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ARTICLE

Extensive Telomere Erosion in the Initiation of Colorectal Adenomas and Its Association With Chromosomal Instability

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Background Telomere shortening, dysfunction, and fusion may facilitate the acquisition of large-scale genomic rearrangements, driving clonal evolution and tumor progression. The relative contribution that telomere dysfunction and/or *APC* mutation play in the chromosome instability that occurs during colorectal tumorigenesis is not clear.

Methods We used high-resolution telomere length and fusion analysis to analyze 85 adenomatous colorectal polyps obtained from 10 patients with familial adenomatous polyposis and a panel of 50 colorectal carcinomas with patient-matched normal colonic mucosa. Telomerase activity was determined using the telomeric repeat amplification protocol. Array-CGH was used to detect large-scale genomic rearrangements. Pearson correlation and Student *t* test were used, and all statistical tests were two-sided.

Results Despite the presence of telomerase activity, we observed apparent telomere shortening in colorectal polyps that correlated with large-scale genomic rearrangements ($P < .0001$) but was independent of polyp size and indistinguishable from that observed in colorectal carcinomas ($P = .82$). We also observed apparent lengthening of telomeres in both polyps and carcinomas. The extensive differences in mean telomere length of up to 4.6 kb between patient-matched normal mucosa and polyps were too large to be accounted for by replicative telomere erosion alone. Telomere fusion events were detected in both polyps and carcinomas; the mutational spectrum accompanying fusion was consistent with alternative nonhomologous end joining.

Conclusions Telomere length distributions observed in colorectal polyps reflect the telomere length composition of the normal originating cells from which clonal growth was initiated. Originating cells containing both short telomeres and *APC* mutations may give rise to polyps that exhibit short telomeres and are prone to telomere dysfunction, driving genomic instability and progression to malignancy.

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Chromosomal abnormalities such as aneuploidy and nonreciprocal translocations are a hallmark of epithelial cancer (1) and are believed to result from a state of chromosomal instability (CIN) (2). CIN is a dynamic process that refers to an accelerated rate of gains and losses of whole chromosomes or segments of chromosomes (3,4). These chromosomal rearrangements occur early during carcinogenesis and are thought to promote cancer progression (5,6).

Among other mechanisms (7), telomere dysfunction is considered to be a major driving force in the generation of chromosomal rearrangements during tumor progression (8–10). Telomeres protect the natural ends of chromosomes by preventing them from being recognized as DNA double strand breaks (11). In somatic cells, telomeres erode at each cell division because of the "end-replication problem" (12,13) but can also shorten as a consequence of stochastic deletion events (14). In the absence of fully functional DNA damage checkpoints, telomeres can shorten to a length at which they become dysfunctional and capable of fusion with other telomeres or with nontelomeric loci (15–17). Telomere fusion can lead to the formation of anaphase bridges and initiate cycles of breakage–fusion–bridge (18). In human cell lines, breakage–fusion–bridge can result in the numerical and structural chromosomal changes generally observed in epithelial cancers (19,20). In vivo, the concept that telomere dysfunction can promote carcinogenesis by initiating chromosome rearrangements is supported by late-generation, telomerase-deficient, p53-mutant mice, in which telomere dysfunction is associated with the development of carcinomas containing nonreciprocal translocations, aneuploidy, regional amplifications, and deletions (8,10).

The role, if any, that telomere dysfunction may play in driving the progression of human colorectal cancer is not entirely clear. It has been well established that telomere shortening, relative to normal mucosa, occurs in colorectal carcinomas (21–23), but no erosion was detected in adenomatous polyps except in regions of

high-grade dysplasia (24,25). In addition, anaphase bridging, used in this context as a surrogate marker of telomere dysfunction, has been reported at the adenoma/carcinoma transition (26). The interpretation of these data was that telomere dysfunction at this transition induces CIN and initiates colorectal carcinoma (25). This view is in contrast with the presence of large-scale genomic rearrangements that are frequently observed in colorectal adenomatous polyps (27–29), including some of the very earliest lesions, where over 90% of 2-mm adenomas displayed evidence of allelic imbalances (5). Furthermore, telomerase activity is detected in colorectal polyps (22,30,31), and this is enhanced in the context of mutations within the adenomatous polyposis coli (*APC*) gene (32). Mutations within *APC* are considered to be initiating events in colorectal tumorigenesis (33,34). *APC* mutation can induce telomerase activity by the Wnt pathway (32,35); this occurs indirectly by Myc controlling hTERT transcription (36) and directly by β-catenin that, with Klf4, binds the hTERT promoter (37). Furthermore *APC* mutation has been implicated in CIN, the mechanism of which has not been resolved and is contentious (38) but may include the role of APC in the regulation of cytoskeletal proteins and mitotic spindles leading to chromosomal segregation defects (39). Alternatively *APC* mutation may impact CIN downstream of activated Wnt signaling, which may include a deregulation of cell cycle checkpoints, including G1/S (40), a DNA-damage checkpoint activated by telomere dysfunction (11).

Given the role that telomerase plays in telomere length maintenance and the possible role that APC plays in CIN, it is difficult to reconcile telomerase activity and *APC* mutations with telomere-driven genomic instability in polyps. We therefore sought to reexamine the role that telomere length may play in colorectal tumorigenesis in the context of *APC* mutation. To do this, we made use of high-resolution telomere length and fusion analysis to analyze telomere dynamics in a panel of adenomatous colorectal polyps derived from patients with familial adenomatous polyposis with defined germline mutations of *APC* and a panel of sporadic colorectal carcinomas.

