcs, with which Ku interacts, associate in vivo with telomeric DNA in several human cell types, and we show that these associations are not significantly affected by DNA-damaging agents. We also demonstrate that inactivation of Ku80 or Ku70 in the mouse yields telomeric shortening in various primary cell types at different developmental stages. By contrast, telomere length is not altered in cells impaired in XRCC4 or DNA ligase IV, two other NHEJ components. We also observe higher genomic instability in Ku-deficient cells than in XRCC4-null cells. This suggests that chromosomal instability of Ku-deficient cells results from a combination of compromised telomere stability and defective NHEJ.

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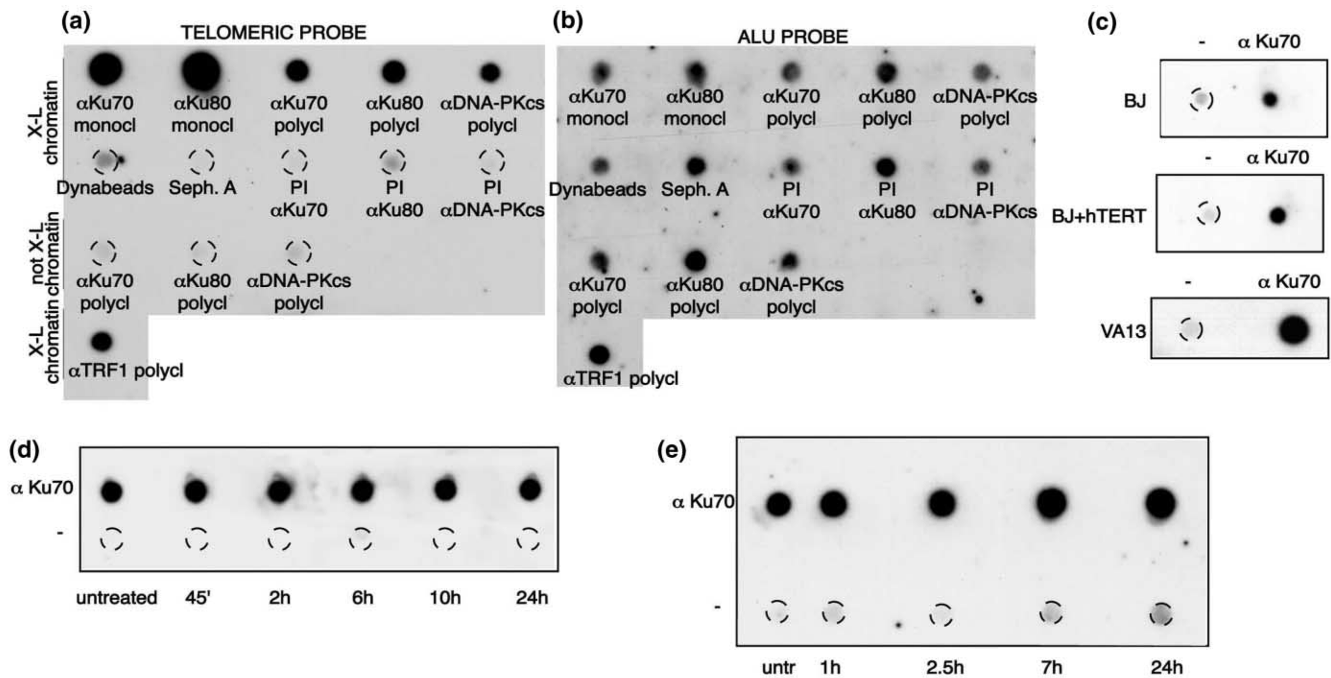
Results and discussion

In *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, inactivation of either Ku subunit leads to shortened stable telomeres [1–2]. Ku has been localized to *S. cerevisiae* telomeres by indirect immunofluorescence and by chromatin-immunoprecipitation (CHIP; [3–4]). In mice, Ku inactivation causes severe radiosensitivity [5]. Ku-inactivated cells also show increased rates of genomic instability [6–8].

