Chapter 5

Discussion and future perspectives
Despite the remarkable advances science has made over the last century, many fundamental questions remain unanswered. In this thesis, the aim is to contribute to a better understanding of telomere biology. Specifically, we examine how a cell is able to distinguish between a double-stranded DNA break (DSB) and a telomere, which resemble each other in structure and share some proteins, but need to be processed in very different ways. The relevance of the adequate distinction between DSBs and telomeres becomes apparent when looking at the consequences of a failure in this process. A DSB is very deleterious for yeast cells, to the extent that one unrepaired DSB is fatal (Resnick and Martin, 1976; Weiffenbach and Haber, 1981). Therefore, tightly regulated repair mechanisms ensure the proper repair of DSBs. However, if a DSB is improperly recognized as a chromosome end and it is repaired as such, telomeric sequence will be added at the broken site. Because genes nearby telomeric sequence are transcriptionally silenced, addition of telomeric sequence at a DSB will lead to silencing of genetic information. Moreover, two human diseases have been associated with truncated chromosomes where telomeric sequence addition occurred, mental retardation and alpha thalassemia (Wilkie et al., 1990; Wong et al., 1997), highlighting the relevance of fundamental research for human health. The opposite scenario, where a telomere is treated as a DSB, also has devastating consequences. Telomere shortening is counteracted by the specialized reverse transcriptase telomerase (Greider and Blackburn, 1985). It is therefore very important to keep chromosome ends available for telomerase-dependent extension and, at the same time, to inhibit the repair mechanisms that act at DSBs. Failure to inhibit DSB repair results in telomere-telomere fusions that can lead to cell death or breakage-fusion-bridge cycles, which are thought to be important in the formation of some cancers (Selvarajah et al., 2006).

A ~40 nt length threshold separates telomeres from DSBs

To answer how a cell discerns between a DSB and a telomere, in chapter 2, we have used two complementary methods. First, we used artificially constructed DNA ends adjacent to telomeric sequence of increasing length, and monitored whether such ends are repaired by telomerase-dependent extension. Second, we developed the iSTEX assay, an inducible method that allows the in vivo monitoring of telomerase-dependent telomere extension events at nucleotide resolution and used it to determine what natural ends were sensed as telomeres and therefore extended by telomerase. Both methods uncovered a length-dependent threshold of about 40 nt of telomeric sequence that establishes the difference between telomeres and DSBs. Below the 40 nt threshold, DNA ends contain too little telomeric sequence and are treated as DSBs and, above it, DNA ends are extended by telomerase. We have also found what proteins are important for the establishment of this length-dependent threshold. Pif1 is important to inhibit telomerase at DNA ends below the DSB-telomere threshold, as evidenced by the increased telomere addition frequency at TG$_{18}$ ends in pif1-m2 cells. On the other hand, the telomerase recruitment function of Cdc13 is required to override the telomerase inhibition by Pif1 and allow extension of
DNA ends above the threshold.

An important question that remains unanswered is what is intrinsically different between the DNA ends below and above the DSB-telomere threshold. One possibility is that Cdc13 is recruited less efficiently to DNA ends below the threshold. *In vitro*, the minimum binding site of Cdc13 is 11 nt in length (Hughes et al., 2000), so Cdc13 could have enough space to bind a TG$_{18}$ end; however, the situation in a living cell might be different. For example, Cdc13 might not be recruited to such short ends. Alternatively, one Cdc13 molecule might be recruited, but multiple Cdc13 molecules might be required for telomere extension. Molecular crowding at such a short end could also affect the binding or function of Cdc13 *in vivo*. Another possibility is that Cdc13 recruitment at DNA ends above the threshold overcomes the telomerase inhibition by Pif1. In this case, when Cdc13 is mutated, telomerase recruitment to the TG$_{34}$ end might be lost. It is also possible that Cdc13 recruitment is unaffected at both sides of the threshold, in which case we can envision at least two options. One is that Pif1 inhibition of telomerase is stronger at DNA ends below than above the threshold. However, we know that Pif1 inhibits telomerase both below and above the threshold, indicating that, unless the strength of inhibition is different below and above the threshold, most likely Cdc13, and not Pif1, is the key player. Alternatively, it will be important to determine whether the other members of the CST complex, Stn1 and Ten1, play a role in the establishment of the DSB-telomere threshold. In short, determining what proteins bind to DNA ends below and above the threshold would help clarify how telomeres and DSBs are differently recognized and processed.

**Development of the inducible STEX assay**

Besides defining the length-dependent DSB-telomere threshold, a major contribution of chapter 2 is the development of the inducible STEX assay (iSTEX). So far, telomere length has been mostly studied by methods that only provide information about the average telomere length of a population of cells. However, iSTEX provides information about telomere extension events on single telomeres at nucleotide resolution. iSTEX can determine to which telomeres telomerase is recruited and, therefore, whether the recruitment of telomerase to the shortest telomeres or the recruitment in general is affected. It also informs about the length of the newly added sequence, and whether the nucleotide addition processivity or the repeat addition processivity are affected. All these aspects are informative to understand what is the cause of telomere length change at a molecular level.

