Telomere capping in non-dividing yeast cells requires Yku and Rap1

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The assembly of a protective cap onto the telomeres of eukaryotic chromosomes suppresses genomic instability through inhibition of DNA repair activities that normally process accidental DNA breaks. We show here that the essential Cdc13–Stn1–Ten1 complex is entirely dispensable for telomere protection in non-dividing cells. However, Yku and Rap1 become crucially important for this function in these cells. After inactivation of Yku70 in G1-arrested cells, moderate but significant telomere degradation occurs. As the activity of cyclin-dependent kinases (CDK) promotes degradation, these results suggest that Yku stabilizes G1 telomeres by blocking the access of CDK1-independent nucleases to telomeres. The results indeed show that both Exo1 and the Mre11/Rad50/Xrs2 complex are required for telomeric resection after Yku loss in non-dividing cells. Unexpectedly, both asynchronously growing and quiescent G0 cells lacking Rap1 display readily detectable telomere degradation, suggesting an earlier unanticipated function for this protein in suppression of nuclease activities at telomeres. Together, our results show a high flexibility of the telomeric cap and suggest that distinct configurations may provide for efficient capping in dividing versus non-dividing cells.

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and Sedivy, 2008). The ways telomeres achieve capping as well as their basic organization are well conserved in eukaryotes. For example, in virtually all organisms, telomeric DNA consists of short tandem DNA repeats, and at the very end of the DNA, the 3′-strand, usually a G-rich strand, protrudes beyond the 5′-end, forming a single-stranded G-tail (ssG-tail; LeBel and Wellinger, 2005). In addition, telomeres comprise proteins specifically associating with the double-stranded and the single-stranded DNA portions, and they fulfil two important functions: first, they assure the complete replication of telomeric DNA and, second, they distinguish chromosomal ends from DNA double-strand breaks (DSB) (Hug and Lingner, 2006; Gilson and Geli, 2007). The lack of recognizing telomeres as DSBs, thus not activating DNA integrity surveillance mechanisms and not allowing canonical DNA repair at them, is thought to be the most essential feature of telomeres (Lydall, 2009).

Given the importance of telomere capping in genome stability, the study of the consequences of dysfunctional telomeres has recently received increased attention. Again, many of the consequences observed after chromosome uncapping are very similar in evolutionary distant organisms such as yeasts and mammals (Verdun and Karlseder, 2007; Palm and de Lange, 2008). For instance, in mammalian and avian cells, the interference with double- or single-stranded telomeric DNA-binding proteins leads to a rapid cell-cycle arrest (Karlseder *et al*, 1999; Hockemeyer *et al*, 2005; Churikov and Price, 2008) and uncapped telomeres attract DNA-damage response factors, such as activated ATM, γH2A-X and 53BP1, which will cluster to form telomere damage-induced foci (Takai *et al*, 2003). Subsequent to their recognition as DSBs, uncapped telomeres are engaged in end-processing reactions and DNA repair processes. The outcomes include either enhanced illegitimate recombination because of increased C-rich-strand degradation and ssDNA accumulation at telomeres (Wang *et al*, 2004; Hockemeyer *et al*, 2006; Wu *et al*, 2006) or telomere–telomere fusions that are accompanied by loss of G-tails (Zhu *et al*, 2003; Deng *et al*, 2009). Such transactions at telomeres can result in abrupt loss of large fractions of telomeric tracts, initiation of destructive fusion-bridge-break cycles, premature senescence and, ultimately, cell death.

In budding yeast, functional capping is dependent on a heterotrimeric complex, composed of Cdc13, Stn1 and Ten1 (the CST complex), that binds the ssG-tails (Garvik *et al*, 1995; Grandin *et al*, 1997, 2001). Loss of function of any CST complex member allows 5′-strand degradation leaving extended overhangs of the 3′-end at the telomeres. They cause a robust cell-cycle checkpoint response and, eventually, cell death (Lydall, 2003). The double-stranded portion of telomeric DNA normally is bound by Rap1, which, at the telomeres, inhibits telomere–telomere fusions (Conrad *et al*, 1990; Pardo and Marcand, 2005). Finally, the yeast Ku complex also contributes to capping: cells lacking either of YKU70

Introduction

Efficient chromosome end capping exerted by telomeres not only is crucial for genome stability and suppression of tumorigenesis, but also allows for normal rates of cell division, delays senescence and protects against ageing (Aubert and Lansdorp, 2008; Deng *et al*, 2008; Jeyapalan

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or *YKU80* display shortened telomeric repeat tracts and ssG-tail accumulation at telomeres (Gravel *et al*, 1998). Furthermore, at elevated temperatures, such cells display hallmarks of activated DNA-damage checkpoints and stop dividing (Fisher and Zakian, 2005).

The mechanisms by which these telomere cap constituents prevent DNA repair attempts from initiating genome instability have just begun to be addressed. The emerging evidence suggests that in most cases, a dysfunctional telomere will be dealt with as a DSB elsewhere in the genome (Longhese, 2008). At such an accidental DSB, both DNA end processing and the choice of the eventual repair pathway used depend on the cell-cycle stage, during which the DSB arises. For example, several studies have unveiled that specific cyclin-dependent kinases (CDK) regulate DSB processing. In yeast, high S-CDK activity in S and G2 phases of the cell cycle stimulates DSB resection and repair by homologous recombination (Aylon *et al*, 2004; Ira *et al*, 2004), whereas in G1, low S-CDK1 activity correlates with preferred repair through NHEJ (Frank-Vaillant and Marcand, 2002; Karathanasis and Wilson, 2002; Ferreira and Cooper, 2004). It is thought that CDK enhances resection by phosphorylation of Sae2 (or its homologues), which co-operates with the Mre11/Rad50/Xrs2 (MRX) complex on the initial trimming of the DSB to generate short, 50–100 base 3'-overhangs (Limbo *et al*, 2007; Sartori *et al*, 2007; Huertas *et al*, 2008). This is followed by a secondary processing that exposes extensive 3'-single-stranded tails and is redundantly executed by either the Sgs1 helicase and the Dna2 nuclease or the 5'-3' exonuclease Exo1 (Mimitou and Symington, 2009). The evidence so far shows that generation of ssDNA at uncapped telomeres requires high activity of the S-CDK and may be limited to late S and G2-M phases (Vodenicharov and Wellinger, 2006). Importantly, this requirement for high CDK1 activity in telomere processing coincides in time with active telomere replication by telomerase, indicating that CDK1 activity may control both telomerase- and recombination-mediated telomere elongation (reviewed in Vodenicharov and Wellinger, 2007). Consistent with this hypothesis, the generation of telomeric G-tails appears to have similar requirements in terms of nucleases and CDK1-dependent Sae2 phosphorylation as the processing events at a DSB mentioned above (Bonetti *et al*, 2009). However, it is currently unknown whether individual telomere cap components are devoted to end protection at different stages of the cell cycle and how the telomeres of non-dividing cells lacking CDK1 activity are protected.