Methods

Samples

DNA samples from colorectal carcinomas and patient-matched normal colonic mucosa were obtained from the Wales Cancer Bank. Specimens from familial adenomatous polyposis patients were obtained from the Institute of Medical Genetics, Cardiff University. Informed consent was obtained according to protocols approved by the Multi-Centre Research Ethics Committee for Wales (ref. MREC 02/09/22 and 12/WA/0071).

DNA Extraction, Single Telomere Length Analysis (STELA), Telomere Fusion Assay, and Telomeric Repeat Amplification Protocol (TRAP)

Genomic DNA from adenomatous polyps was extracted using standard proteinase K/phenol/chloroform protocols after homogenization using a handheld rotor-stator homogenizer (TissueRuptor; Qiagen, Hilden, Germany) (41). For telomere length analysis at 17p and XpYp, we used the modified STELA protocol (15,41). For analysis of telomere fusions, we used a protocol and oligonucleotides described previously (15,17). Quantification of fusion frequencies at the XpYp and 17p telomeres was based on the number of fusion molecules that could be characterized at the DNA sequence level as a function of the number of input DNA molecules into each polymerase chain reaction. We estimated the frequency and 95% confidence interval of normal samples exhibiting no detectable fusion events using the modified Wald method (42). Telomerase activity was detected using the TRAPeze XL Telomerase detection kit (Chemicon International, Billerica, MA).

Array CGH analysis

Array comparative genomic hybridization (CGH) was carried out as a service by Nimblegen (Roche NimbleGen, Madison, WI); copy number gains and losses were identified using the segMNT algorithm included in NimbleScan software. We defined a gain of chromosomal material if the $\log_2$ ratio was 0.5 or greater (adenomatous polyp/normal cell DNA ratio) or loss of chromosomal material if the $\log_2$ ratio was –0.5 or less.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA), and all statistical tests were two-sided. Pearson correlation and the Student *t* test were used. A *P* value of less than .05 was considered statistically significant.

Results

Telomere Erosion in Colorectal Adenomas

We examined telomere length in the early stages of colorectal tumorigenesis. To do this, we used STELA; STELA is capable of detecting extreme telomere erosion and telomere length distributions within the length ranges at which telomere dysfunction can occur (14,15,17,41,43). We analyzed the XpYp and 17p telomeres in a panel of 85 adenomatous polyps, together with patient matched normal colonic mucosa obtained from 10 patients with familial adenomatous polyposis (Figure 1A). The telomere length distributions at 17p and XpYp were similar in each sample analyzed ($P < .0001$, Pearson) (Figure 1A). The overall mean telomere length was statistically significantly shorter in the polyps than in their normal adjacent tissue (4.7 kb in polyps vs 6 kb in normal; $P = .01$, *t* test) (Figure 2B; Supplementary Figure 1A, available online). Of the 85 polyps analyzed, 58 (17p) and 62 (XpYp) exhibited shorter telomere length distributions than their associated normal mucosa, with mean length differentials of 1.4 kb and 1.9 kb, respectively (Figure 1B); of these, more than 64% were statistically significantly different ($P < .05$) (Figure 2D; Supplementary Figure 2B, available online). Several polyps (n = 10 of 85 17p; n = 12 of 85 XpYp) displayed statistically significant telomere lengthening compared with normal mucosa ($P < .05$) (Figure 2D; Supplementary Figure 2B, available online). Strikingly the telomere length distributions varied considerably, with distinct distributions observed in different polyps from the same individuals (Figure 1, A and B). Variation of up to 4.9 kb was observed between polyps in some individuals, and this variation appeared to arise because of different degrees of telomere erosion/elongation compared with the normal adjacent matched samples; for example, individual 2G had polyps exhibiting telomere length differences