We analyzed whether both Ku subunits and the catalytic subunit of DNA-PK (DNA-PKcs) are physically associated with telomeric DNA in vivo in human cells by CHIP [9]. Human HeLa cells were crosslinked with formaldehyde, chromatin was prepared, and immunoprecipitations were performed with antibodies against Ku or DNA-PKcs. Telomeric DNA in the immunoprecipitates was then analyzed by dot-blot hybridization (Figure 1a). In addition to the previously reported interaction of Ku80 with telomeres [10, 11], we found that Ku70 and DNA-PKcs are also associated with telomeric DNA. Therefore, the entire, possibly catalytically competent, DNA-PK complex is associated with the mammalian telomere. Ku could also be crosslinked to telomeric repeats in cells in G1, S, G2, or M phases of the cell cycle (data not shown) and in primary human fibroblasts before or after transduction with hTERT, the catalytic component of telomerase (Figure 1c). Ku is also associated with telomeres in ALT cells (VA13 cells in Figure 1c), cells that maintain their telomeres by homologous recombination (HR)-based mechanisms [12]. In *S. cerevisiae*, the generation of DNA double-strand breaks (DSBs) causes some Ku to dissociate from telomeres [3]. CHIP analysis of human cells irradiated with 10 Gy of ionizing radiation (Figure 1d), or grown in the presence of 50 μg/ml of the radio-mimetic drug phleomycin (Figure 1e), revealed no significant changes in the association of Ku with telomeric DNA.

To investigate the functional relevance of Ku association with telomeres, we studied telomere stability in mouse cells in which one or both alleles of Ku80 or Ku70 had been inactivated. First, we analyzed telomere length in cells derived from Ku80 knockout mice [13]. Mouse telomere length cannot always be reliably studied by conventional Southern blot hybridization techniques ([14–15] and unpublished data). Quantitative fluorescence in situ hybridization (qFISH; [16]) can detect and quantitate the signal generated by hybridization of a fluorescent probe

Figure 1



Ku70, Ku80, and DNA-PKcs are associated with human cell telomeric DNA as detected by CHIP. **(a)** A telomeric probe recognized telomeric DNA when antibodies raised against Ku70, Ku80, and DNA-PKcs were used to immunoprecipitate these antigens crosslinked to chromatin. Little or no telomeric DNA was retrieved when antibodies were not included, when preimmune sera (PI) was used, or when crosslinking was omitted. An anti-TRF1 antibody was used as a positive control. Sera raised against factors not expected to have a specific telomeric localization did not bring down significant amounts of telomeric DNA (data not shown). **(b)** The specificity of the immunoprecipitated DNA was checked by stripping the membrane and rehybridizing with an Alu probe: all the immunoprecipitations brought down similar amounts of Alu DNA, showing that enrichment for telomeric DNA is specific. **(c)** Ku is associated with telomeric DNA in a variety of human cell lines; BJ are human primary fibroblasts, BJ+hTERT are BJ cells into which the hTERT gene has been transduced, and VA13 are ALT cells. **(d,e)** Ku remains associated with telomeric DNA in the presence of DNA damage; in (d), cells were irradiated with 10 Gy of X-rays, and samples were analyzed at the indicated times afterwards; in (e), cells were grown in the presence of 50 μg/ml phleomycin for the indicated times. Control CHIP is comprised of beads alone without Ku antibodies. Quantitation is available as Supplementary material.

at individual telomeres, irrespective of polymorphisms in telomeric and subtelomeric sequences. Primary mouse embryonic fibroblasts (MEFs; population doubling < 3) were obtained from 11 embryos from 4 independent litters derived from heterozygous crosses. We found that $Ku80^{-/-}$ animals had lost ~40% of their telomeric repeats in comparison to $Ku80^{+/+}$ animals, whereas $Ku80^{+/-}$ mice displayed an intermediate reduction (~20%; Table 1). Without exception, within each litter, $Ku80^{-/-}$ animals always had shorter telomeres than $Ku80^{+/+}$ animals, and $Ku80^{+/-}$ animals always displayed intermediate-length telomeres. Analysis of the distributions of telomere fluorescence demonstrates that telomere shortening involves the vast majority of telomeres (see the Supplementary material available with this article online). A similar level of telomere shortening was observed in another Ku80 knockout mouse strain [17] (M.P. Hande, D. Gilley, and D.J. Chen, personal communication).

