Role of Human Ku86 in Telomere Length Maintenance and Telomere Capping

Isabel Jaco, Purificación Muñoz, and María A. Blasco

Molecular Oncology Program, Spanish National Cancer Center (CNIO), Madrid, Spain

ABSTRACT

The role of Ku86 at telomeres has been extensively studied in various organisms; however, a role for Ku86 at human telomeres was unknown because Ku86 deletion is lethal for human cells. Here, we used small interference RNA to decrease Ku86 protein levels in human cells. An ~50% reduction in the amount of Ku86 protein was achieved 72 hours after transfection with Ku86-specific small interference RNAs. This decrease in Ku86 levels resulted in a rapid loss of cell viability characterized by increased apoptosis and decreased mitotic index in the cell population. Importantly, Ku86 knockdown was concomitant with a significant loss of telomeric sequences and with increased chromosomal aberrations, including chromatid-type fusions involving telomeric sequences. These findings demonstrate a role for Ku86 in regulating telomere length and telomere capping in human cells, which, in turn, could impact on cancer and aging.

INTRODUCTION

Telomeres are protective structures at the ends of chromosomes that consist of repetitive DNA (TTAGGG repeats in vertebrates) bound to an array of specialized proteins (1). In mammals, TRF1 and TRF2 proteins bind to double-stranded TTAGGG repeats and regulate telomere length and telomere capping (2–4). TRF1 and TRF2 can recruit to the telomere various DNA repair proteins that participate in nonhomologous end joining of double-strand breaks, such as the components of the DNA-PK complex (Ku proteins and the DNA-dependent protein kinase catalytic subunit or DNA-PKcs; refs. 5–11). Telomeres are proposed to protect chromosome ends through the formation of a higher-order chromatin structure, such as that of the so-called telomeric “T-loops”, which are maintained by the ability of the G-strand overhang at telomeres to fold back and invade the double-strand telomere repeats (12). Loss of telomere function can result from loss of TTAGGG repeats in the absence of telomerase activity (13) and/or from loss or mutation of telomere proteins such as TRF2, Ku86, or DNA-PKcs among others (4, 6, 8, 9, 14, 15). Interestingly, loss of function of either TRF2 or DNA-PKcs shares the outcome of increased frequencies of end-to-end fusions that preferentially involve telomeres produced via leading-strand synthesis. This scenario suggests that these proteins are required for the postreplicative processing of the leading strand telomere, possibly to generate the 3’-G-strand overhang, thus contributing to telomere capping (15).

A role for Ku proteins in telomere protection and telomere length regulation has been demonstrated in various eukaryotic systems, including yeast, plants, and mice. However, the outcomes of Ku86 sequences with other gene sequences.

Requests for reprints: Maria Blasco, Telomeres and Telomerase Group, Molecular Oncology Program, Spanish National Cancer Centre (CNIO), Madrid 28029, Spain.

E-mail: mblasco@cnio.es.

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1 Internet address: http://www.ncbi.nlm.nih.gov/BLAST/

2 Internet address: http://www.ensembl.org/Homo_sapiens/blastview.

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The role of Ku86 at telomeres has been extensively studied in various organisms; however, a role for Ku86 at human telomeres was unknown because Ku86 deletion is lethal for human cells. Here, we used small interference RNA to decrease Ku86 protein levels in human cells. An ~50% reduction in the amount of Ku86 protein was achieved 72 hours after transfection with Ku86-specific small interference RNAs. This decrease in Ku86 levels resulted in a rapid loss of cell viability characterized by increased apoptosis and decreased mitotic index in the cell population. Importantly, Ku86 knockdown was concomitant with a significant loss of telomeric sequences and with increased chromosomal aberrations, including chromatid-type fusions involving telomeric sequences. These findings demonstrate a role for Ku86 in regulating telomere length and telomere capping in human cells, which, in turn, could impact on cancer and aging.

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A role for Ku proteins in telomere protection and telomere length regulation has been demonstrated in various eukaryotic systems, including yeast, plants, and mice. However, the outcomes of Ku86 deletion seem to greatly vary in these different organisms, suggesting significant differences in telomere biology. In particular, yeast defec-
of 30% to 50% were transfected with the selected small interference RNAs using Oligofectamine reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions.

