Analysis of telomeric chromatin using pyrrole-imidazole polyamide probe

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Abstract

Mammalian telomeres consist of a long array of repetitive sequence (TTAGGG) and cap chromosome ends to maintain chromosome stability. Without the telomerase activity that can elongate the telomeres, the telomere length shortens every cell division. Upon reaching a critical short length, the telomeres trigger cellular senescence, which can suppress abnormal cell growth relating to tumorigenesis. Telomerase reactivation circumvents the growth limitation by the telomere-dependent senescence and leads to immortalization. Induction of recombination between telomeres, termed alternative lengthening of telomeres (ALT), can also lead to a similar effect leading to immortalization and tumorigenesis. In this thesis, to investigate telomeres in cancer cells by imaging, proteomics, and RNA interactome, I performed two projects: telomere visualization in tissue sections and isolation of telomere-associated noncoding RNAs.

Initially, I report a development of a telomere-visualization method in mouse and human tissue sections using PI polyamide probes. PI polyamides are DNA binding compounds that can be designed to target predetermined DNA sequences. To visualize telomeres in cultured cells and tissue sections, fluorescent in situ hybridization (FISH) method has been used as a ‘standard’ method. However, this method needs a DNA denaturation step for probe hybridization by harsh heating and formamide treatments, and carries the risk of destroying cellular structures which make it difficult to co-stain telomeres with a protein using an antibody. Because PI polyamides bind to the target sequences in the minor groove of double-stranded DNA without denaturation step, this compound is
compatible with immunostaining and has an advantage compared to FISH method. As collaboration with HiPep Laboratories and Prof. Sugiyama’s group at Kyoto University, a fluorescent PI polyamide probe (HPTH-59) that target the mammalian telomere sequence with high specificity was developed. I showed that HPTH-59 visualized telomeres not only in cultured cells but also in mouse and human frozen tissue sections. Double staining with HPTH-59 and antibodies were performed. By quantitatively measuring the telomere length in clinical tissues from an esophageal cancer patient, I found that a cell population positive for a proliferation marker, Ki-67 protein, had shorter telomeres than do the Ki-67-negative cells in non-tumor tissue sections. From these results, I propose that PI polyamides are promising alternative for telomere labeling in cell biology as well as clinical research.

Next, I investigated the chromatin composition of telomeres. Telomeric DNA is three-dimensionally organized as chromatin, where nucleosomes are associated with non-histone proteins and RNAs. It still remains unclear how the telomeric chromatin structure regulates telomere maintenance because of limited information of telomeric chromatin composition. Although various methods were previously developed to identify telomere-binding proteins, there is still no reliable technique for unbiased identification of both proteins and RNAs including non-coding RNAs (ncRNAs) associated with telomeric chromatin. Since HPTH-59 polyamides bind to telomere DNA with high affinity in a mild condition, I expected that HPTH-59 enable us to analyze telomeric chromatin not only by imaging but also by affinity purification for proteomics and RNAomics. To dissect the telomeric chromatin composition, I show a novel approach of locus-specific chromatin purification using a telomere-targeting PI polyamide, and
Finally, from my studies I emphasize that understanding the nature of telomeric chromatin in different types of cancers from various angles is important for basic and clinical aspects. In addition, I anticipate that PI polyamide will be a powerful tool for chromatin imaging and the chromatin composition analysis of other regulatory sequences.
Chapter 1

Telomere Visualization in Tissue Sections using Pyrrole–Imidazole Polyamide Probes

1.1. Introduction

Telomeres protect the end of chromosomes. In mammalian chromosomes, the telomeres consist of a repetitive sequence (TTAGGG)n to prevent chromosome instability and can be elongated by an enzyme “telomerase”. In differentiated cells that do not have the telomerase activity, the telomere length gradually decreases with every cell division. Upon reaching a critical length, the telomeres can induce cellular senescence, which suppresses further cell division. The senescence is thought to inhibit the abnormal growth associated with tumorigenesis (Blackburn 2010; Zakian 2012; Smogorzewska and de Lange 2004). Reactivation of the telomerase can induce cells to circumvent growth limitation by the telomere-dependent senescence and lead to immortalization (Shay and Bacchetti 1997; Kim et al. 1994). Additionally, induction of recombination between telomeres, termed alternative lengthening of telomeres (ALT), can also lead to a telomere elongation resulting to immortalization from the telomere-dependent senescence (Bryan et al. 1997; Bryan et al. 1995).