In the work presented here, we investigated how telomeres are protected in G1 of the cell cycle. Earlier data showed that the ablation of essential-capping proteins Cdc13 or Stn1 in G1 phase did not affect telomere integrity and cell viability (Vodenicharov and Wellinger, 2006). Thus, we examined telomere resection in G1 phase or in quiescent cells and assessed which components of the telomere cap are most crucial for protection in the absence of active S-CDK1. The results show that in non-dividing cells, resection at telomeres can still occur in principle. However, in this situation, the Yku complex has a central function for blocking nuclease access to telomeres. The results also show that in the absence of Yku, the Mre11 and Exo1 nucleases co-operate to resect telomeres. Surprisingly, we found that the depletion of Rap1 from telomeres leads to DNA degradation in both

non-dividing and cycling cells. Thus, the data establish that in resting cells, multiple activities can impinge on genome integrity after telomere uncapping. They, therefore, highlight a certain specialization among different telomere-capping factors: some may be crucial in replicating cells, whereas others may be required specifically during G1 phase or in cells that have exited the mitotic division cycle.

Results

CST complex is dispensable for telomere protection in G1 phase

Our earlier data show that after inactivation of the essential-capping proteins Cdc13 or Stn1, telomeres remain stable in G1-arrested cells (Vodenicharov and Wellinger, 2006). The third member of the CST complex, Ten1, was reported also to be an OB-fold containing protein (Gao *et al*, 2007) and mediate the CST protective function (Xu *et al*, 2009). We, therefore, tested whether Ten1 substitutes for Cdc13 function and stabilizes telomeres in G1. Using temperature-inducible degron (td) versions of the proteins, we depleted Cdc13 and Ten1 from cycling and G1-arrested cells (Vodenicharov and Wellinger, 2006). In degron inducing conditions, both the Cdc13-td and Ten1-td fusion proteins were rapidly degraded and only 2 h after galactose addition at 37°C, they became virtually undetectable (Figure 1A). Cycling *cdc13-td* and *ten1-td* cells also exhibited rapid telomere resection and ssDNA accumulation, a hallmark of telomere deprotection (Figure 1B). However, telomeres did not show visible signs of degradation when G1-arrested *cdc13-td* or *ten1-td* cultures were exposed to degron-inducing conditions for the same amount of time as cycling cells. Furthermore, telomere integrity was fully preserved after a simultaneous depletion of Cdc13 and Ten1 in G1-arrested cells (Figure 1B). In sharp contrast, the simultaneous induction of *cdc13-td* and *ten1-td* degrons in cycling cells leads to massive telomere degradation: the signal for ssG-tails detected in DNA derived from the double mutant is much more important than that for either of the *cdc13-td* or *ten1-td* single. Together with the fact that telomeres in *stn1-td*-containing cells also remain intact in such an experiment (Vodenicharov and Wellinger, 2006), we conclude that the CST complex as a whole is dispensable for telomere protection in G1.

Direct function for Yku in maintaining stable telomeres in G1

A loss of Yku accelerates resection at a DSB in G1-arrested cells (Clerici *et al*, 2008) and enhances focus formation of ssDNA-binding proteins such as replication protein A and Rad51 (Zhang *et al*, 2007; Barlow *et al*, 2008). We, therefore, wished to test whether Yku prevents telomeric end resection in CST-lacking G1-arrested cells. For this purpose, a degron-conditional allele of *YKU70* was constructed. This Yku degron strain grew at wild-type rates on glucose-containing plates at 23°C and telomere length and structure were unperturbed in these conditions (Figure 2A, Raff, 23°C; Figure 2B glucose). Only 2 h after degron induction, the Yku70-td protein became undetectable (Figure 1A) and telomeres in cycling cells had acquired extended ssG-tails typical for cells lacking Yku (Figure 2A). Strikingly, in G1-arrested cells, a loss of Yku70-td alone also resulted in clearly discernable ssG-tail-specific signals (Figure 2A), and this telomere resection occurred at a

Figure 1 The CST complex is dispensable for capping in G1 phase. (A) Total cellular protein extracts from strains MVY160 (*cdc13-td ten1-td*) and MVY150 (*cdc13-td yku70-td*) were prepared before (Raff, 23°C) or 2 h after degon induction (Gal, 37°C) and analysed by western blotting using antibodies against the DHFR moiety of the degon. *: non-specific cross-reacting band. The stripped membrane was re-probed with anti-Sir2 antibody (bottom panel) as loading control. (B) Yeast strains MVY 63 (wild type), MVY81 (*cdc13-td*), MVY145 (*ten1-td*) and MVY160 (*cdc13-td ten1-td*) were pre-grown in YEP-Raffinose (YPR) at 23°C before splitting. One part was left in YPR at 23°C (lanes 1, 4, 7 and 10); cells in the second were arrested in G1 with α -factor and then shifted to 37°C in YEP-Galactose (YPG) for 5 h (lanes 2, 5, 8 and 11), and cells in the third were shifted to 37°C in YPG for 5 h (cycling, lanes 3, 6, 9 and 12). XhoI-digested genomic DNA was analysed for telomere resection by non-denaturing in-gel hybridization. Native gels (top panel) were hybridized to a telomeric CA probe to detect exposed G-rich single-stranded DNA. The gel was denatured and re-probed with the same probe to control of DNA loading (bottom panel). M: size marker. ds + ssGT: controls for single-stranded G-rich and double-stranded telomeric repeats.

comparable level in a *yku70-td cdc13-td* double mutant strain. It is noteworthy that the timeframes used here for Yku70-td degradation were not long enough to cause significant telomere shortening (Figure 2A). The effects on the terminal DNA structure, therefore, are very much confined to the very terminal area of the telomeres without much change in the overall length of the double-stranded portion.