**Ku86 Knock Down Using Short Hairpin RNA Vectors**

The short hairpin RNA constructs used for targeted silencing of human Ku86 were selected following the recommendations of OligoEngine (Seattle, WA) and cloned into a pSUPER-retro vector (pRS; kind donation from Dr. Rene Bernards, NKI, Amsterdam; ref. 28). Four different short hairpin RNAs were designed from the Ku86 sequence: shKu86 2.7, shKu86 3.4, shKu86 4.2, and shKu86 5.4. The sequences for short hairpin RNAs are: ATG TGA CAT CTC CCT GCA A for shKu86 2.7, GTG GAT AAC CAT GTT TGT A for shKu86 3.4, CCA GGT TCT CAA CAG GCT G for shKu86 4.2, and GAG CAT AGA CTG CAT CCG A for shKu86 5.4.

As controls, cells were transfected with empty pSUPER-retro and pSUPER-retro expressing a short hairpin RNA specific for green fluorescent protein (pRSGFP). Transfections were performed with the FuGENE (Roche, Basel, Switzerland) transfection reagent following the manufacturer’s instructions. After 48 hours, transfected cells were selected using 2 μg/mL puromycin. Seventy-two hours after transfection cells were recovered for the different experiments.

**Western Blot Assays**

Whole-cell extracts and nuclear extracts were obtained at the indicated times after transfection as described (29). Protein concentration was determined by Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany). Two to 20 μg of protein extracts were separated on SDS-8% polyacrylamide electrophoresis gel and transferred to nitrocellulose membranes (Bio-Rad). The antibodies used were an anti-Ku86 monoclonal antibody (Kamiya biomedical company, Seattle, WA), an anti-Ku70 monoclonal antibody (Kamiya biomedical company), an anti-TRF2 polyclonal antibody (SF08, kindly provided by Rene Bernards, NKI, Amsterdam; ref. 28). Four different short hairpin RNAs were designed from the Ku86 sequence: shKu86 2.7, shKu86 3.4, shKu86 4.2, and shKu86 5.4. The sequences for short hairpin RNAs are: ATG TGA CAT CTC CCT GCA A for shKu86 2.7, GTG GAT AAC CAT GTT TGT A for shKu86 3.4, CCA GGT TCT CAA CAG GCT G for shKu86 4.2, and GAG CAT AGA CTG CAT CCG A for shKu86 5.4.

**Telomerase Assay.** S-100 extracts were prepared from mock and siKu86–2 transfected cells, and a modified version of the telomeric repeat amplification protocol assay was used to measure telomerase activity (31). An internal control for PCR efficiency was included (TRAPeze kit Oncor).

**RESULTS AND DISCUSSION**

Next, we determined whether knocking down Ku86 protein levels with Ku86 expression, although Ku86 protein levels were knocked down by this small interference RNA to ~80% of Ku86 protein levels in siScramble-2 transfected cells (Fig. 1A). In contrast, 72 hours after transfection with the siKu86–2 duplex, Ku86 protein levels were decreased to ~53.2 ± 2.1% and 46.1 ± 2.9% of those present in mock-transfected cells and siScramble-2-transfected cells, respectively (Figs. 1, A–C). Using a FITC-labeled siKu86–2 coupled to flow cytometry, we estimated that the efficiency of transfection with the siKu86–2 duplex was on average of 69.1 ± 1.3%, as measured in several independent experiments.

To convert arbitrary units of fluorescence into kilobases, telomere fluorescence values were extrapolated from the telomere fluorescence of LY-R (R cells) and LY-S (S cells) lymphoma cell lines of known lengths of 80 and 10 kb, respectively (30). We established a linear correlation (r² = 0.999) between the fluorescence intensity of the R and S telomeres with a slope of 38.6. The calibration-corrected telomere fluorescence intensity was calculated as described (9).

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**Telomere assay.** S-100 extracts were prepared from mock and siKu86–2 transfected cells, and a modified version of the telomeric repeat amplification protocol assay was used to measure telomerase activity (31).

**Scoring of Chromosomal Abnormalities**

**Quantitative Fluorescence In situ Hybridization.** The indicated numbers of metaphases from each cell culture (~200) were scored for chromosomal aberrations by superimposing the telomere image on the 4', 6-diamidino-2-phenylindole chromosome image in the TFL-telo software (gift from Dr. Peter Lansdorp, The Terry Fox Laboratory, Vancouver, British Columbia, Canada).