Telomere length can be used as a diagnostic marker to detect immortalized cells with short telomeres or ALT cells with much longer telomeres. Thus far, quantitative fluorescent in situ hybridization (Q-FISH) with a peptide nucleic acid (PNA) probe has
been widely used to visualize relative telomere length in individual cells (Lansdorp et al. 1996; Henderson et al. 1996; Levsky and Singer 2003). Many clinical studies of telomere length measurement by Q-FISH have demonstrated that there are types of cancer cells have shortened telomeres (Meeker, Gage, et al. 2002; Meeker, Hicks, et al. 2002; Ferlicot et al. 2003). However, the FISH method requires harsh treatment using heat and 50% formamide for probe hybridization, which carries the risk of destroying cellular structures. Indeed, only a few studies have performed telomere labeling along with immunostaining for a cell marker (e.g., tumor marker) that can provide results with physiological relevance in human tissue sections (Meeker et al. 2004). Additionally, simple and less time-consuming steps and labeling reproducibility, such as clinical studies with numerous samples, are preferable for high-throughput experiments. The ‘gold standard’ for labeling telomeres, FISH, still has issues to be resolved, including for instance, the time required and convenience.

*N*-methylpyrrole (P)–*N*-methylimidazole (I) (PI) polyamides bind to the minor groove of double-stranded DNA without denaturation and can recognize Watson–Crick base pairs (Figure 1) (Trauger, Baird, and Dervan 1996; White et al. 1998; Chenoweth and Dervan 2009; Dervan 2001; Dervan and Edelson 2003; Dervan, Doss, and Marques 2005; Bando and Sugiyama 2006; Blackledge and Melander 2013). PI polyamide is an alternative to the nucleic acid probes, which require denaturing genomic DNA, before probe hybridization. Maeshima et al. a tandem hairpin PI polyamide probe with a fluorophore (TH59) that target human telomere sequences (TTAGGG)n under mild conditions (Maeshima, Janssen, and Laemmli 2001). A hairpin structure is composed of two antiparallel linear compounds folded in a U-shape and is connected to another.
hairpin moiety through a hinge segment (Figure 2). To increase the binding selectivity of TH59, my collaborators developed a method for the robust synthesis of TH59 and derivatives of TH59 with higher specificity for human telomeres in the cell (Kawamoto et al. 2013; Hirata et al. 2014; Kawamoto et al. 2015).

Here, I show that HPTH59-b, which has an optimal hinge connecting two hairpins, visualizes telomeres not only in cultured cells but also in mouse and human frozen tissue sections (Figure 3). The tissue sections were co-stained with HPTH59-b and antibodies for cell-specific markers to compare the telomere lengths in various cell populations in the tissue sections. By quantitatively measuring telomeres in clinical tissue samples from an esophageal cancer patient, I found that a cell population positive for a proliferation marker, Ki-67 has shorter telomeres than those in the Ki-67-negative cells in non-tumor tissue sections. Therefore, PI polyamide provides an advantageous alternative for the measurement of telomere length in clinical research.

Figure 1. A structural model of HPTH59-b binding to DNA.
Figure 2. Chemical structure of HPTH59-b. TAMRA is tetramethylrhodamine. In the box, the base recognition profile of HPTH59-b is shown.

Figure 3. A simple scheme for telomere labeling using HPTH59-b. Green dots indicate signals from telomere repeats.
1.2. Results

*Telomere length measurement by quantitative PI polyamide labeling*

Several studies have shown that telomeres in cancer tissues are often shorter than those in normal tissues (Hastie et al. 1990; Bryan et al. 1998; Sommerfeld et al. 1996), thus, quantitative telomere measurement should make an important contribution to investigations of tumorigenesis. To verify the quantification of telomere length by PI polyamide labeling, I stained the telomeres in cells having different lengths of telomere repeats with fluorescently labeled HPTH59-b (Hirata et al. 2014) and 4', 6-diamidino-2-phenylindole (DAPI) (Figure 4, 5). The telomere lengths of HeLaS3, HeLa1.3, and U2-OS ALT cells are 2–10 kb, ~23 kb, and <3 to >50 kb, respectively (Takai et al. 2010; Scheel et al. 2001).
Figure 4. Telomere labeling with HPTH59-b in human cultured cells.

