Epigenetic Regulation of Telomere Maintenance

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Abstract

As chromatin structures, telomeres undergo epigenetic regulation of their maintenance and function. In plants, these processes are likely of a higher complexity than in animals or yeasts, as exemplified by methylation of cytosines in plant telomeric DNA or reversible developmental regulation of plant telomerase. We highlight the dual role of telomeres from the epigenetic point of view: (i) as chromatin structures that are the subject of epigenetic regulation (e.g. DNA and histone modifications), and (ii) as chromosome domains acting themselves as epigenetic regulatory elements (e.g. in the telomere position effect). Possibly, some molecular tools (e.g. telomeric transcripts) are common to both these aspects of telomere epigenetics. We further discuss the justification for the classical textbook view of telomeres as heterochromatic structures.

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Merging Epigenetics with Telomere Biology

Epigenetics is apparently approaching (if not already passing) its zenith these years. Its importance for shaping cellular phenotypes – programming and possible re-pro-

gramming of the developmental fate of cells – is currently widely recognised, and consequently, this field of research has become rather crowded. As a result of this effort of the large international community, the molecular mechanisms responsible for epigenetic phenomena have been clarified in considerable details, and applications of this knowledge (slowly but surely) are entering practise, e.g. in medicine [reviewed in Bojang and Ramos, 2013], namely in the form of epigenetic drugs [Nebbioso et al., 2012].

Epigenetics is beyond doubt the field in which plant model systems have played a dominant role – because of the unmatched richness and diversity of epigenetic tools found in plants. Epigenetic molecular mechanisms including methylation of cytosines in DNA, covalent modifications of histones and RNA interference (RNAi) pathways are involved in regulation of pivotal cellular processes such as gene expression, replication timing, differentiation, and adaptation of the organism to changing environmental conditions. Plants, as sessile organisms, have developed a complex set of epigenetic tools to cope with suboptimal living conditions and adapt to environmental changes. Some of these mechanisms are unique to plants, the striking example being a motley pattern of cytosine methylation. In contrast, in animals methylated cytosines are located almost exclusively in a CG sequence context, although more recent studies have revealed a fraction of methylated cytosines in non-CG motives in de-differentiated human cells [Ramsahoye et al., 2000; Lister

et al., 2009], and 5-methylcytosines in CHG triplets (where H indicates non-G nucleotides) were detected in genes containing trinucleotide repeats in human normal and tumour tissues [Lee et al., 2010]. The functional significance of these modifications remains elusive.

In plants, methylated cytosines are frequently located in symmetric CG doublets and CHG triplets, as well as in non-symmetric CHH sequences, and correspondingly, 3 plant methyltransferase enzymes have been identified. MET1, a dominant plant methyltransferase, is responsible not only for the maintenance of CG methylation (like its mammalian homolog DNMT1) but also for the stability of the general epigenetic pattern including non-CG methylation, histone modifications and chromatin structure [Mathieu et al., 2007]. Methylation of cytosines in symmetric CHG sites is driven by the plant-specific enzyme CHROMOMETHYLASE 3 (CMT3). DOMAINS REARRANGED METHYLTRANSFERASES 1/2 (DRM1/2), orthologs of mammalian DNMT3a/b, cooperate with CMT3 in maintenance of non-symmetric CHH methylation and in de novo methylation of cytosines in all sequence contexts in the process of RNA-directed DNA methylation (RdDM) [Henderson et al., 2010]. RdDM was also originally described in plants and considered as a plant-specific mechanism. Correspondingly, plant-specific RNA polymerases IV and V participating in this process were described [Herr et al., 2005; Wierzbicki et al., 2012]. Inspired by these discoveries in plants, the involvement of RdDM in gene silencing was then demonstrated in human cells [Kawasaki and Taira, 2004; Morris et al., 2004].

Differences have also been described between plant and animal kingdoms in the other type of epigenetic marks – covalent modifications of histone proteins. The pattern of histone modifications is extremely complex, and its combinatorial character in histone amino acids of a particular nucleosome constitutes the basis of the so-called histone code. This code, as well as the other epigenetic codes, does not show universality comparable to the genetic code. A typical example of the striking differences between plants and animals is methylation of lysine 9 of histone H3 and lysine 20 of histone H4: whereas in *Arabidopsis thaliana* mono-methylated lysine residues are associated predominantly with heterochromatic loci and tri-methylated with euchromatin, the situation in mouse is the opposite, with H3K9me and H4K20me present mainly in euchromatin, and H3K9me3 and H4K20me3 in heterochromatin [reviewed in Fransz et al., 2006].