We next studied telomere length in tissues derived from young adult mice using flow-FISH [18], a technique based on FISH combined with flow cytometry that allows for quantitation of telomeric DNA in large numbers of intact permeabilized G0–G1-gated interphase cells. Flow-FISH analysis of freshly isolated primary thymocytes and splenocytes from $Ku80^{+/+}$ and $Ku80^{-/-}$ mice revealed a reduction in telomeric DNA in the latter (Table 2). These data establish a role for Ku80 in the control of telomere length in mice. The discrepancy between our findings and those of [19] may reflect technical differences in data analysis or differences in mouse breeding or maintenance.

To investigate whether Ku also plays a functional role in the telomeres of embryonic stem (ES) cells, we studied telomere length by qFISH in $Ku70^{-/-}$ ES cells. We found that Ku70 inactivation leads to telomeric shortening. Both the calculated telomere lengths (Table 1) and the histogram plots (see Supplementary material) show that the $Ku70^{-/-}$ ES cells that we have studied have shorter telomeres than $Ku70^{+/+}$ parental cells (~29 kb versus 52 kb, respectively).

Table 1

Quantitative FISH analysis of telomere length.

MEFs Ku80 genotype		Metaphases analyzed	p arm	q arm	all ends
1st litter	+/+		39.0 ± 0.4	53.8 ± 0.6	46.4 ± 0.4
	+/−		25.2 ± 0.3	28.5 ± 0.5	26.8 ± 0.3
	−/−		18.7 ± 0.2	17.6 ± 0.3	18.1 ± 0.2
2nd litter	+/+		36.2 ± 0.4	46.7 ± 0.6	41.4 ± 0.4
	+/−		31.3 ± 0.4	42.5 ± 0.6	36.9 ± 0.4
	−/−		28.4 ± 0.3	39.5 ± 0.5	33.9 ± 0.3
3rd litter	+/+		34.1 ± 0.5	44.0 ± 0.6	39.1 ± 0.4
	+/−		27.7 ± 0.3	39.5 ± 0.5	33.6 ± 0.3
	−/−		25.2 ± 0.4	30.3 ± 0.4	27.7 ± 0.3
4th litter	+/−		29.3 ± 0.3	42.8 ± 0.6	36.0 ± 0.3
	−/−		20.3 ± 0.4	28.5 ± 0.5	24.4 ± 0.3
Mean	+/+	46	36.8 ± 0.3	48.9 ± 0.4	42.3 ± 0.2
Mean	+/−	61	28.4 ± 0.2	38.5 ± 0.3	33.5 ± 0.2
Mean	−/−	62	22.2 ± 0.2	28.0 ± 0.2	25.1 ± 0.1
ES Ku70 genotype					
+/+		17	46.7 ± 0.4	57.5 ± 0.4	52.1 ± 0.3
−/−		17	29.2 ± 0.3	37.4 ± 0.3	33.3 ± 0.2
ES XRCC4 genotype					
+/+		16	41.4 ± 0.2	55.2 ± 0.2	48.3 ± 0.2
−/− (clone 14–10)		16	38.4 ± 0.2	48.8 ± 0.3	43.6 ± 0.2
−/− (clone 9–12)		15	47.7 ± 0.3	56.9 ± 0.4	52.3 ± 0.2

qFISH analysis of early passage primary MEFs from $Ku80^{+/+}$, $Ku80^{+/-}$, and $Ku80^{-/-}$ embryos, together with the qFISH analysis of wild-type ES cells or $Ku70^{-/-}$ or $XRCC4^{-/-}$ ES cells. Values are expressed in telomere fluorescence units (TFU) as mean ± standard error for the p arm, the q arm, and all ends. One TFU corresponds to 1 kb of TTAGGG repeats.