Telomerase-active cells (HeLa1.3 and HeLaS3) and alternative lengthening of telomere (ALT) cells (U2-OS) were stained with DAPI (first column) and HPTH59-b (second column). Merged images are in the third column. The fourth column shows merged images of HPTH59-b staining and differential interference contrast (DIC) images. Note that some U2-OS cells have extra-nuclear telomere signals (yellow arrowhead).
Figure 5. Distribution histograms of telomere signal intensities in HeLa1.3 and HeLaS3 cells. Signal intensities in HeLa1.3 (long telomere) and in HeLaS3 (short telomere) cells are shown in blue and red, respectively. The overlapping area of the two distributions is shown in purple. The numbers of telomere signals measured for HeLa1.3 and HeLaS3 cells are 13171 dots from 280 cells and 10805 dots from 277 cells, respectively. Median values of the intensity distributions for HeLa1.3 and HeLaS3 are 50.0 (blue arrow) and 26.7 (red arrow), respectively. To compare these median values, the Wilcoxon rank sum test was used (P<0.01).
As shown in Figure 4, HeLa1.3 cells exhibited more intense HPTH59-b signals than HeLaS3 cells. Quantitative measurement of their signal intensities normalized to the DAPI signal indicated that the telomere signals of HeLa1.3 cells were significantly higher than those of HeLaS3 (Figure 5). Moreover, quantitative measurement of telomere signals on mitotic chromosomal spreads also showed that the signals at every chromosomal end of HeLa1.3 cells were higher than those of HeLaS3 cells (Figure 6, 7), suggesting that the telomeres of HeLa1.3 are longer than those of HeLaS3 cells. In U2-OS cells, I observed the strongest telomere signals among the three cell lines (Figure 4). Additionally, U2-OS cells had several intense signals even outside the nucleus, which have been reported to be extra-chromosomal telomere DNA in the cytoplasm (Tokutake et al. 1998). These results suggested that the observed signal intensity of HPTH59-b can distinguish different telomere lengths among these cultured cells.
Figure 6. Telomere labeling in chromosomal spreads with HPTH59-b.
HeLa1.3 (1st row) and HeLaS3 (2nd row) cell spreads stained with DAPI (1st column) and HPTH59-b (2nd column). The 3rd column is the merged images.
Figure 7. Distribution histograms of telomere signal intensities in HeLa1.3 and HeLaS3 cell spreads.

Telomere signal intensities in HeLa1.3 (6564 dots from 38 cells) and HeLaS3 (10895 dots from 43 cells) cell spreads are shown in blue and red, respectively.

Median values of signal intensities in non-tumor and tumor tissues are 194 (blue arrow) and 135 (red arrow), respectively. The overlapping area of the two distributions is shown in purple. To compare these median values, the Wilcoxon rank sum test was used (P<0.01).
Telomere labeling in tissue sections

