Suppression of cdc13-2-associated senescence by pif1-m2 requires Ku-mediated telomerase recruitment

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Abstract

In Saccharomyces cerevisiae, recruitment of telomerase to telomeres requires an interaction between Cdc13, which binds single-stranded telomeric DNA, and the Est1 subunit of telomerase. A second pathway involving an interaction between the yKu complex and telomerase RNA (TLC1) contributes to telomerase recruitment but cannot sufficiently recruit telomerase on its own to prevent replicative senescence when the primary Cdc13-Est1 pathway is abolished—for example, in the cdc13-2 mutant. In this study, we find that mutation of PIF1, which encodes a helicase that inhibits telomerase, suppresses the replicative senescence of cdc13-2 by increasing reliance on the yKu-TLC1 pathway for telomerase recruitment. Our findings reveal new insight into telomerase-mediated telomere maintenance.

Keywords: Cdc13; Pif1; Ku complex; replicative senescence; telomerase recruitment

Introduction

Telomeres are composed of G/C-rich repetitive sequences at the termini of eukaryotic chromosomes and play a pivotal role in genome maintenance by “capping” chromosome ends, preventing them from unwanted nucleolytic degradation, homologous recombination, and fusion with neighboring chromosomes (Jain and Cooper 2010). In addition, to overcome progressive telomere shortening due to the end replication problem, telomeres are elongated by a specialized reverse transcriptase called telomerase. In the budding yeast Saccharomyces cerevisiae, telomerase is minimally composed of the protein subunit Est2 and the RNA subunit TLC1 (Singer and Gottschling 1994; Lingner et al. 1997). However, additional accessory proteins, Est1 and Est3, are required for telomerase activity in vivo and are thought to be involved in the recruitment and/or activation of telomerase (Welling and Zakian 2012). Eliminating any of the Est proteins or TLC1 results in an “ever shorter telomeres” (est) phenotype characterized by progressive telomere shortening that ultimately leads to replicative senescence (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay et al. 1996).

Maintaining telomere length homeostasis through the regulation of telomerase is essential for genome stability. Several lines of evidence suggest that the recruitment of telomerase to telomeres involves a direct interaction between the Est1 subunit of telomerase and Cdc13, a protein that binds single-strand telomeric DNA with high affinity (Lin and Zakian 1996; Nugent et al. 1996). Expression of a Cdc13-Est2 fusion protein can support telomere maintenance in an est1A null mutant, suggesting that the main function of Est1 is to bring telomerase to telomeres (Evans and Lundblad 1999). Cdc13 is essential for telomere capping, so a null mutation is lethal; however, an extensively studied point mutant, cdc13-2, is not capping defective but displays an est phenotype (Nugent et al. 1996). The amino acid mutated in cdc13-2, E252, lies within the recruitment domain (RD), which is able to recruit telomerase to telomeres when fused to the DNA-binding domain of Cdc13 (Pennock et al. 2001). The mutation (E252K) results in a charge swap and can be suppressed by est1-60, which encodes a mutant Est1 with a reciprocal charge swap (K444E), suggesting a direct physical interaction between the two proteins (Pennock et al. 2001). Consistent with this idea, purified full-length Cdc13 and Est1 interact in vitro (Wu and Zakian 2011), and structural analysis revealed two conserved motifs within the Cdc13 RD, called Cdc13_EBM_N and Cdc13_EBM_C (referring to N- and C-terminal Est1-binding motifs, respectively), responsible for this interaction (Chen et al. 2018). The Cdc13 E252K mutation resides within the latter motif. Surprisingly, mutations in the Cdc13_EBM_C motif, including E252K, do not abolish the interaction between Cdc13 and Est1 in vitro despite causing a dramatic reduction in Est1 telomere association in vivo (Chan et al. 2008; Wu and Zakian 2011; Chen et al. 2018). Thus, the mechanism by which the Cdc13_EBM_C motif promotes telomerase-mediated telomere extension is still unclear.