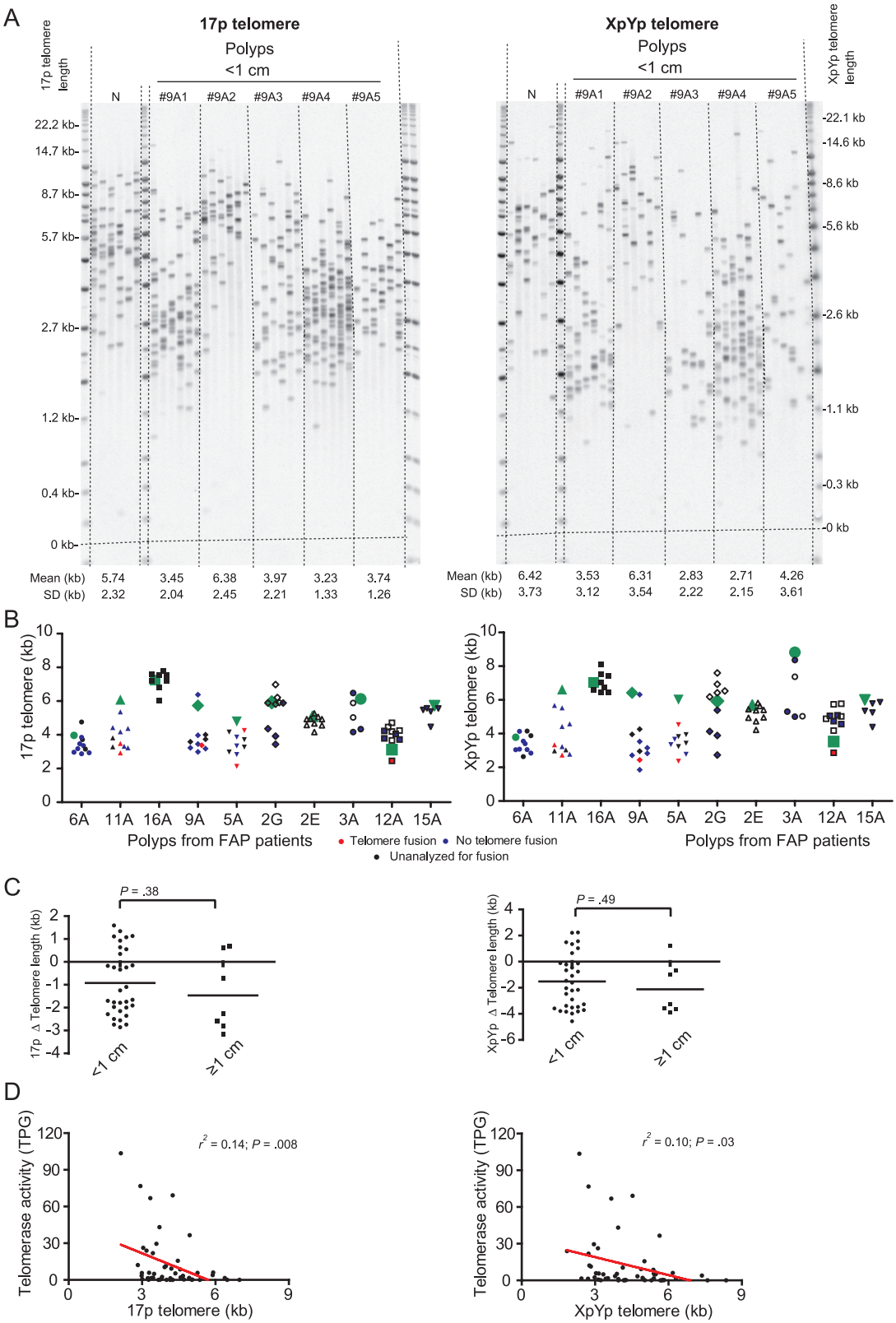

Figure 1. Telomere length distributions in adenomatous polyps are variable. **A**) Single telomere length analysis at the 17p and XpYp telomeres in five polyps, all with a size of less than 1 cm from the same individual, together with patient-matched normal mucosa (N). Mean telomere length and standard deviation are indicated below. **B**) Mean length of the 17p and XpYp telomeres in a panel of adenomatous polyps, together with the patient matched normal tissue (**green**). **Blue symbols** indicate samples that were analyzed for fusion, but no fusions could be detected; **red symbols** indicate samples in which fusion could be detected. **C**) Change in telomere length compared with patient-matched normal mucosa, in polyps less than or greater than 1 cm. **D**) Relationship between telomerase activity displayed as total product generated (TPG) with telomere length at 17p and XpYp. FAP = familial adenomatous polyposis.