Next, to examine whether the telomere labeling by HPTH59-b can be applied to cells in tissues, I prepared frozen tissue sections of a mouse (20-µm thickness) and stained them with fluorescent HPTH59-b (green). Images of lung and brain regions are presented in Figure 8. Intense HPTH59-b foci in the DAPI-staining DNA region were clearly detected in both tissues. To test the telomere-targeting efficiency of HPTH59-b, I stained mouse tissue sections with HPTH59-b and anti-TRF1 (telomere-binding protein) antibody simultaneously and compared the signals. I observed strong signals of HPTH59-b with low background noise, whereas TRF1 signals were weak with high background noise in the brain and lung (Figure 9A). Additionally, substantially more signals were detected by HPTH59-b than by TRF1 (Figure 9A, B). On the other hand, in cultured murine cells (MC12), the intensity and number of HPTH59-b signals were comparable to those of TRF1 signals (Figure 9B). Thus, the HPTH59-b probe might label telomeres in tissue sections more efficiently than immunostaining.
Figure 8. Co-staining with HPTH59-b and TRF-1 antibody in cultured cells and tissue sections. First column, DAPI signal; second column, HPTH59-b signal; third column, merged images of DAPI and HPTH59-b. Enlarged images of the boxed region in the upper part are shown in the lower part.
Figure 9. Co-staining with HPTH59-b and TRF-1 antibody in cultured cells and tissue sections. (A) Mouse embryonic carcinoma cells (MC12) and frozen mouse tissue sections stained with DAPI (blue), HPTH59-b (red) and TRF1 antibody (green). Enlarged images of the boxed region in the upper row are shown in the lower row. (B) Venn diagrams of telomere signals derived from HPTH59-b and TRF1. The number of dots was extracted from images in (A).
Knowing the telomere length of a specific cell population in a tissue, especially at the single-cell level, is very useful. To explore telomere length in a specific cell type, I focused on the telomere length in a germ cell line, which has high telomerase activity. DEAD box proteins (DDX), putative RNA helicases, are specifically and highly expressed in the germ cell lineage in both sexes, and they are widely used as a marker of germ cell lineage (Fujiwara et al. 1994; Toyooka et al. 2000). Testis sections containing gonadal tissues, where primordial germ cells (PGCs) are located, were treated with HPTH59-b (red) and anti-DDX4/MVH antibody (green). Immunostaining with anti-DDX4/MVH antibody specifically labeled the cytosol of PGCs, and HPTH59-b showed clear foci in both the germ cells and somatic cells (Figure 10). The telomere signals in PGCs seemed to be weaker than those in PGC marker-negative cells. The PGC marker-positive cells showed a unique characteristic of nuclear organization: less DAPI staining over the nucleus, which is consistent with a previous report (Yoshioka, McCarrey, and Yamazaki 2009). The quantitative measurement of telomere signals normalized to the DAPI signal suggested that PGCs had slightly longer telomeres than the PGC-negative cells (Figure 11). These results demonstrated that HPTH59-b highlighted telomeres and the antibody marked PGCs simultaneously, thereby demonstrating that double staining with HPTH59-b and antibodies can be used to label telomeres in a specific cell lineage in tissue.
Figure 10. Telomere labeling in mouse germ cells and somatic cells. Neonatal (P0) mouse gonads stained with DAPI (blue), anti DDX4/MVH antibody (germ cell marker; green), and HPTH59-b (red). Enlarged images of the boxed region in the first-row images are shown in the second row.
Figure 11. Distribution histograms of telomere signal intensities in germ cells and somatic cells. I analyzed 19 cells (469 dots) and 19 cells (390 dots) in germ (blue) and somatic (red) cells, respectively. The identical image containing PGCs in Figure 10 is shown at the top. Median values of signal intensities in germ and somatic cells are 220 (blue arrow) and 152 (red arrow), respectively. The overlapping area of the two distributions is shown in purple. To compare these median values, the Wilcoxon rank sum test was used (P<0.01).
Shorter telomeres in human tumor cells

Telomere length is strongly connected to cell immortalization and tumorigenesis. Indeed, telomere shortening has been observed in carcinoma derived from bladder, esophageal, gastric, head and neck, ovarian, and renal cells (Wentzensen et al. 2011; Zhang et al. 2015). To further investigate telomeres in human neoplastic cells, I performed simultaneous labeling in esophageal cancer tissue using HPTH59-b and an antibody for a proliferation marker Ki-67, which is highly expressed in cells that are actively dividing, but it is absent in cells under a quiescent state, such as cells in the G0 phase of the cell cycle (Schluter et al. 1993). I found that some cell fractions in the lesions were still Ki-67-positive (green) and the fluorescent intensity of telomere foci appeared lower than that of Ki-67-negative cells (Figure 12). Using digital image analysis, I quantitatively compared telomere signals (Figure 13). The distribution map of the telomere signals revealed two distinct peaks between tumor and non-tumor tissues. These results suggest that highly proliferating tumor cells have shorter telomeres than non-dividing cells, and HPTH59-b can detect differences in telomere length between tumor and non-tumor cells in the tissue.
Figure 12. Different telomere lengths between human tumor and non-tumor tissue sections. Frozen sections of esophageal tumor/non-tumor tissue stained with DAPI (blue), anti-Ki-67 (growth marker; green) antibody, and HPTH59-b (red). Enlarged images of the boxed region in the upper images are shown in the lower part.
Figure 13. Distribution histograms of telomere signal intensities in tumor and non-tumor tissue sections.