Using an earlier isolated *ts* allele of *YKU70* (Gravel and Wellinger, 2002), we obtained results that recapitulated those obtained with the *yku70-td* allele above (Supplementary Figure S1). Another recently described factor associated with telomere capping is the conserved KEOPS complex (Downey *et al*, 2006). However, we could not find evidence for an implication of KEOPS in telomere resection in G1 (Supplementary Figure S2A). Further, it was proposed that telomerase activity (Chan and Blackburn, 2003) or a telomere-bound telomerase enzyme (Vega *et al*, 2007) may contribute to the protection of short and partially uncapped telomeres. Yku80 interacts with a stem loop in *TCL1* RNA (Peterson *et al*, 2001; Stellwagen *et al*, 2003) to tether telomerase to telomeres in G1 (Fisher *et al*, 2004). Thus, we investigated whether in G1-cells, the cause for telomere deprotection in the absence of Yku is due to an inability of telomerase to localize to telomeres. In cells harbouring the *tlc1-Δ48* allele of *TLC1*, the Yku80-*TLC1* RNA interaction is disrupted, and we thus combined this allele with the *cdc13-td* allele. Surprisingly, telomere resection in such double mutant cells arrested in G1 cells remained undetectable and not significantly different from that seen in *cdc13-td* cells (Figure 2C). Moreover, even in cycling cells, there was no significant enhancement of ssG-tail signals in the *cdc13-td tlc1-Δ48* strain as compared with that in *cdc13-td* cells

(Figure 2C). The same experiments carried out using the reciprocal *yku80-135i* allele yielded indistinguishable results (data not shown). Taken together, these results show that during the G1 phase, the Yku complex assumes an important protective function at telomeres that is independent of its interaction with the telomerase RNA.

Telomere deprotection in G1 does not result in checkpoint activation or reduced viability

A loss of the CST-capping function in cycling cells leads to a rapid Rad9-dependent cell-cycle arrest in G2/M (Garvik *et al*, 1995). Cells lacking Yku and incubated at elevated temperatures activate DNA-damage checkpoints and cease growing (Feldmann and Winnacker, 1993; Teo and Jackson, 2001; Maringele and Lydall, 2002). Thus, we examined whether abrogation of Yku-dependent telomere capping in G1 would lead to DNA-damage responses and loss of viability. *Yku70Δ* strains harbouring either a wild type or the *yku70-30^{ts}* allele on a plasmid were grown at 37°C for approximately six population doublings to inactivate Yku and then divided in two parts. One was left asynchronous, whereas the other was α -factor arrested before incubation for 8 h at 37°C. Cells of each culture were spotted on agar plates and re-grown at either 23 or 37°C to score for viability at permissive or restrictive conditions for the *yku70-30^{ts}* allele, respectively. There was no loss in viability after Yku inactivation in G1-arrested cells, even for the Yku null strain harbouring an empty vector (Figure 3A, leftmost panel). The viability of asynchronously growing *yku70-30^{ts}* strain decreased by a magnitude during the time interval assayed here and the viability of *yku70Δ* cells was reduced even further (Figure 3A, third panel). Similar experiments conducted in a *cdc13-td*

yku70Δ strain harbouring either a wild-type or the *yku70-30^{ts}* allele showed that even if both Yku and the CST complex were inactivated at the same time in G1, viability remained unchanged (Supplementary Figure S2B, Gal). Yet, Yku70 inactivation was clearly deleterious to cells continuously exposed to 37°C on plates (Figure 3A; Supplementary Figure S2B, right side panels).

To assess checkpoint activation in cells lacking Yku, we used cells harbouring the *yku70-td* allele and kept for up

to 5 h at restrictive temperature. Surprisingly, there was no detectable Rad53p phosphorylation, a signature of checkpoint activation, in either G1-arrested or cycling cells (Figure 3B, lanes 8–9). In these conditions, some telomere resection did occur and ssG-tails were detectable, yet overall telomere length was virtually normal (Figure 2A). Combined with earlier data (Gravel and Wellinger, 2002), these results suggest that after loss of Yku-mediated telomere capping, telomere resection does occur even in G1-arrested cells. However, the net outcome is not sufficient for strong checkpoint activation nor does it lead to high levels of cell death, which is consistent with very limited C-strand loss that remains confined to the telomeric repeat tracts.

Exo1 and Mre11 collaborate to resect unprotected telomeres in G1

Given that there indeed is telomere resection in G1, we next sought to determine which exonucleolytic activities are responsible for it. For this, we used the strain with inducible *yku70-td* ablation to examine the contribution of different exonucleases to G1-specific telomere resection. Deletions of selected candidate nuclease genes were combined with the *yku70-td* allele, Yku loss was induced for 5 h in both G1-arrested or cycling cells and the resulting ssG-tail signals quantified (Figure 4). A deletion of *EXO1* completely reverses the ssG-tail-specific signals in both cycling (grey bars) and α -factor arrested (white bars) *yku70-td* cells to yield signals that are similar to those observed in *YKU70^{wt}* cells. *MRE11* deletion also suppresses ssG-tail DNA accumulation after Yku70-td depletion in G1-arrested cells (compare white to black bars in Figure 4B). However, in stark contrast to the situation with an *EXO1* deletion, the *MRE11* deletion did not have any apparent effect on ssG-tail levels in cycling cells. Finally, in *yku70-td sae2Δ* cells, the general pattern for telomere resection in both G1 and asynchronous cells is similar to that observed in *yku70-td* cells. The simplest interpretation of these data is that in G1-arrested cells, Mre11 and Exo1 co-operate in the processing of uncapped telomeric ends. Moreover, they also show that resection is considerably enhanced later in the cell cycle.

Rap1 stabilizes telomeres in quiescent cells and may counteract nucleases in G1

Budding yeast Rap1 binds to double-stranded telomeric repeats in which it negatively regulates telomerase, functions in gene silencing and inhibits NHEJ (Pina *et al*, 2003; Pardo

Figure 2 Yku protects telomeres from degradation in G1 phase. (A) MVY63 (wild type), MVY81 (*cdc13-td*), MVY165 (*yku70-td*) and MVY150 (*cdc13-td yku70-td*) cultures were processed exactly as in Figure 1B. Numbers below each sample indicate mean relative overhang signal intensities with the wild type set as 1 ($n=3$). The differences in ssDNA signal intensities between G1-arrested *yku70-td* and wild type or *cdc13-td* are significant ($P<0.002$), but not that between G1-arrested *yku70-td* and *cdc13-td yku70-td* ($P=0.153$). (B) The 10-fold serial dilutions of strains MVY63 (wild type), MVY81 (*cdc13-td*), MVY165 (*yku70-td*) and MVY150 (*cdc13-td yku70-td*) were spotted on plates in degtron-OFF (glucose 23°C) or degtron-ON (galactose, 37°C) conditions, grown for 3 days and photographed. Rows shown were on respective plates with all controls (see Supplementary Figure S8). (C) MVY63 (wild type), MVY81 (*cdc13-td*), MVY352 (*cdc13-td tlc1-Δ48*) and MVY335 (*tlc1-Δ48*) strains were treated exactly as in Figure 1B and ssDNA at telomeres analysed by in-gel hybridization as above.