In contrast, mutations in Cdc13_EBM_N abolish the Cdc13-Est1 interaction in vitro, yet only result in a modest reduction in Est1 telomere association and short, but stable, telomere length in vivo (Chen et al. 2018). This telomerase recruitment pathway works in parallel with a second pathway involving Sir4, the yKu complex, and TLC1. Double-strand telomeric DNA is bound by Rap1 (Buchman et al. 1988; Conrad et al. 1990), which interacts with Sir4 (Moretti et al. 1994). Sir4, in turn, interacts with the...
Yku80 subunit of the yKu complex (Roy et al. 2004), which binds to the tip of a 48-nm hairpin in TLC1 (Peterson et al. 2001; Stellwagen et al. 2003; Chen et al. 2018). Mutations that abolish the yKu-TLC1 interaction (e.g., tlc1Δ and yku80-135i) result in slightly short but stable telomeres (Peterson et al. 2001; Stellwagen et al. 2003), much like Cdc13EBM,N mutations. Disrupting both the yKu-TLC1 interaction and Cdc13EBM,N-Est1 interaction results in an est phenotype (Chen et al. 2018).

Pif1, a 5′–3′ helicase that is evolutionary conserved from bacteria to humans, directly inhibits telomerase activity at telomeres and DNA double-strand breaks (Schulz and Zakian 1994). Pif1 has both mitochondrial and nuclear isoforms, by altering the first (pif1-m1) and the second (pif1-m2) translational start sites, the functions can be separated (Schulz and Zakian 1994). The pif1-m2 mutant abolishes nuclear Pif1 and, similar to pif1Δ, has elongated telomeres (Schulz and Zakian 1994). In vitro, purified Pif1 reduces telomerase processivity and displaces telomerase from telomeric oligonucleotides (Boulé et al. 2005). In vivo, deletion of PIF1 increases telomere association of Est1, while overexpression of PIF1 reduces telomere association of Est1 and Est2 (Boulé et al. 2005).

We previously showed that a double-strand break adjacent to at least 34bp of telomeric sequence is efficiently extended by telomerase, resulting in the addition of a de novo telomere, but this does not occur in Cdc13EBM,N mutants, such as cdc13-2 (Strecker et al. 2017). Surprisingly, we found that the lack of telomere addition in cdc13-2 cells can be suppressed by the pif1-m2 mutation (Strecker et al. 2017). In this study, we find that pif1-m2 suppresses the replicative senescence caused by the cdc13-2 mutation in a manner dependent on the yKu-TLC1 telomerase recruitment pathway. In addition, pif1-m2 suppresses the replicative senescence caused by disrupting both the yKu-TLC1 and Cdc13EBM,N-Est1 interactions. These observations provide new insight into the complexity of telomerase-mediated telomere maintenance.

Materials and methods
Yeast strains and plasmids
All yeast strains used in this study are listed in Table 1. Standard yeast genetic and molecular methods were used (Sherman 2002; Amberg et al. 2005). The YEp24-CDC13 plasmid was first described in an article from the Hartwell lab, where it was originally designated YEp24-CDC13-161-4 (Garvik et al. 1995). Plasmids pEF54 (pRS415-cdc13-F237A) and pFR96 (pRS415-cdc13-F237A, E252K) were created by site-directed mutagenesis of pBD4317 (pRS415-CDC13, Strecker et al. 2017) using primers designed by NEBaseChanger and the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Cat. No.: E0554S). The mutations were confirmed by DNA sequencing.

Liquid culture senescence assay
Liquid culture senescence assays were performed essentially as previously described (van Mourik et al. 2016). Each senescence assay started with diploid strains. Freshly dissected haploid spores were allowed to form colonies on YPD agar plates after two days of growth at 30°C. Cells from these colonies were serially passaged in liquid culture medium at 24-h intervals. For each passage, the cell density of each culture was measured by optical density (calibrated by cell counting using a haemocytometer), and the cultures were diluted back into fresh medium at a cell density of 2 × 10^5 cells/ml. Cell density was plotted as a function of population doublings.

Telomere Southern blot
Telomere length analysis by Southern blotting was performed essentially as previously described (van Mourik et al. 2018). A 1.8-kb DNA fragment containing telomeric sequences generated from the SsmAI-digestion of plasmid pYT103 (Shampay et al. 1984) was loaded together with each sample. Southern blots were probed with a telomere-specific probe (5′-TGTTGGGTGTGGTGTGGGTGTGGTG-3′).