Telomere signal intensities in tumor (511 dots from 54 cells) and non-tumor (635 dots from 50 cells) are shown in red and blue, respectively. Median values of the signal intensities in non-tumor and tumor tissues are 27.8 (blue arrow) and 16.7 (red arrow), respectively. The overlapping area of the two distributions is shown in purple. To compare these median values, the Wilcoxon rank sum test was used (P<0.01).
1.3. Discussion

To demonstrate and verify the application of PI polyamide to clinical studies, I used PI polyamide HPTH59-b, along with immunostaining, to visualize telomeres in mouse and human frozen tissue sections. My quantitative analysis of the telomere signals also suggested that highly proliferating cells in tumor tissue had shorter telomeres than non-dividing cells in non-tumor tissue. Although the issue of whether telomere shortening is a consequence of cell proliferation during tumor expansion or a cause of tumorigenesis initiation is controversial, HPTH59-b provides a simple and quick detection method for telomere alteration at the single-cell level, thereby contributing to the genetic diagnosis of malignancy and drug response in patients.

Telomere visualization by HPTH59-b has several advantages over the Q-FISH method. First, the sample preparation process is much faster. Even with tissue sections, incubation with HPTH59-b for just 1 hour is sufficient for staining telomeres. Moreover, even when co-staining with HPTH59-b and an antibody for cell-specific markers, the whole procedure can be completed within 4.5 hours. The second advantage is high sensitivity. As shown in Figure 9A, HPTH59-b generated more telomere signals with lower background noise than did immunostaining with a TRF1 antibody, whereas with cultured cells, HPTH59-b showed a staining efficiency comparable to that of a TRF1 antibody. In the case of thicker tissue sections, the probe size might be critical to accessing telomeres, and HPTH59-b (2.67 kDa) is much smaller than antibodies (~150 kDa). The third advantage is that HPTH59-b labeling can be carried out under mild conditions. This probe can also detect telomere sequences in small DNA molecules in the cytoplasm of ALT cells, which have extra-chromosomal circular DNA of telomeric
repeats due to the high activity of recombination (Dunham et al. 2000; Nabetani and Ishikawa 2011). Only a few studies using the conventional FISH method have ever reported the extra-chromosomal DNA in cytoplasm (Tokutake et al. 1998). This is probably because, unlike large genomic DNA, such small circular plasmid-like DNAs may be easily washed away during the harsh hybridization process and/or easily re-annealed, preventing the nucleic acid probes from hybridization. In contrast, HPTH59-b binds to dsDNA TTAGGG repeats without denaturation, and it may have a similar labeling efficiency for the extra-chromosomal and chromosomal telomere repeats.

Two new telomere labeling methods that use genome editing systems have recently been reported. One involves telomere labeling using a transcription activator-like effector (TALE)-based strategy in both fixed and living mammalian cells (Ma, Reyes-Gutierrez, and Pederson 2013; Miyanari, Ziegler-Birling, and Torres-Padilla 2013). Although the TALE-based method is applicable to live-cell imaging, it is time consuming (i.e., for plasmid construction and establishment of stable cell lines expressing the fluorescent-TALE). Moreover, the TALE-based strategy cannot be applied to telomere labeling in tissue sections, especially to clinical samples from patients. On the other hand, HPTH59-b can label telomeres in both cultured cells and tissue sections without constructing plasmids and establishing stable cell lines.

Another recent approach uses the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated caspase 9 (Cas9) technique (Chen et al. 2013). Recently, Deng et al. reported the use of in vitro constituted nuclease-deficient CRISPR/Cas9 complexes as probes (Cas9-mediated fluorescence in situ hybridization,
CASFISH) (Deng et al. 2015). Telomere labeling by this method does not require DNA denaturation and can quickly (15 min) label telomeres in cultured cells and tissue sections. However, the production cost of large amounts of single-guide RNAs (sgRNAs) and dCas9 (nuclease-deficient) protein make it more expensive than the HPTH59-b method, which could be problematic for high-throughput applications, such as those involved in cancer diagnosis.

With the recent development of new technologies, our understanding of chromatin structure and dynamics is deepening (Maeshima et al. 2016). Because our sensitive telomere labeling method can be performed under mild conditions, another interesting application to telomere regions involves super-resolution imaging without harsh treatments. This technique could help to elucidate how telomere chromatin is organized in the cell nuclei. Therefore, telomere visualization using the PI polyamide-based approach discussed here would contribute to telomere biology and related medical science.

This research was reported in a peer-reviewed journal;
1.4. Material and Methods

Synthesis
HPTH59-b was synthesized as reported previously (Hirata et al. 2014).