The last epigenetic mechanism briefly mentioned in this introduction is RNAi. In the breakthrough discovery

of this process, plant models played an important role, too. Although the mechanism of RNAi was elucidated using *Caenorhabditis elegans* in 1998 [Fire et al., 1998], several years earlier posttranscriptional silencing of gene expression – at that time called co-suppression – was described (but not explained) in *Petunia hybrida* [Napoli et al., 1990] and has been shown to have the same mechanistic base as RNAi. Among other RNAi processes studied and unravelled using plant models let us mention, for example, the discovery of RNA-dependent RNA polymerase [Dalmay et al., 2000; Mourrain et al., 2000] (this enzyme activity was detected in humans only recently as a specific pathway for double-stranded RNA production [Maida et al., 2009]), the involvement of small RNA molecules in defence processes against viral infection [Waterhouse et al., 1998], and the direct connection between small RNA molecules, DNA methylation and gene silencing [Jones et al., 1998; Mette et al., 2000].

The control of gene activity, and subsequently, of gene expression networks, which establish the cell phenotype (through their impact on the proteome and, ultimately, the metabolome), occurs at the level of chromatin structure. Therefore, the epigenetic landscape, a term used originally by Conrad H. Waddington as a metaphor to express the interconnected roles of stochastic and deterministic events in cell differentiation, can also be understood (in a narrow sense) as a chromatin landscape. Nucleosomal and higher-order-levels of chromatin structure regulate, for example, the accessibility of DNA to transcription factors, the timing of replication, the activity of transposable elements, and the dynamics of DNA repair, utilising the above-mentioned molecular epigenetic mechanisms as well as additional ones like chromatin remodelling or deposition of variant histones to nucleosomes [Zhu et al., 2012]. It is thus evident that epigenetic processes are not limited only to the gene expression level. For example, the process of nucleosome assembly on newly replicated DNA (i.e. an epigenetic process) is tightly connected with maintenance of genome integrity: in chromatin assembly factor 1 mutants of *A. thaliana* (*fas* mutants), a progressive and specific loss of telomeres and rDNA was observed [Mozgova et al., 2010; Jaske et al., 2013].

Telomeres are terminal, but nevertheless integral parts of chromosomes, and as such they are also formed and function as supramolecular nucleoprotein (chromatin) structures. Their DNA component is usually formed by repetitive DNA whose incomplete end-replication by the conventional replication machinery can be compensated by elongation via a specific ribonucleoprotein com-

plex with RNA-dependent DNA polymerase activity – the telomerase. Besides preventing the replicative shortening, telomeres also protect chromosome ends from being mistaken for unrepaired chromosome breaks. For the latter function, protein components of telomeres are responsible (see below). Whereas nucleotide sequences of telomeres are usually homogeneous, of a low degeneracy (as a result of precise synthesis by telomerase and protection of telomeres against recombination), and the same at all chromosome ends, their adjacent proximal sequences (telomere-associated or subtelomeric), forming a boundary region between a telomere and a distal-most gene, are frequently composed of highly degenerated telomere-like motifs, satellite repeats, and single- or low-copy sequences. These may combine in complex subtelomeric arrays [Fajkus et al., 1995a, b; Sykorova et al., 2001, 2003a].