Figure 3 Yku inactivation does not lead to checkpoint activation and loss of viability. (A) MVY480 (*yku70Δ*) cells transformed with empty vector, *pRS314-KU70* or *pRS314-ku70-30ts* plasmids were pre-grown overnight in Sc-TRP media containing raffinose at 37°C as described in Materials and methods. Half of the culture was then arrested in G1 with α -factor and the incubation continued at 37°C for 8 h for both G1-arrested and cycling cells. The 10-fold serial dilutions from the resulting cultures were spotted and grown on Sc-TRP plates containing glucose at either 23 or 37°C to assess cell viability after *yku70-30ts* inactivation. (B) Rad53 phosphorylation assessed in total protein extracts derived from MVY63 (wild type), MVY81 (*cdc13-td*), MVY165 (*yku70-td*) and MVY150 (*cdc13-td yku70-td*) strains was analysed by western blotting. The cultures were either G1-arrested (top) or asynchronously growing (bottom) in raffinose at 23°C (lanes 1, 4, 7 and 10), in galactose at 37°C for 2 h (lanes 2, 5, 8 and 11) or 5 h (lanes 3, 6, 9 and 12), as indicated. Phleomycin-treated wild-type cells served as a control for Rad53 activation (lanes 13 and 14).

and Marcand, 2005). However, this protein also has a widespread essential function in transcriptional activation (Lieb *et al*, 2001). To directly assess the consequences of Rap1 loss on telomere integrity, we constructed a conditional *rap1-td* allele (Supplementary Figure S3). Cells harbouring this new allele grew slightly slower than wild-type cells, even in degen-uninduced conditions (Supplementary Figure S3B). However, telomere length analyses on DNA derived from *rap1-td* cells after outgrowth for at least 125 generations did not reveal any changes in telomere size or heterogeneity and these cells grew like wild type when cultured in liquid media (data not shown). In degen-inducing conditions (galactose, 37°C), cells containing the *rap1-td* allele stopped growing and their telomeres gradually elongated (Supplementary Figure S3B, S3C). To completely deplete Rap1-td, an incubation time of >2 h in galactose-containing media at 37°C was required (Figure 5A; Supplementary Figure S3A). Therefore, in our experiments, Rap1-td degradation was induced for either 5 or 16 h before carrying out further analyses. After such an induction, the cellular Rap1-td pool fell well below the detection limit on western blots (Figure 5A) and telomere-bound Rap1-td was also lost as assessed by chromatin immunoprecipitation (Supplementary Figure S3D). Unfortunately, *rap1-td* cells did not respond to α -factor stimulation with cell-cycle arrest, even under permissive growth conditions, suggesting that some aspects of Rap1 function in silencing are affected in these *rap1-td* mutants. To analyse effects of Rap1 loss on telomeres in non-cycling cells, we turned to cultures in stationary phase (G0-cells). As validation for a similar behaviour of telomeres in G0 as in G1, *yku70-td* cells exhibited indistinguishable telomeric DNA degradation when arrested in either way (Figures 2A and 5C), whereas telomeres in *cdc13-td* harbouring cells were stable in both (Figures 1A, 2A and 5C). In addition, both

resting G0 cells and G1-arrested *yku70-td* cells shared the same requirement for Exo1 and Mre11 in telomere resection (Figure 4; Supplementary Figure S4).

G0 arrest in all experiments was achieved by growing cells in liquid raffinose media to saturation, typically about 72 h. One half of the culture then received galactose and was shifted to 37°C for 16 h, thereby inducing Rap1-td-loss, whereas the other remained at 23°C for the same time. Cell-cycle exit and G0 accumulation was monitored by FACS analysis of relevant *rap1-td* cultures (Figure 5B). We did not observe signs of cell-cycle re-entry immediately after galactose addition to G0 cultures, suggesting that other nutrients than carbon were limiting cell growth (Supplementary Figure S5). The steady-state levels of Rap1-td were somewhat lower in quiescent G0 cells; however, the Rap1-td protein was completely ablated in these cells after induction of degen proteolysis (Figure 5A). Analysis of DNA extracted from G0-residing *rap1-td* cells under native gel conditions revealed that the telomere-terminal DNA structure remained intact at 23°C, but was severely compromised at 37°C (compare lanes: Raff, 16 h with Gal, 16 h in G0-arrested *rap1-td* cells, Figure 5C). These data show that in non-dividing cells, both Rap1 and the Yku complex are required for an efficient barrier against nucleases.

Telomeric dsDNA-binding proteins in fission yeast and mammals have been reported to have functions in suppressing illegitimate recombination and facilitating replication fork progression through telomeric repeats (Wang *et al*, 2004; Miller *et al*, 2006; Sfeir *et al*, 2009). Thus, the signals observed in native gels after Rap1 loss may represent exacerbated signals for ssG-tails at chromosomal termini, be partially single-stranded extrachromosomal DNA circles or represent other aberrantly shaped DNA structures produced during perturbed telomere replication. However, experiments

Figure 4 Nucleases processing uncapped telomeres in G1. (A) Yeast strains MVY63 (wild type), MVY165 (*yku70*-td), MVY425 (*yku70*-td *mre11*Δ), MVY430 (*yku70*-td *sae2*Δ) and MVY420 (*yku70*-td *exo1*Δ) were processed and extracted DNA analysed as in Figure 1B. (B) Quantification of telomeric ssDNA in same strains as in (A) was carried out as described in Materials and methods. The cultures analysed were as follows: black bars: asynchronous in YPR at 23°C, white bars: G1-arrested YPG at 37°C, grey bars: asynchronous in YPG at 37°C. Mean and s.d. were calculated by averaging values from four independent experiments. The difference in ssDNA signal intensities was significant between cycling wild type and *yku70*-td ($P=0.0082$), *yku70*-td *mre11*Δ ($P=0.0022$) or *yku70*-td *sae2*Δ ($P=0.0016$), but not between cycling wild type and *yku70*-td *exo1*Δ ($P=0.322$) cells. The P -value difference between G1-arrested wild type and *yku70*-td ($P=0.04$) or *yku70*-td *sae2*Δ ($P=0.018$) cells was also significant, but not that between G1-arrested wild type and *yku70*-td *exo1*Δ ($P=0.25$) or *yku70*-td *mre11*Δ ($P=0.1$) cells.

in which DNA from such Rap1-depleted cells was treated with various nucleases confirmed that the signals detected here indeed represented terminal restriction fragment (TRF)-associated ssDNA extensions (Supplementary Figure S6).