Besides Ku, the other known NHEJ components are DNA-PKcs and the XRCC4/DNA ligase IV complex. The involvement of DNA-PKcs in the control of telomere length was addressed previously [20] and by others (D. Gilley and D.J. Chen, personal communication), so we investigated whether the XRCC4/DNA ligase IV complex controls telomere length. When we used qFISH to study two independent $XRCC4^{-/-}$ ES cell clones [21], we found no significant and reproducible effects of XRCC4 loss on telomere length (Table 1). In line with this, Southern blot analysis did not reveal marked differences between the telomere length profiles of DNA ligase IV-defective human 180BR cells [22] and their matched controls (see Supplementary material). Together, these data imply that a defect in NHEJ per se does not cause telomeric shortening, and of the known NHEJ components, only Ku, and possibly DNA-PKcs, have additional specialized roles at telomeres. These results are in agreement with those obtained in *S. cerevisiae* and *S. pombe*, where homologs of Ku70, Ku80, XRCC4, and ligase IV are all necessary for NHEJ, but only defects in Ku70 or Ku80 lead to telomeric shortening.

Telomeres are thought to prevent chromosomal fusions and the consequent risk of genomic instability. Consistent with recent studies [6–8], we found that $Ku80^{-/-}$ MEFs were highly aneuploid and had high levels of chromosomal abnormalities (Table 3). Analysis by qFISH of fused chromosomes in $Ku80^{-/-}$ cells indicated that 73% of the fusion points scored were devoid of detectable telomeric repeats. Cells from heterozygous mice ($Ku80^{+/-}$) had intermediate levels of genomic instability. The study of genomic stabil-

Table 2

Flow-FISH analysis of adult mouse thymocytes and splenocytes.

	Thymocytes			Splenocytes		
Ku80 genotype	MESF	CV	p value	MESF	CV	p value
+/+	148.46	9%	–	150.69	7%	–
−/−	107.01	18%	0.001	129.84	12%	0.04

Telomere length analysis by flow-FISH of primary thymocytes and splenocytes. The inactivation of Ku80 leads to the reduction of the telomeric signal. Values are expressed in thousands of molecules of equivalent soluble fluorochrome (MESF). Values are expressed with their CV (coefficient of variation). The statistical significance of signal differences is expressed by the calculated p value.

Table 3

Cytogenetic analysis.

MEFs genotype	Metaphases analyzed	Aneuploid cells	End-to-end fusions (%)[a]	Fusions lacking TTTAGG repeats[a]	Fragments (%)[a]	Telomeres without detectable TTAGGG repeats[b]
$Ku80^{+/+}$	62	0	1 (1.6%) 1RL	0	3 (48%) 2AC, 1C	0
$Ku80^{+/-}$	61	4 (6.6%)	8 (13.1%) 7R, 1DIC	8 (13.1%)	11 (18%) 10AC, 1C	18
$Ku80^{-/-}$	107	35 (32.7%)	119 (111.2%) 55R, 45RL, 19DIC	78 (72.9%)	102 (95.3%) 54AC, 20C, 29F	306
ES genotype		Chromosomes per metaphase (Mean ± SE)[c]				
$Ku70^{+/+}$	50	40.4 ± 0.1	1 (2%) 1RL	1 (2%)	2 (4%) 2AC	0
$Ku70^{-/-}$	50	41.5 ± 0.2	20 (40%) 16 RL, 4DIC	18 (36%)	10 (20%) 9AC,1C	19
ES genotype						
$XRCC4^{+/+}$	50	40.8 ± 0.1	0	0	0	0
$XRCC4^{-/-}$ (14–10 clone)	50	40.5 ± 0.2	5 (10%) 5RL	0	6 (12%) 6AC	0
$XRCC4^{-/-}$ (9–12 clone)	50	40.3 ± 0.2	3 (6%) 3RL	1 (2%)	2 (4%) 1AC	0

Cytogenetic analysis of metaphase chromosomal spreads of primary MEFs and ES cells. Annotations are as follows:
[a] The percentage represents events per 100 cells.
[b] Telomeres with estimated TTAGGG repeats shorter than 200 bp.
[c] ES cells tend to be intrinsically aneuploid, therefore the chromosome number per metaphase is given.
AC = acentric chromosomes, C = centric chromosomes, DIC = dicentric chromosomes, F = fragments, R = rings, and RL = Robertsonian-like fusions.

ity of $Ku70^{-/-}$ ES cells revealed that they also have increased numbers of chromosomal fusions and fragmentations compared to Ku-proficient ES control cells (Table 3). However, Ku-inactivated ES cells showed less genomic instability than Ku-deficient MEFs. Since the HR pathway appears to play a more prominent role in DNA DSB repair in ES cells than in later development [23], we speculate that the existence of an active HR system may compensate in part for the inactivation of the NHEJ machinery and thus reduce the accumulation of chromosomal aberrations in ES cells as compared to MEFs.