**Chromosome Orientation-Fluorescence In situ Hybridization.** Chromosome orientation-fluorescence in situ hybridization (CO-FISH) was performed as described previously (15). Metaphase spreads were photographed on a Leitz Leica DMRB fluorescence microscope.

**Telomere Length Analysis**

**Quantitative Fluorescence In situ Hybridization.** Metaphase and interphase nuclei were prepared for quantitative FISH and hybridized as described (9). To correct for lamp intensity and alignment, images from fluorospheres (fluorescent beads; Molecular Probes) were analyzed using the TFL-Telo software (gift from Dr. Peter Lansdorp, The Terry Fox Laboratory, Vancouver, British Columbia, Canada). Images were captured using Leica fluorescent microscope. Under this capturing conditions, the fluorescence of individual telomeres as short as 500 bp can be detected and quantified. The TFL-Telo software was used to quantify the fluorescence intensity of telomeres from at least 10 metaphases of each data point. The images of metaphases were captured on the same day, in parallel, and scored blindly.

**Statistical Analysis**

**Student’s t Test.** A Student t test with “two-tails,” “two-samples of unequal variance” (or Welch’s correction), was used to calculate the statistical significance of the observed differences. GraphPad Prism v.3.0a and Microsoft Excel v.2001 were used for the calculations.

For Student’s t test the differences are considered significant for P < 0.05, very significant for P < 0.01, highly significant for P < 0.001, and extremely significant for P < 0.0001.

Next, we determined whether knocking down Ku86 protein levels affected the expression of Ku86-interacting proteins, such as Ku70 and TRF2. For this, we have measured Ku70 and TRF2 proteins levels in mock-transfected cells, as well as in cells transfected with the siKu86–2 and the siScramble-2 duplexes 72 hours after transfection. TRF2 protein levels did not significantly change in siKu86–2-transfected cells and were 106.7 ± 6.3% and 107.5 ± 15.7% of TRF2 levels present in mock-transfected cells and siScramble-2-transfected cells.
cells, respectively, 72 hours after transfection (Fig. 1, B and C). Ku70 protein levels, however, decreased to 69.4 ± 5.2% and 66.3 ± 1.25% of the Ku70 levels in mock-transfected and siScramble-2–transfected cells, respectively, 72 hours after transfection (Fig. 1, B and C). The fact that Ku70 protein levels were decreased in Ku86-interfered cells is in agreement with previous observations indicating that the stability of both proteins is linked (24).

SiKu86–2 duplexes were also used to knock down Ku86 protein levels in two additional human tumor cell lines, SAOS and U2OS (Materials and Methods). Ku86 and Ku70 protein levels were significantly decreased in SAOS and U2OS cell lines transfected with the siKu86–2 duplexes compared with the corresponding controls transfected with the siScramble-2 duplex (Fig. 1D).

To confirm the results obtained with small interference RNA duplexes, additional Ku86 sequences were targeted using four independent short hairpin RNA vectors: shKu86 2.7, shKu86 3.4, shKu86 4.2, and shKu86 5.4 (Materials and Methods; Fig. 2). All of the shKu86 vectors were able to reduce Ku86 protein levels in HeLa cells 72 hours after transfection (Fig. 2A). The shKu86 5.4 construct was selected for additional analysis, because it showed the highest decrease of Ku86 protein levels (see above). The loss of viability of siKu86–2–transfected cells but not of siKu86–1– and siScramble-2–transfected cells is in agreement with the fact that the siKu86–2 duplex was the most efficient in knocking down Ku86 protein levels (see above). The loss

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**Fig. 1.** A, quantification of Ku86 protein levels corrected by actin levels at the indicated times after transfection with siKu86–1, siKu86–2, or siScramble-2 duplexes. The number of independent experiments is indicated for each data point (n); bars, ±SE. B, representative example of Western blots showing Ku86, Ku70, and TRF2 protein levels at 48, 72, and 96 hours after mock-transfection (NT) or transfection with siKu86–2 (S) and siScramble-2 (S). Actin was used as a loading control for total extracts. C, quantification of average levels of Ku86, Ku70, and TRF2 proteins 72 hours after transfection with siKu86–2 and after correction with the corresponding loading controls. The number of independent experiments is indicated (n); bars, ±SE. D, representative images of Ku86 and Ku70 protein levels 72 hours after transfection with siKu86–2 or siScramble-2 duplexes in SAOS and U2OS cell lines. At right, quantification of Ku86 and Ku70 proteins levels after correction with the actin control is also shown.