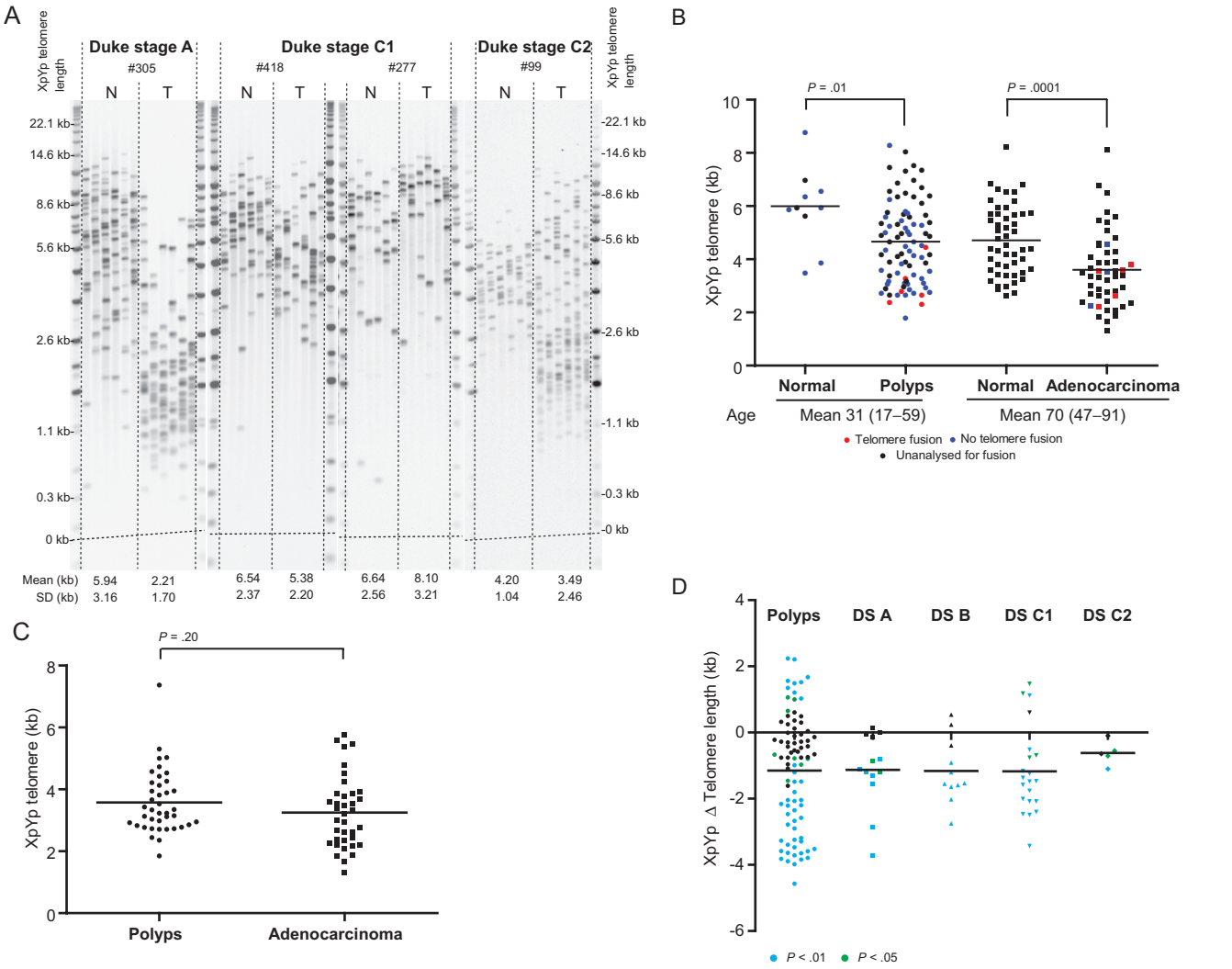

Figure 2. Telomere length distributions in colorectal carcinomas are similar to that observed in adenomatous polyps. **A**) Single telomere length analysis showing matched normal mucosa (N) and tumor (T). Mean telomere length and standard deviation are indicated below. **B**) Overall XpYp telomere length in normal, adenomatous polyps, and carcinomas. Mean age and range are indicated below. **C**) The telomere XpYp lengths of adenomatous polyps and carcinomas that display statistically significant differences from the normal ($P < .05$, *t* test). **D**) Change in XpYp telomere length of polyps and carcinomas broken down by Dukes stage compared with matched normal mucosa; **color coding** refers to the statistical significance of the difference. All statistical tests were two-sided.

between +1.7 kb to −3.2kb (Figure 1B). The heterogeneity of the telomere length profiles generated with STELA can reflect the clonality of the cell populations analyzed (41,44): the telomere length profiles observed in polyps ranged from homogeneous distributions consistent with clonal growth to heterogeneous profiles that may reflect either oligoclonality or subclonal diversification within the polyp (Supplementary Figure 1B, available online)(45). This will also reflect the proportions of neoplastic and nonneoplastic cells in different polyps, a situation that would result in an overestimation of the telomere length of the clonal tumor population. The degree of telomere erosion in the polyps was not correlated with their size (Figure 1C). Telomerase activity was readily detected in the colorectal polyps analyzed (n = 50), and a weak inverse correlation with telomere length was observed at both XpYp ($P = .03$) and 17p ($P = .008$) (Figure 1D).

We examined XpYp and 17p telomere length in a panel of 50 colorectal carcinomas, together with patient matched normal colonic mucosa, thereby allowing a direct comparison with the adenomatous polyps. As previously reported (21,23,46–48), the telomere length distributions were statistically significantly shorter at both chromosome ends in carcinomas compared with the normal adjacent tissue (XpYp: 3.6kb in carcinomas vs 4.7kb in normal tissue samples; $P = .0001$) (Figure 2, A and B; Supplementary Figure 1A, available online). A total of 41 of 50 carcinomas (82%) exhibited shorter XpYp telomeres, with a mean 1.5 kb shorter than their associated normal tissue (n = 35 of 45 [78%] for 17p; mean difference of 1.2 kb). Telomere length did not statistically significantly differ between the different tumor stages (XpYp: 3.3 kb in Dukes stage A samples, 3.2 kb in Dukes stage B, 4 kb in Dukes stage C1, and 3.5 kb in Dukes stage C2; $P = .30$).

Telomere length decreased as a function of age in normal mucosal tissue samples at a rate of 26 bp/year (±11bp/year; $P = .03$) (Supplementary Figure 1C, available online). This accounts for why the normal matched adjacent tissues of the polyps (patients

mean age = 31) have longer telomeres than the normal matched samples of the carcinomas (patients mean age = 70) (Figure 2B).

We compared the telomere length distributions of the carcinomas with the adenomatous polyps that exhibited statistically significant telomere erosion compared with patient-matched normal tissue ($P < .05$, t-test; n = 40 or 85 polyps and n = 37 of 50 carcinomas). Importantly, this showed no statistically significant difference in mean telomere lengths between the polyps and the carcinomas at both telomeres analyzed ($P = .20$ at XpYp; $P = .82$ at 17p) (Figure 2C; Supplementary Figure 2A, available online). Furthermore, there was no statistically significant difference in the overall degree of telomere erosion between the polyps and the carcinomas (XpYp mean difference = 1.2 kb in the polyps and 1.1 kb in all carcinomas, 1.1 kb in Dukes stage A, 1.2 kb in Dukes stage B, 1.2 kb in Dukes stage C1, and 0.62 kb in Dukes stage C2) (Figure 2D; Supplementary Figure 2B, available online). The biggest single telomere length difference between a sample and the normal matched tissue was 4.6 kb at XpYp telomere, and this was observed in a polyp.

Short Telomeres and Fusion in Adenomas and Colorectal Carcinomas

We have previously shown that short dysfunctional telomeres can be subjected to fusion with other telomeres and nontelomeric loci in experimental cells undergoing crisis in vitro (15,17) and in chronic lymphocytic leukemia (43,49). Importantly, we observed that telomere erosion and dysfunction in chronic lymphocytic leukemia can precede clinical progression, a situation that appeared similar to that observed here in adenomatous colorectal polyps. We therefore investigated whether the short telomeres detected in colorectal polyps and carcinomas were capable of fusion. Using our single molecule telomere fusion assay, we determined the baseline of fusion at the XpYp and 17p telomeres in normal colorectal tissue. We analyzed a total of 4.7 million diploid genome equivalents from seven matched normal samples and found no verifiable fusion events, representing an estimated frequency of 4.3×10^{-7} ($\pm 5.9 \times 10^{-7}$) (42); this frequency was consistent with our previous observations in normal cells (15,49). We detected fusion in six of 45 adenomatous polyps and in five of eight colorectal carcinomas involving the XpYp or 17p telomeres (Figures 1B, 2B, and 3A); fusion events were verified by sequence analysis (Figure 3B). Interestingly, the frequencies of fusion in the six polyps and in the five carcinomas displaying fusions were comparable (mean fusion frequency of the six polyps = 4×10^{-6}; mean fusion frequency of the five carcinomas = 6×10^{-6}; $P = .25$) (Figure 3A). In one carcinoma sample, a clonal telomere fusion event was identified involving the 17p telomere only (Supplementary Figure 2C, available online).