Moreover, iSTEX has two improvements with respect to the classic STEX assay (Teixeira et al., 2004). First, the use of an inducible system largely facilitates the execution of the experiment because it eliminates the need of the nearly 100% mating efficiency between the donor (telomerase positive) and the recipient (telomerase negative) strains required in the original STEX assay (Teixeira et al., 2004). This level of mating efficiency is rarely observed. Second, the use of the *tlc1-tm* template mutant ensures that all telomere extension events are mediated by telomerase. To detect telomere extension events, the
classic STEX relies on the imperfect nature of the telomere repeats added by the yeast telomerase. The telomeric sequences are aligned to a reference sequence. The sequence of the extended telomeres does not align to the non-extended ones, in an otherwise clonal population. Those misalignments are called divergent events and are the readout for telomere extension events. However, telomere divergence events can arise from artefacts generated during PCR amplification, cloning and sequencing (Claussin and Chang, 2016). To overcome this issue, iSTEX uses a telomerase template different from the wild type, ensuring that all telomere extension events are dependent on telomerase and, therefore, avoiding false positives. Moreover, iSTEX excludes telomeric divergence events arising from recombination. Deletion of RAD52 is the common way to exclude recombination events but, when combined with telomerase-negative cells, results in accelerated replicative senescence (Le et al., 1999), which can render the assay more challenging. Because tlc1-tm telomeric sequence is not present in the cell before the start of the experiment, deletion of RAD52 is not required to exclude recombination events. It is formally possible that a recombination event takes place once the telomeres have been extended by the mutant telomerase. This is however unlikely, since the experiment allows only one cell cycle, and it would in any case represent a negligible fraction of the total divergent events.

Cdc13-independent telomerase recruitment to chromosome ends

In the course of the studies described in chapter 2 we found that, while cdc13-2 mutation inhibits telomere addition at TG₃₄ ends, as expected, further mutation of PIF1 allows telomerase-dependent extension of these ends. This is striking because the cdc13-2 mutant carries a point mutation in the telomerase recruitment domain, which makes it behave like a telomerase-null mutant (Nugent et al., 1996). In fact, recruitment of the telomerase subunits Est1 and Est2 to the telomeres has been recently shown to be largely impaired in cdc13-2 cells (Chen et al., 2018). This suggests that telomerase can be recruited in a Cdc13-independent manner to extend the telomeres, probably through the Yku complex, which can interact with the telomerase RNA subunit (Stellwagen et al., 2003) and has been proposed to recruit telomerase (Hass and Zappulla, 2015). Alternatively, the interaction between Cdc13 and Est1 might not be completely lost in cdc13-2 cells, and mutation of PIF1 allows just enough telomerase recruitment to prevent senescence.

Preliminary studies to understand how those cdc13-2 pif1-m2 telomeres are maintained show that, contrary to cdc13-2 cells, cdc13-2 pif1-m2 cells do not senesce and, although shorter than in pif1-m2 cells, telomeres are stable in length (data not shown).

Non-essential G-quadruplex-mediated telomere protection

Chapter 4 explores the in vivo role of yeast telomeric G-quadruplexes. To do so, we made use of the tlc1-tm mutant because of the characteristic telomeric sequence that it introduces to the chromosome ends, which does not meet the requirements for the
formation of G-quadruplexes. Our *in vitro* and *in vivo* experiments (circular dichroism and viability rescue experiments of telomere capping-deficient *cdc13-1* strains upon stabilization of G-quadruplexes) are in line with the hypothesis that these telomeres are impaired in G-quadruplex formation. However, these experiments do not rule out the possibility that the *tlc1-tm* phenotype is unrelated to G-quadruplex formation. On the one hand, although widely used in G-quadruplex studies, circular dichroism is an *in vitro* method to measure the ability of oligonucleotides to fold into G-quadruplex structures, which does not necessarily represent the *in vivo* situation. On the other hand, G-quadruplex stabilization was achieved by deletion of *PIF1*. *PIF1* deletion was chosen to stabilize G-quadruplexes because it is the best G-quadruplex unwinding helicase described so far (Paeschke et al., 2013). However, the many functions that Pif1 carries out complicate the interpretation of the results, as discussed in chapter 4. Other methods to stabilize G-quadruplexes, like deletion of the *SGS1* helicase (Sun et al., 1999), overexpression of the G-quadruplex binding protein Stm1 (Hayashi and Murakami, 2002) or addition of the G-quadruplex stabilizing ligand PhenDC (Piazza et al., 2010) resulted in inconclusive results (data not shown). The use of a G-quadruplex specific antibody (Biffi et al., 2013) combined with G-quadruplex stabilizing ligands and chromatin immunoprecipitation could provide more robust data about the G-quadruplex forming potential of these telomeres. Yet, our data support the previously reported rudimentary telomere protection role by G-quadruplexes when Cdc13 is affected (Smith et al., 2011). The fitness of *tlc1-tm* cells, indistinguishable from wild-type cells, indicates that the G-quadruplex-mediated telomere capping function is not essential.