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**Fig. 2.** A, quantification of Ku86 protein levels corrected by actin levels at 72 and 96 hours after transfection with the indicated shKu86 retroviral vectors. The number of independent experiments is indicated for each data point (n); bars, ±SE. B, quantification of Ku86 and Ku70 protein levels corrected by actin levels at the indicated times after transfection with the shKu86 5.4 retroviral vector. The number of independent experiments is indicated for each data point (n); bars, ±SE.
of viability in siKu86–2–transfected cells was coincidental with a marked decrease in their mitotic index compared with mock-transfected cells (NT) and with siScramble-2–transfected cells (Fig. 3B). In addition, this loss of viability was also coincidental with increased apoptosis as indicated by an increment in poly(ADP-ribose) polymerase cleavage in the siKu86–2–transfected cells compared with mock-transfected cells (NT) and with siScramble-2–transfected cells at different times after transfection (data not shown). Similarly, Annexin V staining at 48, 72, and 96 hours after transfection with the corresponding small interference RNA duplex showed increased apoptosis in siKu86–2–transfected cells compared with cells transfected with the siKu86–2 duplex or with mock-transfected cells (NT; Fig. 3C). As a positive control, cells were treated with staurosporine (Sta) treatment is also shown in the figure. B, representative images of mock-transfected (NT) as well as siKu86–2–transfected SAOS and U2OS cells 72 hours after transfection. Notice the lower density of cells in the siKu86–2–transfected cultures. Percentage of confluency 72 hours after transfection is indicated. E, quantification of mitotic index as percentage of metaphases of the total number of nuclei counted (n = 300). Mitotic index was measured 72 hours after transfection with the indicated shKu86 retrovirus upon treatment of the cells with colcemide. pRSGFP is a control pSUPER-retro vector with sequences against green fluorescent protein.

A similar loss of viability, as determined by a decreased mitotic index, was observed when additional Ku86 sequences were targeted in HeLa cells using four independent short hairpin RNA vectors: shKu86 2.7, shKu86 3.4, shKu86 4.2, and shKu86 5.4 (Fig. 3E).

In summary, knocking down Ku86 protein levels to ~50% of the normal levels with the siKu86–2 duplex leads to a decrease in cell viability, which is concomitant with increased cell death and decreased cell proliferation. This loss of cell viability is not apparent when Ku86 levels are ~80% (siKu86–1 duplex in Fig. 1A) suggesting the existence of a threshold value for Ku86 protein levels beyond which cells start losing their viability. Altogether, these results indicate that Ku86 is required for the viability of human cells, in agreement with previous reports using a human Ku86 knockout cell line (24). The important role of Ku86 in human cells is in contrast with the fact that Ku86-deficient mice are viable (20), thus suggesting differences in the role of Ku86 in humans and mice (see below).

Knocking Down Ku86 Protein Levels in Human Cells Results in Telomere Shortening. To study the impact of knocking down human Ku86 protein levels on telomere length, we performed quantitative FISH with a PNA-telomere probe on metaphases prepared from cells transfected with either siScramble-2 or siKu86–2 duplexes at 72
hours after transfection (Fig. 4A; Materials and Methods). A clear decrease in telomere length was observed in metaphases prepared from siKu86−2−2−2 transfected cells compared with cells transfected with siScramble-2 duplex at 72 hours after transfection (Fig. 4A). The decrease in telomere length was highly significant (Student’s t test P < 0.0001) after comparing >2,240 telomere values for each condition. The telomere shortening observed in siKu86−2−2 transfected cells was concomitant with an increase in signal-free ends (chromosome ends with no detectable TTAGGG signal as indicated by quantitative FISH). In particular, siKu86−2−2−2 transfected cells showed a total of 10.7% of signal-free ends compared with 4.3% in the case of siScramble-2−2 transfected cells (Fig. 4A).