Molecular Structure of Telomeric Fusions in Colorectal Adenomas and Carcinomas

To characterize the telomere fusions that we detected in colorectal adenomas and carcinomas, we isolated and sequenced across the fusion point in a total of 50 fusion events (Figure 3B; Supplementary Figure 3, available online). In doing so, we observed that 14 chromosome ends were involved, at different frequencies, in these fusions (Figure 4A). Microhomology was observed at the fusion point in 78% of the fusion events, with a mean 2.3 nts of perfect homology between the participating telomeres (Figure 4B). All the fusion events involved a deletion of one (29%) or both (71%) telomeres into the telomere-adjacent DNA up to the limits of the assays (Figure 4C) (17). In addition to interchromosomal fusion events, we also managed to characterize four 17p–17p fusions in carcinomas that were consistent with fusion between sister chromatids; all of these exhibited an extensive deletion of the subtelomeric DNA (Figure 3B).

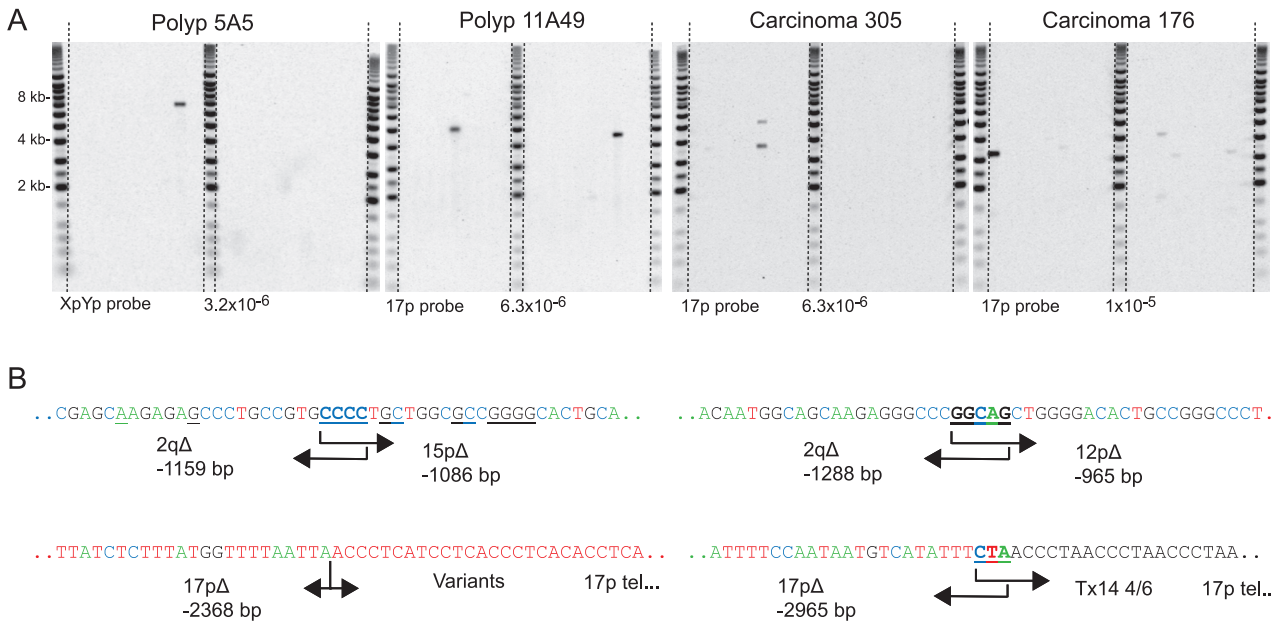

Figure 3. Telomeres in both adenomatous polyps and carcinomas are capable of fusion. **A**) Examples of telomere fusion assays in colorectal adenomatous polyps and carcinomas. **B**) Examples of the DNA sequence of telomere fusion events isolated from polyp samples and carcinomas; this includes two 17p-17p fusion events. The two participating telomeres are indicated together with the extent of deletion in the subtelomeric DNA or the number of telomere repeats (T) or variants as indicated. Homology between the two participating telomeres is **underlined**.