With the exception of some lower organisms with short telomeres (e.g. budding yeasts and some protozoa), the major part of telomeres is folded into nucleosomes [Makarov et al., 1993; Tommerup et al., 1994; Fajkus et al., 1995a] which are regularly spaced but show a 30–40 bp shorter periodicity than the bulk chromatin in the same organism [Lejnine et al., 1995]. All chromatin repeat length values – 156, 166 and 177 bp – observed in telomeres of various eukaryotes [Fajkus and Trifonov, 2001] are multiples of the nucleosome DNA helical repeat of 10.4 bp [Cohan et al., 2006], representing 15–17 times this value. Digestion of telomeric chromatin with micrococcal nuclease results in extensive subnucleosomal cleavage of short (mono- and di-nucleosomal) chromatin fragments [Makarov et al., 1993; Tommerup et al., 1994; Fajkus et al., 1995a]. This likely reflects interplay between telomeric DNA and histones: while wrapping of DNA around histone octamers is intrinsically determined by DNA bendability [Trifonov, 2010], telomeric repeats (mostly 6–8 bp long) do not fit the nucleosome DNA helical repeat of 10.4 bp. Weak nucleosome positioning then results in sliding and lower thermodynamic stability of telomeric nucleosomes [Fajkus et al., 1995a]. Sliding between multiple isoenergetic positions results in high mobility of telomeric nucleosomes under in vitro conditions [Rossetti et al., 1998]. However, the observations of tight and regular nucleosome packing in micrococcal nuclease digestion experiments [Fajkus et al., 1995a], as well as by electron microscopy [Nikitina and Woodcock, 2004], atomic force microscopy, and in reconstitution experiments under near-physiological conditions [Galati et al., 2012] rather point to a stable and periodic structure under in vivo conditions. A possible explanation of the ap-

parent contradiction between the lack of nucleosome positioning signals in telomere DNA sequences and yet their regular chromatin structure was provided by the so-called columnar model [Fajkus and Trifonov, 2001] in which the DNA is continuously wound around columns of histone octamers which are stabilised by interactions between them. Octamer-to-octamer stacking contacts stabilize the overall structure cooperatively, preventing the whole nucleosome structure from sliding.

Recent results show that non-histone chromatin proteins also participate in regulation of telomere homeostasis. Loss of the high mobility group protein HMGB1 results in telomere shortening in *Arabidopsis* [Prochazkova Schruppova et al., 2011] and mice [Polanska et al., 2012]. In mouse embryonic fibroblasts, HMGB1 interacts with telomerase reverse transcriptase (TERT) and telomerase RNA (TR) subunits, and its loss resulted in a marked decrease in telomerase activity and in the level of TR. Interestingly, loss of the closely related protein HMGB2 showed opposite effects [Polanska et al., 2012].

The functionally best characterised components of mammalian telomeric chromatin are telomere-specific proteins, which are the key performers of basic telomere roles – to distinguish between natural chromosome ends and unrepaired chromosome breaks and the corresponding inhibition of the DNA damage response at telomeres, and to ensure chromosome end-replication via recruitment of telomerase and by facilitating progress of replication forks through telomeres [Palm and de Lange, 2008; de Lange, 2009; Sfeir et al., 2009; Nandakumar et al., 2012; Sfeir and de Lange, 2012]. It is not quite clear whether telomere-specific proteins are able to bind telomeric nucleosomes, or whether they compete with histones for binding to telomeric DNA. The rare contributions to this field deal with the proteins TRF1 and TRF2, which are able to bind the duplex part of vertebrate telomeres and are components of the telomeric complex called shelterin [de Lange, 2005]. TRF1 is able to specifically recognize telomeric binding sites located within nucleosomes, forming a ternary complex. The formation of this complex is strongly dependent on the orientation of binding sites on the nucleosome surface, rather than on the location of the binding sites with respect to the nucleosome dyad [Galati et al., 2006]. The telomeric protein TRF2 negatively regulates nucleosome density during nucleosome assembly by a cell cycle-dependent mechanism that increases internucleosomal distance [Galati et al., 2012]. Chromatin structure thus participates in the establishment of a telomeric capping complex in the nucleosomal context.

In plants, a telomeric complex analogous to shelterin awaits full description in spite of many candidate proteins [for reviews see Rotkova et al., 2009; Peska et al., 2011] and promising recent results of functional and interaction assays of telomere repeat binding proteins [Prochazkova Schruppfova et al., 2014]. Motivation to study telomere protein complements in plants, and plant telomere biology in general, arises from the peculiarities of the plant model system. Among these, the developmental plasticity of plant cells, reflected by their totipotency, is most notable. This implicates reversible regulation of telomerase activity – which we actually demonstrated some time ago [Fajkus et al., 1998; Riha et al., 1998]. Molecular players responsible for reversible telomerase regulation in plant cells pose an attractive target for possible biomedical applications of telomere biology and are sought primarily at the levels of protein components of plant telomeres and regulation of the basic telomerase subunits – TERT and TR.