Results and discussion
Mutation of PIF1 suppresses the replicative senescence caused by the cdc13-2 mutation
To investigate how telomere addition is possible in a cdc13-2 pif1-m2 genetic background, we serially passaged cells to determine whether they would senesce. For these experiments, we used strains from our previous study (Strecker et al. 2017): cdc13Δ or cdc13Δ pif1-m2 cells, kept alive by the presence of a high-copy plasmid expressing wild-type CDC13 and the URA3 gene, transformed with an additional high-copy plasmid containing either CDC13 or cdc13-2. These cells also carried a deletion of RAD52 to prevent homologous recombination-mediated telomere maintenance (Claussin and Chang 2015). We then counterselected the first plasmid by growing cells on media containing 5-fluoroorotic acid (5-FOA), which is toxic to cells expressing URA3. 5-FOA-resistant colonies were subsequently serially passaged on agar plates (Figure 1A). Senescence was apparent for cdc13-2 PIFI cells already after the first passage, whereas CDC13 and cdc13-2 pif1-m2 strains did not show any sign of senescence even after the fourth passage. We analyzed the telomere length of these strains and found that, consistent with previous studies, pif1-m2 has increased telomere length compared with wild type (Schulz and Zakian 1994) while the telomeres are very short in the cdc13-2 mutant (Lendvay et al. 1996; Nugent et al. 1996). Interestingly, cdc13-2 pif1-m2 telomeres are approximately wild-type in length, albeit more heterogeneous, and stable throughout the course of the experiment (Figure 1B). Our findings indicate that telomerase-mediated telomere extension can occur in cdc13-2 pif1-m2 cells, allowing cells to maintain telomere length homeostasis and avoid replicative senescence. Because the strains used for this experiment have an unusual genotype (Table 1), relevant for our previous study (Strecker et al. 2017) but not for this study, we performed all subsequent experiments in a different strain background (W303), where none of the genes were overexpressed.

The yKu-TLC1 telomerase recruitment pathway is necessary to maintain telomere length in cdc13-2 pif1-m2 cells
We hypothesized that the yKu-TLC1 pathway may become essential for telomere length homeostasis in cdc13-2 pif1-m2 strains. To test this possibility, haploid meiotic progeny derived from the sporulation of CDC13/cdc13-2 PIF1/pif1-m2 YKU800/yku80-135i and CDC13/cdc13-2 PIF1/pif1-m2 TLC1/tlc1A48 heterozygous diploids were serially propagated in liquid culture for several days (Figure 2, A and B). The yku80-135i and tlc1A48 alleles disrupt the interaction between the yKu complex and TLC1 (Peterson et al. 2001; Stellwagen et al. 2003). As expected, cdc13-2 cultures grew slower as the experiment progressed and cells senesced, but growth was eventually restored upon the emergence of survivors that utilize recombination-mediated mechanisms to maintain telomeres (Lendvay et al. 1996). In contrast, the cdc13-2 pif1-m2
strains did not senesce, confirming our previous observations in a different strain background (S288C for strains used in Figure 1 as opposed to W303 for all other strains used in this study). The cdc13-2 pif1-m2 yku80-135i and cdc13-2 pif1-m2 tlc1A48 triple mutants showed a pattern of senescence and survivor formation, indicating that the yKu-TLC1 telomerase recruitment pathway is required for telomere length homeostasis in cdc13-2 pif1-m2 cells. The yku80-135i and tlc1A48 alleles caused cdc13-2 and cdc13-2 pif1-m2 strains to senesce faster, but the reason for this is currently unclear.