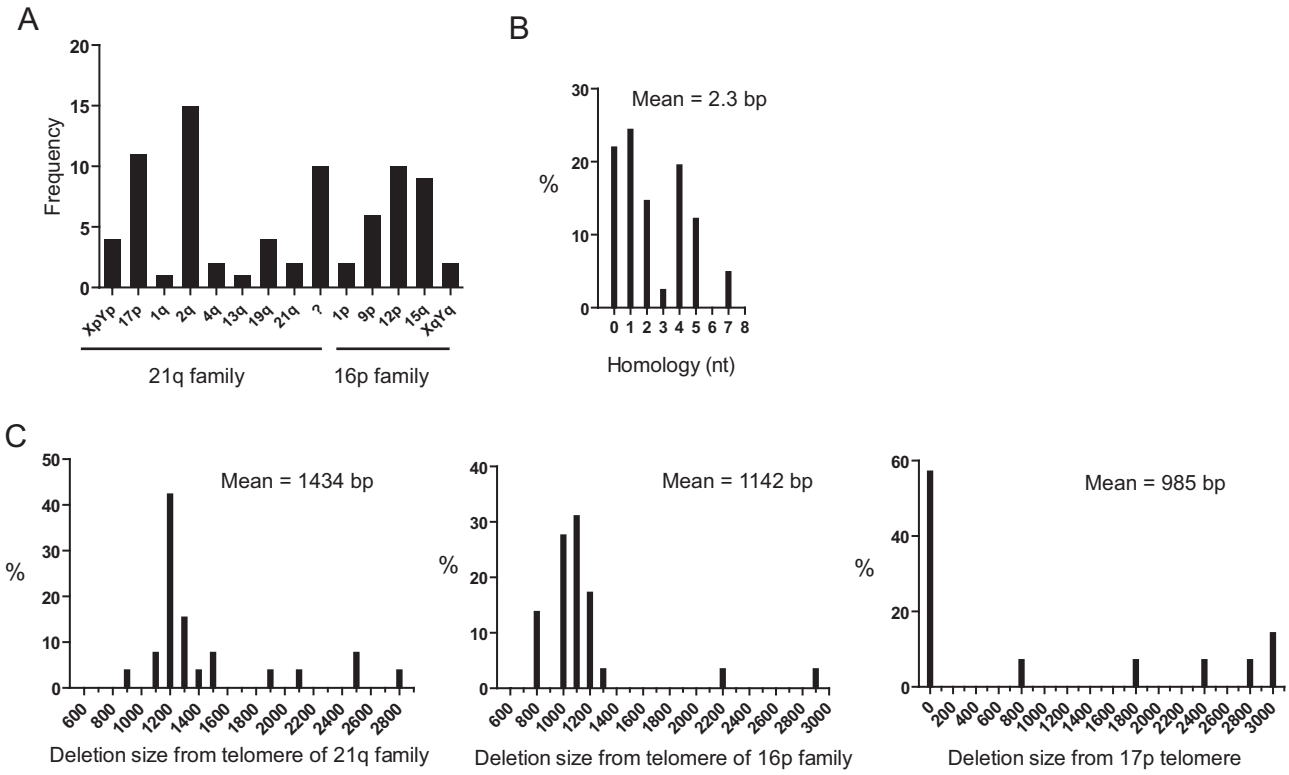

Figure 4. Telomere fusion in colorectal adenomatous polyps and carcinomas displays a distinct mutational profile. **A**) Histogram depicting the frequency of fusion distributed across the different telomeres analyzed. These included XpYp; 17p; 1q, 2q, 4q, 13q, 19q, and 21q for the 21q family of telomeres; 1p, 9p, 12p, 15q, and XqYq for the 16p family of telomeres; and an additional previously uncharacterized chromosome end related to the 21q family of telomeres (denoted with a "?") **B**) Histogram depicting the extent of homology at the fusion point. **C**) Histograms depicting the subtelomeric deletion from the start of the telomere repeat array.

Telomere Erosion and Aneuploidy in Adenomas

We examined whether the short dysfunctional telomeres observed in adenomas were associated with aneuploidy. We analyzed 20 colorectal adenomatous polyps harboring different telomere length distributions with array CGH (Figure 5A). For each patient analyzed, we selected polyps displaying short telomeres and a polyp from the same patient showing a substantially longer telomere length distribution, and a further four polyps with long telomeres from three other patients were also tested. We observed evidence of aneuploidy in eight polyps, all of which displayed at least the loss or gain of a whole chromosome or a large portion of a chromosome (Figure 5A). All polyps displaying aneuploidy harbored short telomere length distributions (Figure 5, A–C); indeed, the presence of aneuploidy in adenomas was statistically significantly correlated with telomere shortening ($P < .0001$) (Figure 5D).

Discussion

A key theme to emerge from our data was that telomere length is extremely variable between different polyps obtained from the same individuals, with polyps displaying both longer and shorter distributions that were independent of polyp size. The extent of the differences in telomere length between patient-matched normal tissue and adenomatous polyps was considerable, with apparent gains in mean telomere length compared with matched normal tissue of up to 2.2 kb and reductions of up to 4.6 kb compared with matched normal tissue (Figures 1C and 2D). If one makes the supposition that these large reductions in length arise as a consequence of replicative telomere erosion during clonal expansion within an adenomatous polyp, then at a mean rate of erosion of 90 bp per population doubling, as determined in cells that do not express telomerase (41,44), a reduction of 4.6 kb would represent more than 50 population doublings of cell division. However, because telomerase was readily detected in the majority of polyps, the rate of telomere erosion will be less, or indeed it may even be negligible (50), in which case the number of cell divisions required to generate a polyp exhibiting the observed length differentials will be considerably greater than 50 population doublings. There is little information about the number of cell divisions that are expected to generate a cellular mass the size and composition of an adenomatous colorectal polyp. However, the hypothetical total mass of cells that could be generated after a minimum of 50 cell divisions of clonal expansion from a single cell is orders of magnitude larger than that of a typical polyp. It is therefore difficult to reconcile the extent of telomere erosion described here with replicative telomere erosion occurring as a function of cell division during the generation of a polyp.