Plant Telomeres as Subjects of Epigenetic Regulation

As chromatin structures, telomeres pose targets for epigenetic molecular mechanisms including DNA methylation, histone modification, chromatin remodelling or RNAi. In the case of telomeres, however, the ultimate result of these processes is not a change in expression of a target gene, but rather a change of telomere length or structure which may be reflected in modulation of telomere protective function.

Methylation of Cytosines in Plant Telomeres

Telomeric DNA is composed of short tandem repetitive sequences, the primary structure of which is relatively conserved. In vertebrates, telomeres consist of TTAGGG repeats, while in most plants the telomeric repeat has one more T (TTTAGGG). Nevertheless, sequence variability of plant telomeres is relatively high, as the vertebrate telomeric sequence has also been identified in species of the order Asparagales [Adams et al., 2001; Sykorova et al., 2003c]. Plants of the genus *Allium* even lack canonical telomeric sequences at chromosome termini [Pich et al., 1996; Sykorova et al., 2006], and a similar pattern was also observed in representatives of the genera *Cestrum*, *Vestia* and *Sessea* (family Solanaceae) [Sykorova et al., 2003b]. Telomere sequence diversity has been described also in algae [Fulneckova et al., 2012, 2013].

As mentioned above, plant cells possess an enzymatic machinery which enables them to methylate cytosines in all sequence contexts, and thus non-symmetrically located cytosines in CCCTAAA telomeric repeats are potential targets for methylation. The first evidence for telomeric methylcytosines in *A. thaliana* was presented in 2008 [Cokus et al., 2008]. In that study, bisulfite-converted DNA was analysed by shotgun sequencing, and this sensitive high-throughput approach revealed a pattern of DNA methylation in so far inaccessible genome regions including telomeres. According to this data, telomeric cytosines are methylated to a level dependent on the cooperative activities of the enzymes DRM1, DRM2 and CMT3. Moreover, there is an evident preference for methylation among telomeric cytosines: the third (inner) cytosine in the CCCTAAA sequence is methylated most frequently (to about 10%), while methylation of the other cytosines is significantly lower (less than 1%). The presence of methylated cytosines in telomeric repeats in *A. thaliana* leaves and in *Nicotiana tabacum* cells in culture was confirmed by an independent approach based on the hybridisation of membrane-bound bisulfite-converted DNA with radioactively labelled probes. In the *Arabidopsis* study [Vrbsky et al., 2010], a TTTAGGG probe homologous to the telomeric repeat with all cytosines methylated (and thus resistant to bisulfite treatment) was used. In tobacco cells, methylation of telomeric cytosines was determined by a degenerate probe TTTAGRR (R = A or G) better reflecting the pattern of methylation (this probe hybridised with bisulfite-treated telomeric repeats in which the inner cytosine was methylated and the outer cytosines were either methylated or non-methylated) [Majerova et al., 2011b].

In many organisms including plants and humans, telomeric repeats are located not only at the chromosome termini but even inside chromosomes, forming so-called interstitial telomeric sequences (ITSs). These sequences are relatively abundant in the *A. thaliana* genome, encompassing 20–70% of total telomeric repeats (the estimation is dependent on the methodology used and the conditions applied for sequence filtering) [Uchida et al., 2002; Gamez-Arjona et al., 2010]. While genuine telomeric repeats are relatively perfect, ITSs and telomere-associated sequences show high levels of imprecise (degenerated) repeats, and can thus be distinguished by the stringency of hybridisation conditions or directly from DNA sequencing data. Definitely, the presence of a significant amount of telomeric repeats outside the chromosome termini complicates analysis of telomeric DNA methylation and chromatin properties and interpretation of data (see