Human tissues
The use of human tissues was approved by the committees of the National Center for Global Health and Medicine (#NCGM-G-001766-00) and was in accordance with the Declaration of Helsinki of the World Medical Association. Participants provided written informed consent. I analyzed one case of a patient who had a surgical operation at the National Center for Global Health and Medicine Hospital. Tissues were prepared from regions diagnosed as esophageal squamous cell carcinoma and adjacent normal tissue according to the manufacturer’s protocol. Briefly, excised tissue was flash frozen in cold acetone with optimal cutting temperature (OCT) compound (Sakura Finetek Japan). Tissue sections (10 µm) were prepared by microtome and placed on the slide glass for telomere staining.

Telomere staining of HeLaS3, HeLa1.3, and U2-OS cells with HPTH59-b
HeLa cells were maintained at 37°C under 5% CO₂ atmosphere in DMEM containing 10% fetal bovine serum (FBS). For polyamide staining, cells were grown on coverslips coated with poly-lysine. The cell coverslips were washed in phosphate-buffered saline (PBS) twice and fixed with 1.85% formaldehyde in PBS. The fixed cells on coverslips were stained with HPTH59-b and then mounted as described previously (Kawamoto et al. 2013). Section images were recorded with a DeltaVision microscope and deconvolved to eliminate out-of-focus blur to obtain clearer pictures. The deconvolved
images were projected (‘Quick Projection’ tool) to obtain the maximum intensity of telomere signals. Non-deconvolved pictures were used for quantitative analysis of HPTH59-b signals.

Telomere staining of mouse tissue sections with HPTH59-b

Postnatal 0 (P0) mice were fixed in 4% paraformaldehyde in PBS overnight at 4°C and placed in 30% sucrose in PBS for 1 day at 4°C. Mouse whole bodies were then embedded in optimal cutting temperature (OCT) compound/30% sucrose (2:1) and incubated for 1 h at room temperature. Embedded samples were stored at -80°C until sectioning. Samples were sectioned (20 µm) using a CM3050S cryostat (Leica) and kept at -30°C until use. All experimental protocols were approved by the Animal Committee of the National Institute of Genetics and carried out according to the guidelines to minimize the pain and discomfort of the animals.

Preparation of human tumor/non-tumor tissue sections

Before staining, sections were incubated in HEN buffer (10 mM HEPES pH 7.5, 1 mM EDTA, 100 mM NaCl) overnight at 37°C. The sections were permeabilized with 0.1% Triton X-100 for 10 min and briefly washed twice with TEN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl). For blocking, the sections were treated with Normal Goat Serum (NGS) in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) for 30 min at room temperature. After a brief rinse with TE buffer, the sections were incubated with 10% NGS, 15 nM HPTH59-b, and 0.5 µg/mL DAPI for 2 h at 37°C. After washing five times for 3 min with TEN200 buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 200
mM NaCl), the sections were mounted and image acquisition was performed as described above.

**Co-staining with polyamide and antibody**

The procedure from sample preparation to blocking was the same as that described above. The sections were incubated with 10% NGS, 15 nM HPTH59-b, 0.5 µg/mL DAPI, and either anti-DDX4/MVH antibody (Abcam #ab13840, 1:500 dilution) or anti-Ki-67 antibody (Oncogene #NA59, 1:20 dilution) for 2 h at 37°C (Maeshima et al. 2006), following the process from washing to image acquisition as described above.

**Quantification of telomere signals**

Images of HeLa1.3 and HeLaS3 cells and human/mouse tissue sections were recorded with a DeltaVision microscope under identical conditions. Non-deconvolved images were projected and used as source images. Extraction of each telomere signal was performed as follows. The background fluorescent signals of HPTH59-b and DAPI in the source images were subtracted using the ‘Rolling ball’ tool (Fiji) (Schneider, Rasband, and Eliceiri 2012). By setting an arbitrary threshold value of HPTH59-b intensity, the telomere spots were contoured. The maximal signal intensities of HPTH59-b and DAPI were extracted from each telomere spot, and each HPTH59-b signal was then normalized with DAPI signals. The Wilcoxon rank sum test was used for statistical analysis, which was performed with R software (R core Team 2016).
Chapter 2

2.1. Introduction

Telomeres protect the chromosome ends so that the ends are not recognized as DNA double-strand breaks (DSB) and also do not trigger DNA damage response (DDR).
2.2. Results
2.3. Discussion

I demonstrated that
2.4. Material and Methods

Synthesis
Acknowledgement

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