The telomere length profiles observed in polyps were indistinguishable from those observed in the carcinomas. Apparent elongation and erosion was observed in carcinomas, the extent of which was similar to that observed in polyps. Furthermore, focusing only

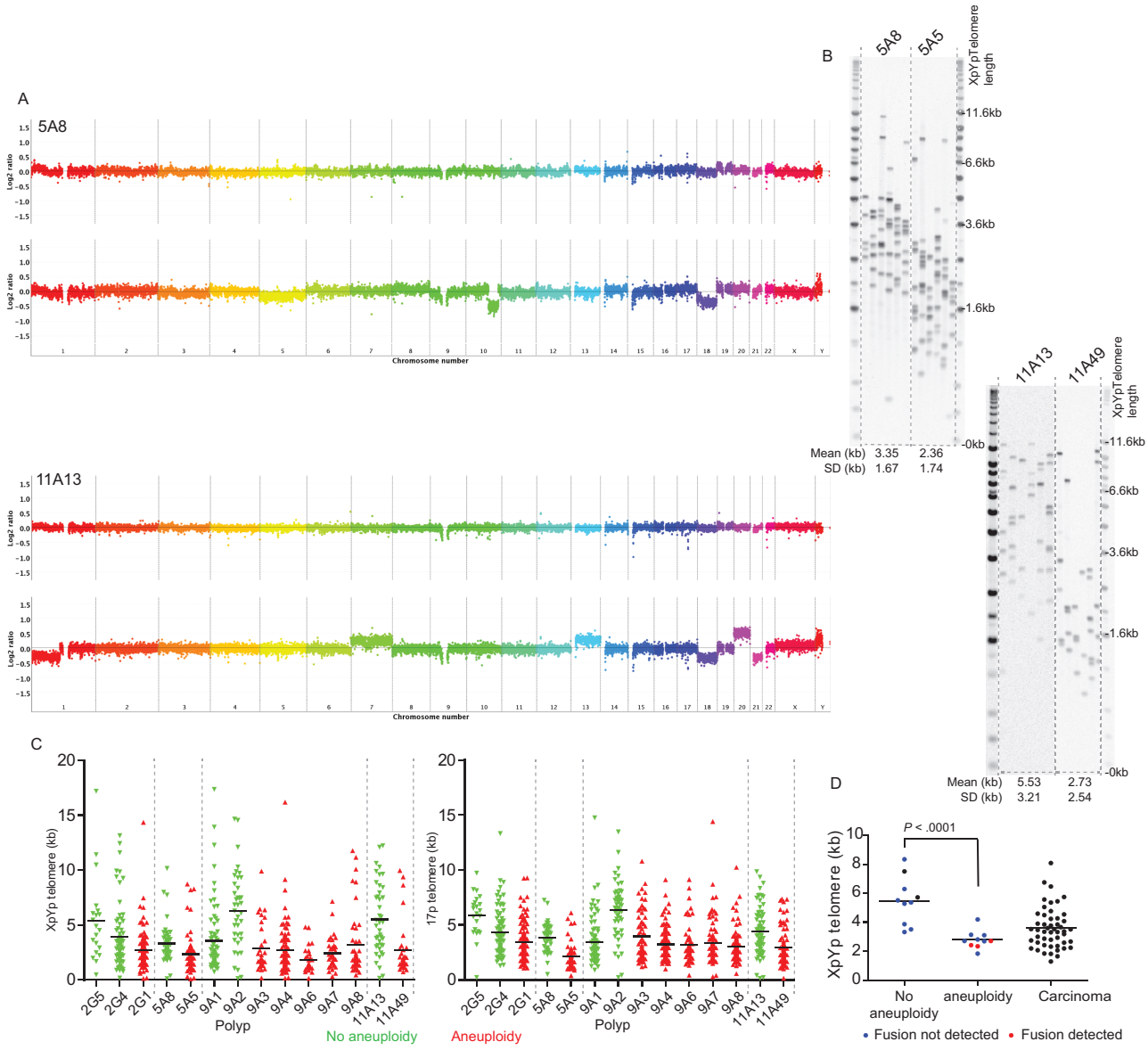

Figure 5. Telomere shortening is associated with large-scale genomic rearrangements. **A**) Array CGH (NimbleGen 135k whole genome array) on polyps display long and short telomeres, as shown by single telomere length analysis (**B**). **C**) Scatter plots of telomere length at the XpYp and 17p telomeres in polyps analyzed with array CGH; **green** indicates where no genomic rearrangements could be identified, and **red** indicates in which clear copy number changes could be identified. **D**) Scatter plot depicting the telomere length of polyps where copy number changes could or could not be identified.

on samples that displayed statistically significant telomere shortening compared with the matched normal tissue, there was no difference between polyp and carcinoma samples. Our data therefore demonstrate that the telomere length distributions observed in colorectal carcinomas are in place before the adenoma/carcinoma transition.

Telomere fusion was detected in adenomatous colorectal polyps and carcinomas. Sequencing of telomere fusion events revealed a distinct mutational profile marked by short patches of microhomology at the fusion points and deletion of the subtelomeric regions. This profile was indistinguishable from those that we have observed previously in sporadic fusion events in normal cells, cells undergoing crisis in vitro (15, 17), and during the progression of chronic lymphocytic leukemia (49); these data indicate a common mechanism of telomere fusion. In human and mouse cells, the fusion between short dysfunctional telomeres is independent of ligase IV and may require Mre11 (51). Together these data are consistent with error-prone alternative-NHEJ processes being involved in the fusion of short dysfunctional telomeres in colorectal tumor cells.