below). To distinguish between genuine telomeres and ITSs in *A. thaliana*, the restriction endonuclease *Tru1I* (recognition site TTAA) was used leaving telomeres intact but cutting in ITSs, and subsequent analysis using an antibody against methylcytosine revealed methylation only in ITSs [Vaquero-Sedas et al., 2011], conflicting with previous observations [Cokus et al., 2008; Vrbsky et al., 2010]. Nevertheless, methylation of telomeric cytosines in the study of Vrbsky et al. [2010] was verified by hybridization under high stringency conditions, and sequencing data were presented which demonstrated directly the presence of methylated cytosines in 13 perfect telomeric repeats in the centromere-proximal part of the 1L chromosome arm telomere. Further, the pattern of methylation density was the same as that described by Cokus et al. [2008], i.e. with the inner cytosine as the most frequently methylated. Essentially the same results were obtained in our independent study [Ogrocka et al., 2014]. In this context, *N. tabacum* and *Nicotiana* species generally seem to be very useful models for analyses of the epigenetic properties of plant telomeric chromatin for several reasons: (i) to our knowledge, no significant fraction of ITSs is present in the tobacco genome [Majerova et al., 2011a]; (ii) tobacco plants and cell cultures are amenable to most molecular biology approaches; (iii) tobacco is a plant well established in telomere biology, and some fundamental findings such as the first evidence of telomerase activity in plants [Fajkus et al., 1996], characterization of telomeric nucleosomes [Fajkus and Trifonov, 2001] and mapping of telomere-subtelomere junctions [Fajkus et al., 1995b] have been made using this model. In the future, utilization of other methodical approaches and analysis of telomeric cytosine methylation in a broader set of plant models is necessary to make unambiguous and generally applicable conclusions in this field.

Epigenetics of Plant Telomeric Histones

Analysis of epigenetic modifications of telomeric histones faces the same difficulties as studies of DNA methylation – hybridization of immunoprecipitated chromatin fractions (ChIP) with telomeric probes provides aggregated information on marks located at both telomeres and ITSs. For mammalian (especially mouse) models, there are papers reporting the heterochromatin-specific histone marks associated with telomeric and subtelomeric chromatin and their importance for telomere maintenance and stability [reviewed in Blasco, 2007]. Several recent reports dealing with human telomeres, however, question the unequivocal view of telomeres as heterochromatic structures: (i) the level of heterochromatic

marks was surprisingly low in human fibroblast telomeres [O’Sullivan et al., 2010], and (ii) the telomeres of human T-cells were associated with euchromatic marks while heterochromatic H3K9me3 was under-represented [Rosenfeld et al., 2009]. Nevertheless, further data were recently presented supporting the significance of the heterochromatic character of human telomeres for proper telomere function and genome integrity [Canudas et al., 2011; Postepska-Igielska et al., 2013].

In the *A. thaliana* model, hybridisation of ChIP fractions under stringent conditions – to suppress signals from ITSs – revealed both types of epigenetic modification at telomeric chromatin which manifests its dual (bivalent) character termed by the authors ‘intermediate heterochromatin’ [Vrbsky et al., 2010]. On the other hand, in separate analyses of *Tru1I*-sensitive (enriched in ITSs) and -resistant (enriched in genuine telomeres) fractions, telomeric histones were associated with euchromatin-specific modifications while ITSs were marked like heterochromatin [Vaquero-Sedas et al., 2011]. Abundant euchromatic marks at *A. thaliana* telomeres were confirmed by the same group by processing of available ChIP-sequencing data for sequence precision [Vaquero-Sedas et al., 2012]. Since only a limited number of reports are available dealing with the epigenetic analyses of plant telomeric chromatin, mostly using only a single model plant, *A. thaliana*, this topic is open and waiting for new comprehensive data.

Telomere Maintenance in Organisms with a Changed Epigenetic Status

The influence of modulation of the epigenetic pattern on telomere stability and protective function has been studied extensively in mammalian models. Results of Maria Blasco’s group showed that loss of heterochromatin-specific epigenetic modifications, including DNA methylation, resulted in telomere elongation and increased telomere recombination in mouse and human cells [reviewed in Blasco, 2007], and conversely, in cells with loss of telomerase function (thus with shorter telomeres) reduced H3K9me3 loading at telomeres and subtelomeres was detected [Benetti et al., 2007]. These data support an interconnection between the heterochromatic character of telomeres and their correct operation. Nevertheless, in a similar study with mouse epigenetic mutants no change of telomere length was detected [Roberts et al., 2011].

In plants, telomere homeostasis was analysed in hypomethylated *N. tabacum* culture cells and in *A. thaliana* plants. Telomeres of tobacco cells cultivated in the presence of hypomethylation-inducing drugs maintained

their lengths despite significantly increased telomerase activity and both global and telomere hypomethylation [Majerova et al., 2011b]. On the other hand, in *A. thaliana* plants hypomethylated either genetically (mutants with loss of function of enzymes essential for the maintenance of a stable DNA methylation pattern) or chemically (via germination in the presence of hypomethylation drugs), telomerase transcription and activity were at wild type levels, but telomeres were significantly shorter. This short telomere phenotype was stably transmitted to the plants segregated from the mutant background and to the next generation of plants influenced by the hypomethylation drugs during germination [Ogrocka et al., 2014]. These results illustrate the differences in epigenetic regulation of telomere homeostasis not only between animal and plant models, but even among plant species.