The question arises then, why are telomeric G-quadruplexes conserved? If organisms of different complexities, from ciliates to humans, have telomeric G-quadruplexes, it is reasonable to think that they have been evolutionary conserved for a reason. Although G-quadruplex structures do not seem to play an indispensable role when it comes to telomere protection, they might influence other telomeric aspects. One could also speculate that G-quadruplexes were ancient chromosome end protecting structures until telomeres became specialized, then got obsolete and only worked as a backup telomere capping mechanism. However, it seems unlikely that such complex structures, as G-quadruplexes are, were only conserved to work when other telomere protecting structures were lacking.

**Characterisation of a Rap1-free telomere**

*tlc1-tm* telomeres show a number of interesting characteristics, the most remarkable probably being that *tlc1-tm* telomeres are not bound by the major telomere binding protein Rap1. Accordingly, telomere length homeostasis of *tlc1-tm* telomeres is altered, leading to long and heterogeneous sized telomeres. However, although longer than wild-type telomeres, *tlc1-tm* telomeres are shorter than those obtained in cells harbouring a *RAP1* C-terminal deletion or *RIF1 RIF2* double deletion (Kyrion et al., 1992; Wotton and Shore, 1997), which might indicate the presence of residual Rap1 at mutant telomeres. A related question is how *tlc1-tm* telomeres remain stable in length. This might be achieved by Rif1, which can
bind the telomeric DNA in a Rap1-independent manner (Mattarocci et al., 2017), or by
the combination of telomerase-dependent extension, facilitated by the absence of Rap1,
and rapid telomere erosion, due to the lack of telomere protection. This is in line with the
strongly increased telomere extension frequency of _tlc1-tm_ telomeres and the very rapid
senescence and telomere shortening very early after telomerase depletion, that can be
explained by the absence of Rap1 at these telomeres, rendering them unprotected and very
dependent on telomerase-mediated extension. Interestingly, when _tlc1-tm_ telomeric repeats
are placed internally, followed by wild-type repeats at the distal ends, the bulk telomere
length increases approximately to the same extent as the length of the internally placed
mutant tract. Therefore, the mutant repeats are not sensed by the protein-counting model
that regulates telomere length (Marcand et al., 1997). Moreover, the fact that Rap1-free
_tlcltm_ cells are perfectly viable implies that the Rap1 essential function is not its telomeric
function.

Rap1 binding to _tlc1-tm_ telomeres was determined by chromatin immunoprecipitation
followed by qPCR. However, because _tlc1-tm_ telomeres are long, the chromatin shearing
process required prior to immunoprecipitation could affect the result. Generation of
shorter _tlc1-tm_ telomeres by, for example, further mutation of _TEL1_, could help overcome
this problem. In addition, electrophoretic mobility shift assays (EMSA) with purified Rap1
would further confirm that Rap1 does not bind _tlc1-tm_ sequence. Mutagenizing the DNA
binding domain of Rap1 to find a Rap1 mutant able to bind _tlc1-tm_ sequence would be
useful for a complementation assay, which would confirm whether the absence of Rap1
is responsible for the observed phenotype. Determining the protein composition of _tlc1-
-tm_ telomeres would also be important. In the absence of Rap1, the recruitment of Rif1
and Rif2 (Hardy et al., 1992; Wotton and Shore, 1997) and the components of the Sir
complex (Moretti et al., 1994; Moretti and Shore, 2001) should be affected. We plan to
use biotinylated oligonucleotides with wild-type or mutant sequence incubated with yeast
protein extracts, followed by mass spectrometry to unbiasedly identify what proteins
are bound to mutant telomeres. Assessing telomere fusion occurrence would also be of
interest, since Rap1 protects chromosome ends from NHEJ events (Marcand et al., 2008).
In short, the finding of a Rap1-free telomere opens many questions. Probably the most
important one that needs to be addressed is how telomeres are regulated in the absence of
their major binding protein.

The general aim of the work presented in this thesis was to determine what
minimally constitutes a telomere in _S. cerevisiae_. We propose that Cdc13 and a telomeric
sequence of about 40 nt or longer are essential elements of telomeres. G-quadruplexes, on
the other hand, do not seem to play an important role at the chromosome ends. As often
happens, while trying to find an answer, many new questions arise, leaving plenty of room
for further research.
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