We have described recently that one of the early events of DNA-damage–induced apoptosis is a dramatic loss of telomere sequences in...
those cells undergoing apoptosis (33). The telomere shortening observed in the siKu86–2–transfected cells, however, is not likely to be the consequence of the increased apoptosis in these cells, because we used metaphase spreads (cells that have started mitosis) to measure telomere length. This conclusion is additionally supported when using the quantitative FISH technique on fixed interphase nuclei, which permits the measurement of telomere length in all of the viable cells of the population and not only in those that have undergone cell division (Materials and Methods). Again, telomere fluorescence was significantly decreased in siKu86–2–transfected cells at 72 hours after transfection, compared with the cells transfected with the siScramble-2 duplex (Fig. 4B). Student’s t test including >7,665 telomere fluorescence values for each cell population showed that the difference in telomere length between the siScramble-2–transfected cells and the siKu86–2–transfected cells was extremely significant (P < 0.0001). This telomere shortening was reflected by the decrease in the frequency of telomeres with >800 arbitrary units of fluorescence, as well as by the increase in the frequency of telomeres with <400 arbitrary units of fluorescence (see green and red lines in Fig. 4B). Interestingly, it is predominantly the loss of extra-long telomeres that seems to account for the differences in telomere length between the siScramble-2–transfected cells and the siKu86–2–transfected cells.

These results were confirmed when additional Ku86 sequences were targeted using short hairpin RNA vectors. In particular, HeLa cells transfected with shKu86 5.4, which we have shown previously to have 59 ± 7.3% and 65 ± 7.5% of Ku86 and Ku70 protein levels, respectively, compared with pRSGFP-transfected cells at 72 hours after transfection, also showed a highly significant shortening of telomeres as shown in Fig. 4C (Student’s t test P < 0.0001).

In summary, these results show that a decrease in the Ku86 protein levels leads to a significant and rapid telomere shortening in human cells, thus suggesting that Ku86 is very important for telomere length maintenance in human cells. This finding is in contrast with Ku86 deletion in mice, which leads to telomerase-mediated telomere elongation (17, 23). These differential effects of Ku86 abrogation on telomere length in human and mouse cells suggest important differences in telomere biology between these two species and, in particular, on the roles of Ku86 at the telomere.

Next, we used in-gel hybridization in native conditions to examine the integrity of the telomeric G-strand overhang in Ku86 knocked-down cells (Materials and Methods). However, no significant difference in the intensity of the G-strand–specific signals was detected between siKu86–2–transfected cells and cells transfected with the siScramble-2 duplex (data not shown). Interestingly, the G-strand overhang has been shown to be elongated in Ku-deficient Saccharomyces cerevisiae mutants, as well as in Ku86-deficient plants (18, 34), but to retain its normal length in Ku86-deficient mice (5, 9, 23). The differential impact of Ku86 deletion on the G-strand overhang integrity, again suggests differences in telomere biology among these various organismal systems, and, in particular, on the role of Ku86 at telomeres.

**Normal Levels of In Vitro Telomeric Repeat Amplification Protocol Telomerase Activity in Human Cells with Knocked Down Ku86 Protein Levels.** It has been shown recently that human Ku70/ Ku86 complex interacts with the catalytic component of telomerase, hTert, thus suggesting that Ku proteins may also regulate telomerase activity in human cells (35). To investigate whether the telomere shortening shown by human cells knocked down for Ku86 was the result of down-regulation of telomerase activity, we measured telomerase activity levels using the telomeric repeat amplification protocol assay in mock-transfected and in siKu86–2–transfected cells (Materials and Methods). However, telomerase activity was similar in mock-transfected cells and in cells transfected with the siKu86–2 duplex (data not shown), indicating that knockdown of Ku86 protein in human cells does not alter the in vitro activity of the telomerase complex. This does not exclude, however, that human Ku86 could regulate the action of telomerase at telomeres, as it has been shown before for mouse and plant Ku86 proteins (17, 23). In this regard, the fact that cells knocked down for Ku86 have shorter telomeres than the noninterfered controls may suggest that Ku86 has a role in the recruitment of telomerase to human telomeres or in the protection of telomeres from exonuclease activities.