Telomeric Transcripts I

Discoveries following the elucidation of the crucial role of RNA molecules in regulation of processes via RNAi mechanisms have transformed the general view of chromatin function. Heterochromatin was traditionally considered as an inactive part of the genome encompassing silent transposons, repetitive elements and inactive genes. Telomeres belonged to this category. Like a bolt from the blue, the report of Volpe et al. [2002] showed that components of the RNAi pathway are essential for proper heterochromatin formation in fission yeast because this requires transcription of heterochromatin. Five years later, it was established that mammalian telomeres are transcribed from subtelomeric regions toward telomeres and that these TERRA transcripts (telomeric repeat-containing RNA) associate with the chromosome termini [Azzalin et al., 2007], thus identifying telomeric RNA as a novel functional component of telomeric chromatin [Schoeftner and Blasco, 2008]. This work is now considered as the first proof of transcription of telomeres, but even earlier reports indicated this possibility [Morcillo et al., 1988; Rudenko and Van der Ploeg, 1989; Solovei et al., 1994]. TERRA, like other long, non-coding transcripts, is believed to have the capacity to epigenetically influence the sequences from which it arose, telomeres and telomeric chromatin.

A positive correlation between telomere length and TERRA transcription was presented by Schoeftner and Blasco [2008]. According to their observations, loss of heterochromatin-specific epigenetic marks in mouse cells led to a long telomere phenotype accompanied by an increased level of TERRA transcripts. On the other hand,

telomere elongation repressed TERRA transcription by increased H3K9me3 density in telomeric chromatin and by heterochromatin protein HP1 α in human cells lines, although the length of TERRA molecules increased upon telomere elongation [Arnoult et al., 2012]. TERRA transcription accelerated telomere shortening in *Saccharomyces cerevisiae* [Pfeiffer and Lingner, 2012], and high TERRA levels were detected in patients suffering from ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome with short telomeres [Yehezkel et al., 2008].

Data reporting the influence of TERRA transcripts on telomeric chromatin and telomere homeostasis in plants are limited, but a complex pattern was observed in *A. thaliana*. While in mammals telomeric transcripts arising only from the subtelomeric region were found, in *Arabidopsis* transcripts originated in both subtelomeres (TERRA) and telomeres (ARRET, antisense telomeric transcripts) were detected – at least at some chromosome termini [Vrbsky et al., 2010]. Moreover, a significant – maybe dominant – fraction of TERRA and ARRET is transcribed from ITSs. A portion of the telomeric transcripts is processed to small RNA molecules which are crucial for the maintenance of methylation of telomeric cytosines via the RdDM pathway (see above). Such processing of telomeric transcripts to small RNAs is not a plant-specific phenomenon; in mouse embryonic stem cells, 24-nt long telomeric transcript molecules were found to be involved in formation of the telomeric heterochromatin [Cao et al., 2009]. Regarding telomeric chromatin structure, the pattern of telomeric histone modifications was essentially the same in *A. thaliana* wild type plants and in mutants with a loss of function of RNA-dependent RNA polymerase in which the level of telomeric siRNAs and telomeric cytosine methylation were significantly reduced. More detailed analysis revealed that close to the telomere-subtelomere boundary, the loading of heterochromatin-specific marks was significantly reduced in the mutant background [Vrbsky et al., 2010], providing evidence for the cooperation of multiple epigenetic mechanisms in the maintenance of plant telomeric and subtelomeric chromatin structure.

Telomeres as Epigenetic Agents

Telomeres are not only targets for epigenetic processes such as DNA methylation and histone modifications, but themselves exert effects on other chromatin domains both in *cis*- and *trans*-positions.