Collectively, these experiments show that Rap1 depletion from telomeres leads to uncapping and C-strand degradation by exonuclease(s) in dividing and quiescent G0 cells. Thus, blocking nucleolytic activities at telomeres even in non-dividing cells appears to be a shared responsibility of multiple telomere-capping factors, underscoring the vital necessity for tight control over nuclease action at telomeres.

ssDNA accumulation in *rap1*-td cells requires EXO1 but does not activate DDR

To assess whether Exo1 was also required for ssDNA generation in *rap1*-td cells, the *EXO1* gene was deleted in cells harbouring the *rap1*-td allele and cycling or G0 stationary *rap1*-td and *rap1*-td *exo1* cultures were analysed for telomere resection after Rap1 loss. Once again, *rap1*-td cells displayed extensive telomeric DNA degradation in both cycling and G0-resting cells (Figure 6A, lanes 6 and 8). This telomere resection observed in cycling *rap1*-td cells was largely dependent on Exo1 as no signals are detected on DNA derived from *rap1*-td *exo1* cells (Figure 6A, lane 10). Surprisingly, this was not the case for resting cells in G0: *rap1*-td *exo1* cells exhibited ssG-tail signals as strong as *rap1*-td cells (Figure 6A, lane 12). These results indicate that after loss of Rap1 from telomeres, the resection occurring in non-dividing cells is independent of *EXO1*. This is in stark contrast to the situation after Yku loss in which C-strand degradation in both G0- and G1-arrested cells is dependent on *EXO1* (Figure 4; Supplementary Figure S4).

The above data strongly suggest that Rap1 and Yku govern two independent and parallel pathways that restrict nucleases in non-dividing cells. To verify this prediction, we deleted Yku70 in *rap1*-td cells and examined the effect in G0 stationary cultures. At permissive conditions, the *rap1*-td *yku70* cells exhibited short telomeres with long ssG-tail signals characteristic for Yku null cells (Figure 6B, lane 7). Upon degran induction and loss of Rap1, telomeres elongated, telomere degradation increased and their general pattern was consistent with the notion that Rap1 and Yku function in separate pathways in G0 telomere protection (Figure 6B, lane 8). We also monitored checkpoint activation through Rad53 phosphorylation in the *rap1*-td and *rap1*-td *yku70* cells. Given the very strong ssG-tail signals observed in *rap1*-td cells and that the checkpoint gets activated in cycling *yku70*Δ cells at 37°C (Teo and Jackson, 2001; Gravel and Wellinger, 2002; Maringe and Lydall, 2002), we expected to easily detect phosphorylated forms of Rad53 in these strains. Surprisingly, however, only a very low level of Rad53 phosphorylation was observed in *rap1*-td cells (Figure 6B, lane 6). Moreover, the *rap1*-td *yku70* mutants displayed only a slightly stronger Rad53 activation than that observed in the *yku70*Δ strain (compare lanes 8 and 4, Figure 6B), data that again are consistent with an additive effect of Rap1 and Yku inactivation.

As the experiments above were carried out with G0 stationary cultures, we also wished to examine Rad53 status in cycling *rap1*-td cells, in which the cell-cycle block induced by checkpoint activation should normally lead to accumulation of cells with fully replicated DNA in G2/M. An exponentially growing *rap1*-td culture was shifted for 16 h to 37°C in galactose, re-diluted into fresh galactose-containing media and the incubation continued for 3 h. Strikingly, phosphorylated Rad53 could not be detected in these cells (Figure 6C, lane 7). This lack of Rad53 phosphorylation was not due to a general inability of Rad53 phosphorylation, because the radiomimetic drug phleomycin could induce strong Rad53 phosphorylation in this same culture (Figure 6C, lane 8). Furthermore, FACS analyses of these *rap1*-td cultures did not reveal any G2/M accumulation (Figure 6D); yet these cell populations had undergone about 5–6 population doublings during the overnight incubation at restrictive

Figure 5 Rap1 ablation in G0 cells leads to massive telomere degradation. (A) MVY401 (*rap1-td*) cells were grown to mid-log phase in YPR at 23°C and the culture was divided into two. Galactose was added to one half to induce Rap1-td loss in cycling cells for 5 or 16 h at 37°C. The second half was allowed to grow till saturation at 23°C (typically 60 h) before addition of galactose and degon induction for 16 h at 37°C. Whole-cell protein extracts from these cultures were analysed by western blotting using antibodies against the DHFR moiety of the Rap1-td degen (upper panel) or anti-HA antibodies recognizing the HA-tagged Ubr1 (middle panel). The stripped filter was re-probed with anti-Sir2 antibody as a loading control (bottom panel). (B) FACS analyses of relevant cultures in (A). (C) DNA was extracted from cycling or stationary MVY81 (*cdc13-td*), MVY401 (*rap1-td*) or MVY165 (*yku70-td*) cells incubated in conditions described in (A) and analysed by non-denaturing in-gel hybridization.

conditions (data not shown). In fact, the FACS profiles after Rap1-td loss show a significant increase in G1-cells, consistent with a loss of Rap1-dependent gene transcription important for cell-cycle entry (Figure 6D). The absence of robust Rad53 phosphorylation and checkpoint activation in *rap1-td* cells is at odds with the visibly strong ssG-tails signals in these cells. For instance, in cycling *cdc13-td* cells, similarly strong ssG-tail signals are accompanied with robust Rad53 phosphorylation at 2 and 5 h after degen induction (Figures 1A, 2A and 3B). To understand the basis of this apparent discrepancy, we sought to obtain a more precise estimate of the length of the single-stranded tracts formed at telomeres after ablation of different capping proteins. Genomic DNA prepared after an exposure for 6 h to restrictive conditions was digested with a mix of four base-pair recognition restriction endonucleases that digest the bulk DNA to an average of < 100 bp fragments, but does not cleave telomeric repeats and ssDNA. The length of the TRFs generated in this way should be sensitive to the extent of single-strand extension. Indeed, when we combined this approach with native in-gel hybridization, TRFs derived from *cdc13-td* cells were longer and ssDNA signal significantly stronger than those of *yku70-td* cells (Figure 6E, native). However, there was little, if any, difference in the length of ssDNA tracts derived from *rap1-td* and *yku70-td* cells (Figure 6E). Furthermore, when genomic DNA from the same strains was hybridized under non-denaturing conditions to Y'-specific oligonucleotide probes in a slot-blot assay, only the *cdc13-td* samples yielded readily detectable hybridization signals (Figure 6F). Parallel control analysis of XhoI-digested DNA samples revealed telomere degradation patterns in all degen strains tested (Supplementary Figure S6D). Although our data cannot exclude that in the absence of Rap1 or Yku a few telomeric ends might

become extensively resected, they show that the overall quality of DNA resection is comparable in *rap1-td* and *yku70-td* cells and that these products rarely contain Y' sequences.