The presence of end-to-end chromosome fusions in adenomatous polyps provides direct evidence that the excessive telomere shortening observed in premalignant tissue can lead to dysfunctional telomeres. Moreover, the positive correlation between telomere erosion and the presence of large-scale, clonal, genomic rearrangements indicates that telomere dysfunction promotes CIN early in the formation of a polyp. Although this is correlative, it is consistent with similar observations in other tumor types (49)

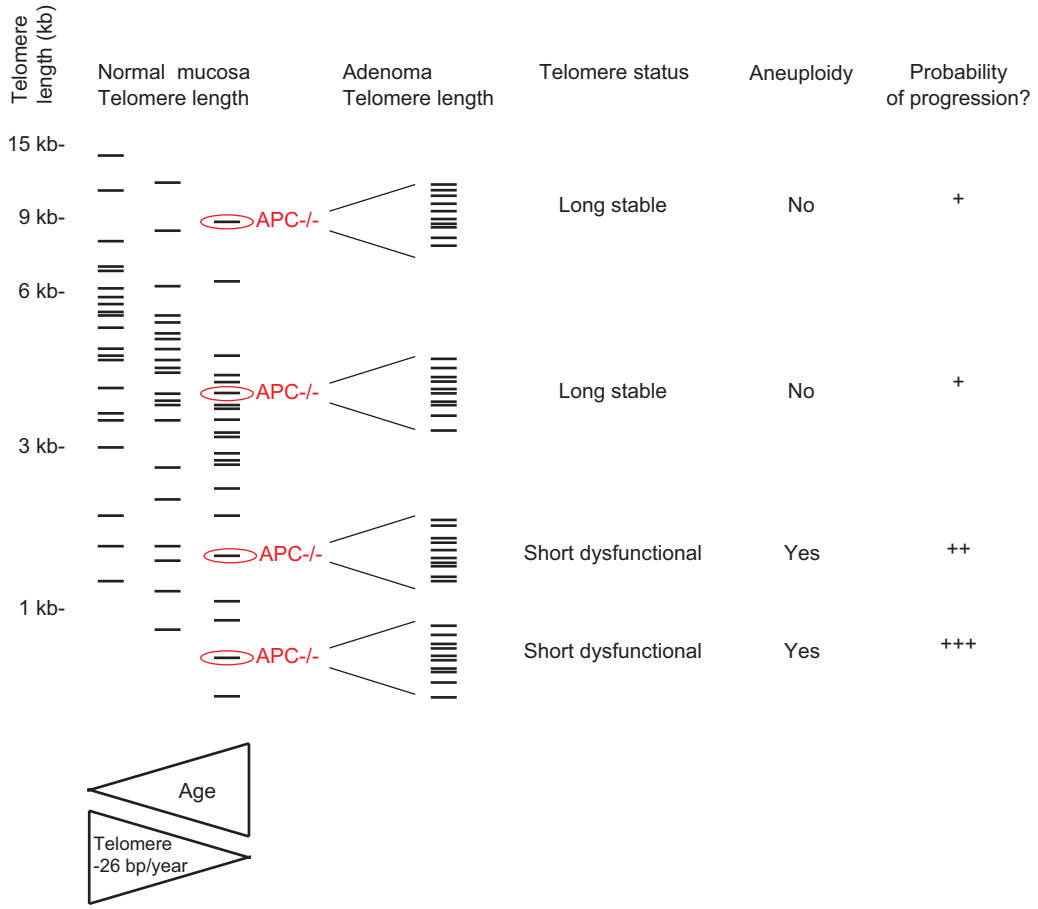

Figure 6. Schematic illustrating our model to explain telomere length profiles in colorectal adenomatous polyps.

as well as a large body of evidence using mouse models showing that the fusion of short dysfunctional telomeres can drive genomic instability and large-scale genomic rearrangements that can facilitate tumor progression (8–10). The majority of the chromosomal aberrations that we observed in adenomas, such as the gain of 13, 7 and 20q and losses of 18, 5, and 1p, have been previously reported in many studies and have been detected in both adenomas and carcinomas (27,52–54). The recurrence of particular chromosomal aneuploidies in adenomas and their maintenance in carcinomas show that these specific imbalances are not random and give a selective advantage for the tumor progression.

CIN is detected in colorectal adenomas irrespective of size (55); indeed CIN can be detected in very small polyps (5). Furthermore, markers of proliferation are detected in polyps of all sizes (55). In our dataset, telomere shortening in adenomas was not correlated with the size of the polyps. Different polyps of the same size from the same patients harbored statistically significantly different telomere length distributions. This observation implies that the degree of telomere erosion in adenomas may not result only from an increased proliferative capacity.

This study has limitations. Both the adenoma and carcinoma samples were not cell purified and will have contained variable amounts of nontumor or nonadenoma cells, including stromal cells and infiltrating lymphocytes. These would have the effect of increasing the apparent telomere length heterogeneity and mean telomere length, thus the extent of telomere shortening documented here is likely to be an underestimate. Furthermore the adenomas analyzed in this study were derived from familial adenomatous polyposis patients; future studies should verify our findings in sporadic adenomas.

To account for our data, we propose that the telomere length profiles observed in colorectal adenomas are dictated by the telomere length of the originating cells. Normal colorectal mucosa display heterogeneous telomere length distributions, with cells containing telomeres within the length ranges observed in the colorectal polyps (Figure 1A). The route by which normal cells suffer telomere erosion is influenced by age and likely arises from differences in the replicative histories and levels of telomerase expression of cells within these tissues. In this situation, *APC* mutation and activation of the Wnt pathway in colonic epithelial cells with long telomeres give rise to adenomas with long telomeres; these telomeres are stable and are not prone to fusion. *APC* mutation in cells with short telomeres gives rise to adenomas with short telomeres that have a propensity to undergo fusion; the resulting CIN leads to large-scale genomic rearrangements that can drive the progression to malignancy (Figure 6). Our data therefore indicate that telomere erosion precedes the initiation of colorectal adenomagenesis, and this may provide a mechanism that may contribute to the age-associated profile of colorectal carcinomas.

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