Telomere Position Effect

As a classical example in this direction, the telomere position effect (TPE) should be mentioned. The silencing effect of telomeres on transcriptional activity of adjacent genes (or transgenes) has been first described in the yeast *S. cerevisiae* [Gottschling et al., 1990]; when a gene was placed near a telomere, its transcription was repressed. Genes under the influence of these TPEs can switch between repressed and transcriptionally active states, each of which is quasi-stable for many cell generations. In fact, TPE can be considered as a specific case of the so-called position-effect variegation described in *Drosophila* as the mosaic expression of genes juxtaposed to heterochromatin [Henikoff, 1992]. Yeast telomeres exert TPE not only at the level of gene transcription, but also on the timing of replication origin activation [Ferguson and Fangman, 1992]. Early experiments further showed that TPE could be alleviated by transcription of a telomere, using an inducible promoter which was introduced adjacent to a telomere of a single chromosome arm such that transcription could be induced toward the end of the chromosome. Transcription proceeded through the entire length of the telomeric tract and caused a modest reduction in the average length of the transcribed telomere. Transcription of the telomere substantially reduced the frequency of cells in which an adjacent reporter gene was subject to TPE, without compromising the stability of the chromosome [Sandell et al., 1994]. The importance of this result became evident only recently in connection with the observation of TERRA telomeric transcripts (see above). Besides yeasts and *Drosophila*, TPE has been identified in several other organisms, including mammals. The first systematic study of TPE in human cells showed that HeLa clones containing a reporter gene adjacent to a newly-formed telomere showed 10 times lower expression of the reporter gene than control clones generated by random integration [Baur et al., 2001]. Expression could be restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of hTERT (human telomerase reverse transcriptase) cDNA resulted in telomere elongation and an additional decrease in expression of the reporter gene in telomeric clones. This dependence of TPE on telomere length suggested a novel mechanism modifying gene expression throughout the replicative life-span of human cells, suggesting that telomere shortening could be involved in human diseases not only as a barrier against tumour progression (by generating DNA damage signals which induce replicative senescence), but also as an age-dependent regulator of subtelomeric gene expression. Among natural human subtelomeric genes, however,

only the interferon-stimulated gene 15 (*ISG15*), located 1 Mb from the end of chromosome 1p, has been found to be regulated by telomere length, while the other genes distal to *ISG15* did not show any TPE [Lou et al., 2009]. A role for increased *ISG15* expression in increased inflammatory response in aging was hypothesized, but not demonstrated. The loss of TPE, however, was recently found to contribute to the pathogenesis of facio-scapulo-humeral dystrophy (FSHD), a disease associated with the contraction of a D4Z4 tandem repeat array at the 4q subtelomere. This D4Z4 array functions as an insulator and repressor that interferes with enhancer-promoter communication and protects transgenes from position effects [Ottaviani et al., 2009, 2010], and upon contraction of the array this insulator function is lost. Furthermore, it was found that the *DUX4* (double homeobox 4) gene, located within each of the D4Z4 units, is the primary candidate for FSHD pathogenesis [Jones et al., 2012]; it is upregulated over 10-fold in FSHD myoblasts and myotubes with short telomeres, and its expression is inversely proportional to telomere length [Stadler et al., 2013]. Thus, FSHD may be the first known human disease in which TPE contributes to an age-related phenotype.

At the molecular level, the features of TPE are dependent on a specific higher-order organization of the telomeric chromatin and chromatin-associated proteins. For example, repression of a subtelomeric reporter gene in human cells was alleviated by increasing the dosage of the TRF1 protein or by trichostatin A. Derepression upon trichostatin A treatment correlated with the delocalization of HP1 α and HP1 β proteins. In contrast, 5-azacytidine, a demethylating agent, or sirtinol, an inhibitor of the Sir2 family of deacetylases, had no apparent effect on telomeric repression [Koering et al., 2002]. Contrary to the results of studies with human cell lines, TPE in mouse ES cells was not reversed by trichostatin A, and prolonged culturing resulted in extensive DNA methylation and complete silencing of telomeric transgenes which could be reversed by treatment with 5-azacytidine. Thus, TPE appears to involve a 2-step process in which the initial repression is subsequently reinforced by DNA methylation [Pedram et al., 2006]. TPE – as an epigenetic phenomenon – is dependent on genetic background, as was nicely illustrated by introduction of the same linear human artificial chromosome into genetically distinct cell lines and animal models [Weuts et al., 2012]. Telomere lengths and de novo subtelomeric DNA methylation in this construct adapted to distinct genetic backgrounds, and expression of subtelomeric genes was inversely correlated with telomere length and subtelomeric methylation.