Collectively, these data show that after loss of Rap1 from telomeres, there are at least two resection activities active at telomeres: one is *EXO1* independent, acting in resting G0 cells, and one that is *EXO1* dependent, acting in cycling cells. Similar to the situation in Yku cells, the latter situation seems not to be sufficient to activate the DNA-damage checkpoint, suggesting a rather limited terminal C-strand resection.

Discussion

Abrogation of chromosome capping in various experimental models triggers DNA-damage and repair responses comparable with those occurring after DSB formation (Longhese, 2008; Palm and de Lange, 2008). Although after a DSB these responses are essential to restore genome integrity, at dysfunctional telomeres, they result in terminal DNA degradation, telomere-telomere fusions and gross chromosome rearrangements thereby fuelling genetic instability, a common feature of human cancer cells. However, the outcomes of telomere dysfunction differ for cells in different cell-cycle stages. In *Saccharomyces cerevisiae*, for example, the abrogation of the CST complex does not lead to telomere uncapping in G1 phase-arrested or quiescent G0 cells, whereas for actively cycling cells, this leads to extensive C-strand loss, DNA-damage responses and ultimately cell death (Vodenicharov and Wellinger, 2006; this study). The data also suggest that, at uncapped telomeres, tightly regulated CDK1-dependent repair attempts are not the only determinants for the cell-cycle-specific processing patterns

Figure 6 Telomere degradation in *rap1-td* cells does not induce a DNA-damage response. (A) Stationary (G0) (lanes 3, 4, 7, 8, 11 and 12) or exponentially growing cultures (lanes 1, 2, 5, 6, 9 and 10) of MVY63 (wild type, lanes 1–4), MVY401 (*rap1-td*, lanes 5–8) and MVY450 (*rap1-td exo1*, lanes 9–12) strains were incubated for 16 h either in YPR at 23°C (lanes 1, 3, 5, 7, 9 and 11) or in YPG at 37°C (lanes 2, 4, 6, 8, 10 and 12) to deplete Rap1. Analysis of genomic DNA derived from indicated strains and experimental conditions was as before (Figure 1B). In parallel, protein samples were prepared from the same cultures and subjected to western blot analysis with anti-Rap1 antibody (bottom) to monitor degra proteolysis. (B) Stationary (G0) MVY63 (wild type), MVY 480 (*yku70Δ*), MVY401 (*rap1-td*) and MVY 550 (*rap1-td yku70Δ*) cells were incubated for 16 h either in YPR at 23°C (lanes 1, 3, 5 and 7) or in YPG at 37°C (lanes 2, 4, 6 and 8) to deplete Rap1. DNA samples were collected and analysed as in (A) and protein samples were probed on western blots for Rap1-td degradation and for Rad53 activation. (C) Asynchronous MVY63 (wild type) and MVY401 (*rap1-td*) cultures growing at 23°C in YPR or grown for 16 h at 37°C in YPG to induce the *rap1-td* degra were mock-treated (–) or treated (+) with 10 g/ml phleomycin for 1 h before extraction of proteins and analysis with anti-Rad53 antibodies on western blot. (D) FACS analysis of same strains as in (A) grown asynchronously for 16 h either in YPR at 23°C or in YPG at 37°C. (E) Exponentially growing MVY63 (wild type), MVY 81 (*cdc13-td*), MVY 165 (*yku70-td*) and MVY401 (*rap1-td*) strains in YEP-Raffinose at 23°C were shifted to 37°C for 6 h to induce degra. DNA derived from the cultures before and after degra induction was digested with a mixture of AluI, HaeIII, HinfI and MspI before in-gel hybridization analysis under native (left) and denaturing (right) conditions using a telomeric CA probe. (F) Genomic DNA from the same strains and conditions as in (E) was applied in native form (upper row) or after denaturation (lower row) to a nylon membrane in a slot-blot apparatus and hybridized to Y'-specific radiolabelled oligonucleotide probes (upper panel); the DNA on the same filter was then denatured and rehybridized to a probe specific for the *TRP1* locus (bottom panel).

(Shrivastav *et al*, 2008). We propose that both the proliferative state and the functional specialization of telomere proteins contribute to the distinct requirements for efficient capping in non-dividing versus dividing cells.

Functional specialization of telomeric proteins in telomere protection

Genomic DSBs are actively processed in a CDK1-induced manner immediately after S-phase entry (Aylon and Kupiec,

2005; Doksan *et al*, 2009). Of note, there is a direct causal relationship between DNA replication, enhancement of DSB processing and checkpoint activation (Zierhut and Diffley, 2008; Doksan *et al*, 2009). In contrast, CST-lacking uncapped telomeres remain intact during G1 and throughout most of S phase (Vodenicharov and Wellinger, 2006). These data indicate that the essential function of CST is restricted to a narrow time window correlating with telomere replication in late S phase. Hence, we propose that the CST complex performs a rather specialized function in protection that is intimately linked to the passage of the replication fork. The specialized function of CST in capping replicating telomeres is likely directly connected to its function in coordination of conventional semiconservative DNA replication with telomerase-mediated telomere elongation (Qi and Zakian, 2000; Grossi *et al*, 2004). This is consistent with mounting evidence suggesting that the CST complex is a specialized RPA-like complex (Gao *et al*, 2007). Interestingly, CST-like complexes recently have been discovered in a variety of organisms, including mammals, which underscores the functional conservation of the replication-coupled type of telomere capping (Casteel *et al*, 2009; Miyake *et al*, 2009; Surovtseva *et al*, 2009; Wan *et al*, 2009). It has also become clear that not all functions of the CST complex are simply a reflection of its ssDNA-binding activity (Vodenicharov and Wellinger, 2006), and there is increasing evidence for cell-cycle-regulated post-translational modifications of at least some proteins. For example, CDK1-mediated Cdc13 phosphorylation at tyrosine T308 is necessary for wild-type levels of telomere elongation by telomerase and negatively affects Cdc13 stability (Li *et al*, 2009; Tseng *et al*, 2009).