In light of the differences between the effects of Ku and XRCC4 on telomere length, we carried out a comparative cytogenetic analysis of ES cells lacking XRCC4 or Ku70. In agreement with previous work [24], we found higher levels of chromosomal aberrations in the $XRCC4^{-/-}$ clones than in the parental cells. However, despite the XRCC4 deletion having been introduced into the same ES cell strain by the same technique and in the same laboratory as the Ku70 mutation [21, 25], $Ku70^{-/-}$ cells had five times more chromosome fusions and more than twice the number of fragmented chromosomes than $XRCC4^{-/-}$ cells. Furthermore, and in line with the telomere measurement data, telomere signals were detectable at most chromosomal fusion points in $XRCC4^{-/-}$ cells, but not at those of $Ku70^{-/-}$ cells. Since previous work has established that inactivation of Ku or XRCC4 impairs DNA NHEJ to a similar extent [25, 26], these data suggest that the extensive chromosomal instability of Ku-deficient cells is not due to a NHEJ defect alone but reflects a combination of defective NHEJ and impaired telomere function.

In conclusion, we have found that Ku70, Ku80, and DNA-PKcs associate with telomeric DNA in a variety of human cell types and under conditions in which telomeres are maintained by either telomerase-based or HR-based mechanisms. In addition, we have found that loss of Ku leads to telomeric shortening in MEFs, adult tissues, and ES cells. These findings support the view that the function of Ku in telomere length control is conserved from yeast to man. However, unlike the situation in yeast, we have found that, at least by the assay methods we used, the association of human Ku with telomeres is apparently resistant to the generation of DNA damage. Furthermore, our data (see Supplementary material) and those of [19] reveal that the loss of Ku in mouse does not lead to the generation of lengthened telomeric single-stranded overhangs. Although the reason for these differences is not yet apparent, they could be linked to DNA-PKcs, which is present in mammals but not in yeasts. Furthermore, although other explanations are possible, our cytogenetic analysis of XRCC4- and Ku-deficient cells sug-

gests that the telomeric shortening induced by the lack of Ku acts in addition to the impairment of repair of endogenous DNA damage to cause the high rates of genomic instability observed. Finally, our observation that $Ku80^{+/-}$ mouse cells have somewhat shortened telomeres and increased genomic instability compared with wild-type controls raises the possibility that inactivation of a single allele for a Ku subunit could lead to heightened genomic instability in humans.

Materials and methods

Mice and cells

Ku80 mice [13] were crossed with 129/Sv mice, and F1 $Ku80^{+/-}$ mice (129/Sv × C57BL/6-albino) were intercrossed to produce $Ku80^{+/+}$, $Ku80^{+/-}$, and $Ku80^{-/-}$ mice. The colony was maintained by the intercross of these progeny and used for experiments. MEFs were isolated from E13.5 embryos derived from intercrosses of $Ku80^{+/-}$ mice. Primary thymocytes and splenocytes were isolated from $Ku80^{+/+}$ and $Ku80^{-/-}$ mice at 6–8 weeks of age. The generation and culture of $Ku70^{-/-}$ ES cells and $XRCC4^{-/-}$ ES cells was as described previously [21, 25].

Telomere length measurements and chromosome analysis

For qFISH analysis, chromosome spreads prepared from mouse embryonic fibroblasts (MEFs) were hybridized with a Cy-3-labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probe. Quantitative analysis of digital images was performed as previously described [16]. Chromosomal aberrations were monitored. All studies were carried out blind. Two experimental set-ups, one in Vancouver and one in New York, were used, and, when the same samples were analyzed, consistent results were achieved. Flow-FISH was used to measure telomere length in a large number of splenocytes and thymocytes [18]. Telomere lengths in the human DNA ligase IV-defective cell line 180BR [22] and its matched control were studied by Southern blot analysis with a telomeric probe.

Chromatin immunoprecipitations (CHIPs)

In vivo crosslinking and chromatin purification was performed as described in [9]. A detailed methodology is supplied as Supplementary material.

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