In contrast to many other organisms with extensive gene-poor subtelomeric regions, telomeres in *A. thaliana* are directly adjacent to transcriptionally active genes. In this configuration, telomeres are more similar to silenced transposons inserted in gene-rich regions than to pericentromeric heterochromatin [Vrbsky et al., 2010]. This is also reflected in the intermediate heterochromatin features of telomeres and the very limited expansion of repressive histone H3 modifications to telomere-associated regions [Bernatavichute et al., 2008; Vrbsky et al., 2010]. Correspondingly, TPE has not been observed in *A. thaliana*. Also, the absence of replicative telomere shortening during plant development [Fajkus et al., 1998; Riha et al., 1998] probably excludes the possibility of a modulation of subtelomeric gene activity by telomere truncation similar to that observed for human *ISG15* or *DUX4* genes (see above). *A. thaliana* with its relatively short telomeres of intermediate heterochromatin character may, of course, represent a specific case and not provide general conclusions. TPE could possibly be present in plants with long telomeres and large subtelomeric heterochromatin blocks, like e.g. tobacco [Fajkus et al., 1995a, b]. Although very limited information is available here, a stable maintenance and expression of subtelomeric insertions has been demonstrated in tobacco [Iglesias et al., 1997], without any marks of TPE. The question of TPE in plants thus requires further investigation.

In addition to TPE, a recent report suggests that short telomeres can affect transgene expression at non-telomeric sites in the mouse [Roberts et al., 2013]. Using multiple generations of *Terc* knockout mice (lacking a functional gene coding for the RNA subunit of telomerase), the authors showed that inheriting shorter telomeres from one parent increased the likelihood of transcriptional silencing at a non-telomeric transgene inherited from the other parent, and that reduced transgene expression during embryonic development was associated with the inheritance of shorter telomeres rather than lack of *Terc*. The activity of a non-telomeric transgene further decreased in the next generations and was associated with increased DNA methylation and also with genetic changes, including variations in copy number at the transgene array. Although the mechanism of this *in trans* effect of short telomeres is unknown, it apparently involves both genetic and epigenetic pathways.

Telomeric Transcripts II

Considering the functions of telomeric transcripts, we could find ourselves in a vicious circle: telomeric transcripts as epigenetic factors may modulate the struc-

ture of telomeric chromatin and influence telomere homeostasis, as discussed in the preceding paragraphs, while on the other hand TERRA transcripts are derived from telomeres and in this context may represent a tool for telomere-mediated epigenetic regulation. A striking example is an interconnection between TERRA level and telomerase activity. Inhibition of telomerase by TERRA was observed in in-vitro experiments in human [Redon et al., 2010] and in mouse [Schoeftner and Blasco, 2008] cells. On the other hand, telomerase activity was not dependent on the TERRA level in vivo in yeast [Pfeiffer and Lingner, 2012] or in human cancer cells [Farnung et al., 2012]. Recent experiments demonstrated that heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), which is involved in RNA biogenesis and telomere maintenance, may alleviate inhibition of telomerase by TERRA, providing a possible explanation for the contradiction between the in vitro and in vivo data [Redon et al., 2013].

To our knowledge, only one report dealt with the connection between TERRA and telomerase activity in plants. In tobacco suspension culture cells cultivated in the presence of hypomethylation drugs, a decrease or an increase of TERRA levels was observed depending on the drug used, while in both cases telomerase activity increased significantly [Majerova et al., 2011b]. These results suggest that factors other than changed TERRA transcription are involved in modulation of telomerase activity in this system. Plant models would offer possibilities to study the potential influence of elongated or shortened telomeres on TERRA transcription because *Arabidopsis* mutants exhibiting these phenotypes – long or short telomeres – are available. However, there is an obstacle which is common in plant telomere biology: transcripts are derived from both genuine telomeres and from ITSs, and although it is possible to analyse transcription from the respective telomeres specifically by PCR [Vrbsky et al., 2010], evaluation and interpretation of the data would be complicated. Promising results in this field will likely depend on novel approaches and studies of other model species.

Conclusions

The epigenetics of telomeres associates problems of both epigenetics and telomere biology, but – due to the relative universality and essentiality of telomeres – provides findings and perspective discoveries of fundamental importance. We are already aware of the variety of

colours (or flavours) of plant chromatin, as well as of the alternative telomeres and variant telomere-maintenance mechanisms in plants. So the coming steps will not be simple but surely exciting.

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