Unlike the CST complex, Yku may encircle duplex DNA ends in a way that could obstruct replication (Walker *et al*, 2001). Therefore, this complex may be actively removed from telomeric ends to allow passage of replication forks and post-translational modifications may contribute to this regulation (Zhao and Blobel, 2005; Postow *et al*, 2008). Nevertheless, the complex appears to have a devoted function in telomere protection in non-dividing cells and this G1-specific protection inhibits DNA processing and degradation at telomeres, perhaps in a similar manner as NHEJ-proteins prevent DSB processing (Zhang *et al*, 2007; Clerici *et al*, 2008; Wu *et al*, 2008; Zierhut and Diffley, 2008). However, after loss of Yku in G1, telomere resection is rather limited and the deprotected G1 telomeres do not induce a DNA-damage response, nor is there a reduced viability (Figures 2 and 3). At a DSB, resection and initiation of a checkpoint response in G1 is positively correlated with the actual number of DSBs and four DSBs are sufficient to elicit a robust response (Zierhut and Diffley, 2008). Assuming that Yku loss renders all 32 chromosomal ends accessible to degradation, the results presented here indicate that the signal emanating from uncapped telomeres is far less pronounced than the one generated by DSBs. The data thus support the notion that upon Yku loss, there is very limited degradation exclusively at the tips of telomeric tracts. This limitation may be brought about by other telomeric proteins, such as Rap1 (see below), or by the heterochromatic nature of telomeric chromatin.

Our results thus strongly suggest that at telomeres, Yku is an important factor for blocking initiation of resection. In Yku-lacking cells, Exo1 is essential for resection in all cell-cycle phases, whereas Mre11 appears to contribute

significantly to initial telomere resection only in G1 (Figure 4). Thus, G1-specific telomeric resection requires both Exo1 and Mre11, but in cycling cells, Mre11 is not required. Our results, therefore, highlight the distinct requirements for processing activities at endonuclease-induced DSB versus deprotected telomeres. In terms of the specific activity of the MRX complex, our results here closely parallel those obtained for processing of HO endonuclease-induced DSB (Ivanov *et al*, 1994; Lee *et al*, 1998; Clerici *et al*, 2008). In fission yeast, the telomere-specific function is shared between the Mre11 and Dna2 nucleases (Tomita *et al*, 2004), suggesting a possible function for the latter in telomere processing in budding yeast as well. It should be noted that although telomere processing can take place independently of CDK1 activity (Figures 2 and 4), the latter either potentiates this process or increases the number of initiating events at telomeres.

Our analyses of telomere resection in G1 failed to confirm an earlier suggested putative protective function for telomerase (Vega *et al*, 2007). Although we could confirm the decreased maximum permissive temperature of *cdc13-1* cells bearing *tlc1-Δ48* or *ku80-135i* alleles (MV & RW, unpublished), no increases in ssDNA signals could be detected, arguing against an increased accessibility of telomeres to nucleases in the absence of G1-bound telomerase (Figure 2).

Important function for Rap1 in preserving telomeric DNA end structure

Perhaps the most striking discovery of our work is that a loss of Rap1 results in the generation of ssG-tails both in dividing and non-dividing cells (Figures 5 and 6). Earlier work had established that Rap1 is a negative regulator of telomerase (Marcand *et al*, 1997) and inhibitor of NHEJ (Pardo and Marcand, 2005). Our work here confirms that after Rap1 loss, telomeres do lengthen (Figures 5 and 6). Although one could invoke mechanisms of unrestrained telomerase-mediated G-strand elongation as source for the striking ssG-tail signals, we think this scenario is unlikely, particularly for resting G0 cells. First, Est1, an essential co-factor for telomerase in yeast, is actively degraded upon exit of G2/M and the protein presumably is not present in G0 (Osterhage *et al*, 2006). Second and consistent with the above, telomerase appears not being able to elongate telomeres in G1-arrested cells (Marcand *et al*, 2000; Osterhage *et al*, 2006). Therefore, the Exo1-independent ssG-tails formed in G0 cells most likely are the net outcome of terminal C-strand degradation activities. In the case of cycling cells, the appearance of the ssG-tail signals is dependent on yeast Exo1 (Figure 6). Although we cannot exclude a contribution of telomerase in the telomere lengthening phenotype, this latter result suggests that for these cells as well, the bulk of ssG-tails were created by C-strand degradation. However, it is important to emphasize that degradation at individual telomeres in Rap1-depleted cells appears not very extensive. First, TRF sizes, even after prolonged time periods in the absence of Rap1 did not decrease, but rather increase. Second, in contrast to the situation after interference with CST, depletion of Rap1 did not lead to checkpoint activation (see below). Third, the size of ssDNA resection products was similar in *rap1-td* and *yku70-td* cells (Figure 6E). And finally, hybridization experiments failed to reveal resection into Y' sequence in DNA from *rap1-td* cells (Figure 6F). Consistent with the idea that Rap1

inhibits only distal C-strand degradation, yeast cells, in which the telomeric repeat sequences are replaced with vertebrate repeats that do not normally bind Rap1, longer ssG-tails and increased Cdc13 association with telomeres were detected (Alexander and Zakian, 2003; Bah *et al*, 2004). This enhanced degradation may stem directly from perturbations in telomeric chromatin in the absence of Rap1, which may facilitate nuclease access to telomeric ends. Such a scenario is supported by observations that several C-terminus Rap1 mutants that affect telomere silencing exhibit elevated ssDNA levels at telomeres (Supplementary Figure S7).

Lack of checkpoint activation in Rap1-deprived cells

Remarkably, despite the presence of apparently high amounts of ssDNA, Rap1 depletion did not evoke strong checkpoint responses. It is still unclear how many telomeres become functionally uncapped after Rap1 or Cdc13 inactivation and what would be the threshold level of purely telomeric ssDNA to activate the checkpoint. Processing at an HO-induced DSB occurs at different rates at different ends and only a few are extensively resected (Zierhut and Diffley, 2008). Our data are consistent with these results (Figure 6). Thus, in *rap1*-td cells, only a few telomeric ends may be subject to extensive resection and most ends bear relatively short overhangs not reaching into subtelomeric sequences. Another possibility is that in *rap1*-td cells, the combined presence of the other telomere-capping factors CST and Yku attenuates the checkpoint response (Michelson *et al*, 2005). Likewise, the deletion of *Schizosaccharomyces pombe* Taz1 protein results in telomere-uncapping and elongated G-rich overhangs without apparent checkpoint activation (Ferreira and Cooper, 2001; Miller *et al*, 2005). Finally, although *rap1*-td cells are able to activate Rad53 in response to DNA-damaging agents (Figure 6B), we cannot exclude that some factors specifically required for checkpoint activation at terminal forks are affected directly or indirectly in these cells (Doksani *et al*, 2009).