**Cells Knocked Down for Ku86 Show Increased Chromatid-type Fusions With Telomere Signals at the Fusion Point and Preferentially Involving Telomeres Produced by Leading-strand Synthesis.** To investigate the putative role of human Ku86 in telomere capping, we studied chromosomal aberrations spontaneously arising in siKu86–2–transfected cells, as well as in mock-transfected cells and cells transfected with the control siScramble-2 duplex. For quantification of chromosomal aberrations we used two independent techniques, telomere quantitative FISH and chromosome orientation FISH (Materials and Methods). First, we performed telomere quantitative FISH on >200 metaphases from each siKu86–2– and siScramble-2–transfected cell, as well as from the corresponding mock-transfected cells, at 72 hours after transfection (Materials and Methods; Table 1). siKu86–2–transfected cells showed a 2-fold increase in interchromosomal chromatid-type fusions compared with the mock-transfected cells and the cells transfected with the siScramble-2 duplex, 0.02 and 0.01 fusions per metaphase, respectively (Table 1; Fig. 5, for example). Of notice, these chromatid-type fusions always showed telomere signals at the fusion point as determined by quantitative FISH (indicated with an arrow in Fig. 5). It is unlikely that these fusions resulted from an intact chromatid fused to one with critically short telomeres, because properly capped/normal-length telomeres are protected from DNA repair events. Therefore, the existence of end-to-end chromatid fusions with telomeres at the fusion point suggests that they were produced by telomere uncapping rather than by critical telomere shortening. These results are in agreement with the fact that Ku86–interfered cells show normal levels of telomerase activity (see above), as well as with previous findings showing that Ku86-deficient mice show increased end-to-end fusions with long telomeres at the fusion point (9, 22, 23). Also in agreement with this notion, siKu86–2–transfected cells did not show significantly increased frequencies of

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**Table 1 Analysis of chromosomal aberrations in mock, siScramble-2, and siKu 86–2–transfected cells using quantitative FISH**

<table>
<thead>
<tr>
<th>Metaphase no</th>
<th>Chromosome fusions* (TTAGG)</th>
<th>Chromosome fusions* (+TTAGG)</th>
<th>Chromatid fusions* (TTAGG)</th>
<th>Chromatid fusions* (+TTAGG)</th>
<th>Sister Chromatid fusions* (TTAGG)</th>
<th>Breaks and Fragments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT (72 h)</td>
<td>250</td>
<td>0.03</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Scramble (72 h)</td>
<td>150</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Ku86 (72 h)</td>
<td>340</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. Increased chromosomal instability was observed in cells presenting low levels of Ku86, particularly a 2-fold increase in chromatid-type telomere fusions was observed in siKu86–2–transfected cells compared to the mock and the siScramble-2 controls. These fusions always contained telomere signals (+TTAGG) repeats at the fusion point.

* Frequency of chromosomal aberrations per metaphase.
dicentric chromosomes lacking telomere signals at the fusion point compared with the mock-transfected cells (Table 1; Fig. 5, for example).