Taken together, the findings reported here strongly suggest that in addition to the cell-cycle regulation of DNA repair activities, the functional specialization of telomere-capping proteins determine the fate of unprotected telomeres during different phases of the cell cycle. The outcomes of telomere dysfunction in higher eukaryotes also appear to differ depending on the cell-cycle stage (Konishi and de Lange, 2008; Dimitrova and de Lange, 2009), thus highlighting the conserved nature of these processes. Our data indicate that telomere capping may rely on separate sets of proteins depending on the state in which a cell resides and suggest possible important differences in telomere cap composition and structure in dividing versus non-dividing cells. Cancer can arise from either quiescent or proliferating cells (Wang and Dick, 2005; Blagosklonny, 2006). Oncogenic mutations that increase self-renewal capacity of cells can either turn a quiescent stem cell into a proliferating cancer stem cell or a proliferating cell into an immortal cell. Thus, our findings have relevant implications for understanding the initiation of an early pre-cancer stage in resting cells. In such cells, repair of DNA damage may be altered and they may have accumulated telomeric damage that could potentate the process of their immortalization once they enter cycles of proliferation.

Materials and methods

Strains and plasmids

S. cerevisiae strains used are isogenic to the W303 background. Degron strains were constructed, verified and maintained as published (Sanchez-Diaz *et al*, 2004). Primer sequences used to construct degron strains are available upon request. All strains were normally maintained in YEP-Dextrose or YEP-Raffinose (YPR) at 23°C and shifted to 37°C in YEP-Galactose to induce telomere uncapping as indicated. Gene disruptions were made using a one-step PCR-mediated method (Brachmann *et al*, 1998). The *tlc1*-Δ48 or *ku80*-135i alleles were introduced by a two-step gene replacement procedure. The centromeric (*CEN*, *TRP1*) plasmids bearing either the *YKU70^{wt}* or the *yku70-30ts* gene were earlier described (Gravel and Wellinger, 2002). Complete genotypes of all strains used in this study are listed in Supplementary Table S1. To score for cell viability shown in Figure 3A; Supplementary Figure S2B, respectively, indicated strains were pre-grown for six generations at 37°C in YPR. This is needed to allow loss of telomeric repeats after Yku-inactivation to yield a very short tract and after which effects of Yku loss can be documented (Gravel and Wellinger, 2002). In case of *cdc13*-td *yku70*Δ cells, G1-arrested or cycling cultures were divided in two and galactose or glucose was added to induce or suppress *cdc13*-td, respectively. After 8 h, serial dilutions of each test culture were spotted on agar plates containing glucose and grown at either 23 or 37°C to score for viability at permissive or restrictive conditions for the *yku70-30^{ts}* allele.

Cell-cycle arrest and flow cytometry

To synchronize cells in G1, logarithmically growing cultures were incubated with 0.5 μg/ml α-factor for 3.5 hours at 23°C. For analyses of G0 cells, logarithmically growing overnight cultures were incubated for at least more 60 h (typically a total of 72 h), before carrying out the indicated treatments. For cellular DNA content analyses, sample aliquots were withdrawn from the cultures at indicated time points and fixed in 70% ethanol. Propidium iodide stained cells were analysed on an FACSscan flow cytometer (Becton and Dickinson Biosciences).

In-gel hybridization analyses, non-denaturing slot blot and ssDNA quantification

Total yeast genomic DNA was isolated using a modified glass bead procedure (Wellinger *et al*, 1993) aided by a FastPrep-24 (MP Biomedicals, Inc.) cell disruptor. Non-denaturing in-gel hybridization conditions, telomeric CA oligonucleotide and pCT300-derived probes were as described (Wellinger *et al*, 1993; Dionne and Wellinger, 1996). To control for equal loading, DNAs in the gels were denatured and re-probed with CA oligonucleotide. As a rule, a segment of the denatured gel ranging from 0.8 to 1.6 kbp and containing the TRFs is shown in most of the figures as a loading control. For ssDNA quantification, the signal from the entire lane on the native gels was integrated, except for Figure 4; Supplementary Figure S4, in which only the TRF signals were taken into account. Quantifications of telomeric ssDNA signals were carried out using a Storm Phosphor imager (GE-Healthcare) equipped with ImageQuant software. To calculate the relative overhang signals, the normalized signal was arbitrary set at 1 for the wild-type strain grown under given experimental conditions and the signal levels in various mutants were determined accordingly. This procedure ensures that the obtained relative signals can be directly compared between lanes, gels and independent experiments, irrespective of DNA loading differences. *P*-values for differences between ssDNA signals in different strains and conditions were determined using unpaired two-sample two-tailed *t*-test.

For estimation of the length of ssG-tails in various strains, genomic DNA extracted 6 h after degron induction was treated with a mixture of AluI, HaeIII, HinfI and MspI four-base cutters, before non-denaturing in-gel hybridization with telomeric CA oligonucleotides probe. Slot-blot hybridization in native conditions was carried as described by Garvik *et al* (1995), except that the hybridization was at 55°C using a mix of two Y'-specific oligonucleotides complementary to the G-rich telomeric strand. After denaturation, the membrane was hybridized to a random-primer-labelled *TRP1* probe located near *CENIV*.

For all figures in which composite blots or minimal band areas (westerns) are shown, larger areas or full gels are reproduced in the Supplementary Figure S8.

Protein extraction and western blot analysis

Whole-cell protein extracts were prepared as described (Knop *et al*, 1999) and samples analysed on 8% SDS-PAGE, followed by electroblotting onto HybondECL membrane (GE-Healthcare). Cdc13-td, Ten1-td and Yku70-td were detected using a mouse monoclonal antibody against the DHFR degon moiety (Abnova, clone 2B10, 1:150). Rap1-td was detected using a rabbit polyclonal antibody directed against the carboxy-terminus of Rap1 (Santa Cruz, 1:500). The same membranes were stripped and re-probed with an anti-HA antibody (Roche Diagnostics, clone 12CA5, 1:400) to monitor Ubr1 induction and with anti-Sir2 (Santa Cruz, 1:250) to control for equal loading. Phosphorylated Rad53 was detected either using rabbit polyclonal sera kindly provided by Dr N Lowndes, or a mixture of goat polyclonal antibodies directed against the N- and C-termini of Rad53 (Santa Cruz, 1:500).

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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