The increase in chromatid-type fusions involving telomeres at the fusion point in the Ku86 knockdown cells could suggest a role for this protein in the postreplicative processing of telomeres produced by leading-strand synthesis, as has been proposed for DNA-PKcs and TRF2 (15). In particular, Bailey et al. (15) demonstrated that deficiency in either TRF2 or DNA-PKcs (a Ku-interacting protein) lead to an increase in end-to-end chromatid-type fusion specifically involving leading-strand telomeres (telomeres generated through leading-strand synthesis). This finding indicated that both TRF2 and DNA-PKcs are required for the postreplicative processing of this strand to generate a proper telomere capping structure. Such processing could be the formation of a G-strand overhang, which, in turn, could allow the formation of T loops (15). Therefore, the current notion is that the leading-strand telomere has to be processed to form a proper capping structure. The chromosome orientation FISH technique allows identification of telomeres produced by leading-strand DNA synthesis (Materials and Methods; ref. 15). Therefore, to study the involvement of telomeres produced by leading-strand synthesis in the chromatid-type fusions present in the Ku86 knockdown cells, we performed chromosome orientation FISH on >90 metaphases from both siKu86–2–transfected cells and from the corresponding mock-transfected cells 72 hours after transfection. All of the chromatid-type fusions present in siKu86–2–transfected cells were of the leading-to-leading type and showed a frequency of 0.022 fusions per metaphase compared with 0.01 in the mock-transfected cells (Fig. 5, for example; arrow indicates telomeres produced by leading-strand synthesis at the fusion point). The frequency of leading-to-leading chromatid-type telomere fusions in siKu86–2–transfected cells is comparable with that described previously for single DNA-PKcs deficiency (15), thus suggesting a role for Ku86 in the processing of the leading-strand telomere, possibly to generate the 3’-G-strand overhang and contribute to telomere capping (15). Of note, the occurrence of telomere fusions in Ku86 knocked-down cells is significantly lower than the frequency of end-to-end chromosome fusions (Robertsonian-like fusions) described previously for Ku86-deficient mice (8, 9, 22, 23). In addition, a role for mouse Ku86 in leading-strand processing has not been demonstrated to date. It is likely that the fact that mouse chromosomes are acrocentric could account for these differences between humans and mice. In particular, whereas end-to-end fusions of human chromosomes are very unstable, end-to-end fusions of mouse chromosomes are stably transmitted in culture (36). Chromosome breaks and fragments were also significantly increased in the Ku86 knocked-down cells compared with the controls (Table 1; NT versus siKu86–2, \( P = 0.03; \) siScramble-2 versus siKu86–2, \( P < 0.0001 \)). The increased frequencies of breaks and fragments in siKu86–2–transfected cells are in agreement with the known role of Ku86 in nonhomologous end-joining of double-strand DNA breaks.

In summary, these results show an ~2-fold increase in chromatid-type fusions in the Ku86 knocked-down cells compared with the controls, thus indicating a role for human Ku86 in telomere capping and, in particular, in the capping of telomeres produced by leading-strand synthesis. **Concluding Remarks and Significance.** The results presented here suggest a role for Ku86 in telomere length maintenance and telomere capping in human somatic cells. In particular, Ku86 knockdown by using siKu86 duplexes results in significant telomere shortening and increased chromatid-type fusions involving telomeres with detectable TTAGGG signals. It is possible that part of these effects could be also mediated by the expected down-regulation of the Karp1 transcript, which encodes for a longer isoform of Ku86 in human cells (37). The fact that most of the chromatid-type fusions present in human cells knocked-down for Ku86 involve telomeres produced by leading-strand synthesis suggests a role for human Ku86 in the proc-
ROSSING OF TELOMERES PRODUCED BY LEADING-STRAND SYNTHESIS, SIMILAR TO THAT PROPOSED BEFORE FOR DNA-PKCs AND TRF2 (15). IN ADDITION, Ku86 KNOCKDOWN RESULTS IN A FAST LOSS OF TELOMERIC SEQUENCES. THIS IS IN MARKED CONTRAST TO Ku86 DELETION IN MICE OR PLANTS, WHICH RESULTED IN TELOMERASE-DEPENDENT TELOMERE ELONGATION, POINTING TO SIGNIFICANT DIFFERENCES IN TELOMERE BIOLOGY BETWEEN DIFFERENT ORGANISMS.

THE ROLES OF HUMAN Ku86 IN TELOMERE LENGTH MAINTENANCE AND TELOMERE CAPPING, PREDICT THAT HYPOMORPHIC MUTATIONS OF Ku86 COULD DRAMATICALLY IMPACT ON AGING AND CANCER IN HUMANS. FINALLY, THROUGH ITS SIMULTANEOUS ROLES ON TELOMERE BIOLOGY AND DOUBLE-STRAND BREAK REPAIR, Ku86 DEPLETION MAY INCREASE THE SENSITIVITY TO DNA DAMAGE AGENTS IN A SYNERGISTIC MANNER, BECAUSE SHORT TELOMERES HAVE BEEN SHOWN TO RESULT IN INCREASED SENSITIVITY TO IONIZING RADIATIONS (29).

OF NOTE, IMPORTANTLY, THE RESULTS PRESENTED HERE AGREE WITH FINDINGS REPORTED RECENTLY BY MYUNG ET AL. (38), USING A DIFFERENT EXPERIMENTAL APPROACH.

REFERENCES
Role of Human Ku86 in Telomere Length Maintenance and Telomere Capping

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