The abundance of TLC1 RNA is reduced to 30% and 48% in yku80-135i and tlc1A48 mutants, respectively, compared with wild-type cells (Zappulla et al. 2011). In addition, disrupting the yKu-TLC1 interaction causes mislocalization of TLC1 to the cytoplasm (Gallardo et al. 2008; Pfingsten et al. 2012). It is possible that reduced abundance and/or mislocalization of TLC1, rather than disruption of the yKu-TLC1 telomerase recruitment pathway, is responsible for the senescence of cdc13-2 pif1-m2 yku80-135i and cdc13-2 pif1-m2 tlc1A48 triple mutants. Sir4 is also required for the yKu-TLC1 recruitment pathway, but deletion of Sir4 does not affect TLC1 abundance (Hass and Zappulla 2015), and there is no evidence that sir4A affects TLC1 localization. We find that cdc13-2 pif1-m2 sir4A triple mutants also senesce (Figure 2C), although the "dip" in the senescence curve was more shallow (note the difference in scale on the y-axis). The shallow dip is consistent across multiple isolates of cdc13-2 pif1-m2 sir4A (nine isolates) as well as cdc13-2 sir4A (six isolates). The presence of the shallow dip in cdc13-2 sir4A indicates that this effect is due to sir4A, and is unrelated to the pif1-m2 suppression of cdc13-2 senescence. This effect of sir4A has also been observed with respect to the senescence of mre11A yku80A double mutants, which was attributed to increased recombination and amplification of Y′ subtelomeric elements (Liu et al. 2021). While these experiments leave open the possibility that reduced abundance and/or mislocalization of TLC1 plays a role in the senescence of cdc13-2 pif1-m2 cells with an additional yku80-135i, tlc1A48, or sir4A mutation, the simplest interpretation of our findings is that recruitment of telomerase via the yKu-TLC1 pathway is indeed required for telomere length homeostasis in cdc13-2 pif1-m2 cells.

Combining mutations that disrupt the Cdc13 EB-N–Est1 interaction (e.g., cdc13-F237A) and the yKu-TLC1 interaction leads to replicative senescence (Chen et al. 2018). We tested whether the pif1-m2 mutation could suppress this replicative senescence and found that it can: cdc13-F237A tlc1A48 strains senesce while cdc13-F237A pif1-m2 tlc1A48 strains do not (Figure 2D). Similarly, pif1-m2 can suppress replicative senescence of a cdc13-E252A, E252K mutant that disrupts both the Cdc13EB-N and Cdc13EB-C motifs (Figure 2E).

Table 1 Yeast strains used in this study

| Strain name | Genotype | Source |
|-------------|----------|--------|
| DDY3768     | MATa INC-m2 urs3-52 lys2-801 ade2-101 trp1-1-A63 his3-2 A200 leu2-3,112::natMX rad52::HIS3 VII-L-TGC4-AR COS-LS2 ura3-1::hphMX cdc13::kanMX YEp24-CDC13 pRS425-CDC13 | Strecker et al. (2017) |
| DDY3778     | MATa INC-m2 urs3-52 lys2-801 ade2-101 trp1-1-A63 his3-2 A200 leu2-3,112::natMX rad52::HIS3 VII-L-TGC4-AR COS-LS2 ura3-1::hphMX cdc13::kanMX YEp24-CDC13 pRS425-CDC13-E252K | Strecker et al. (2017) |
| DDY3783     | MATa INC-m2 urs3-52 lys2-801 ade2-101 trp1-1-A63 his3-2 A200 leu2-3,112::natMX rad52::HIS3 VII-L-TGC4-AR COS-LS2 ura3-1::hphMX cdc13::kanMX YEp24-CDC13 pRS425-CDC13 | Strecker et al. (2017) |
| DDY3793     | MATa INC-m2 urs3-52 lys2-801 ade2-101 trp1-1-A63 his3-2 A200 leu2-3,112::natMX rad52::HIS3 VII-L-TGC4-AR COS-LS2 ura3-1::hphMX cdc13::kanMX pif1-m2 YEp24-CDC13 | Strecker et al. (2017) |
| VSY20       | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::natMX/CDC13 pif1-m2/PIF1 yku80-135i::kanMX/YKU80 | This study |
| VSY7        | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::natMX/CDC13 pif1-m2/PIF1 tlc1A48::kanMX/TLC1 | This study |
| EFSY142     | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::natMX/CDC13 pif1-m2/PIF1 sir4A::hphMX/TLC1 | This study |
| EFSY73      | MATa MATa ade2-ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::kanMX/CDC13 pif1-m2/PIF1 tlc1A48::hphMX/TLC1 pRS415-cdc13-F237A | This study |
| FRY867      | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::kanMX/CDC13 pif1-m2/PIF1 tlc1A48::hphMX/TLC1 pRS415-cdc13-F237A/E252K | This study |
| CAY2        | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS est1-1::HIS3/EST1 pif1-m2/PIF1 | This study |
| MCY815      | MATa MATa ade2-ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS mec1-1::MEC1 tel1::URA3/TEL1 pif1-m2/PIF1 | This study |
| EFSY8       | MATa MATa ade2-ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::natMX/CDC13 pif1-m2/PIF1 rif1::HIS3MX/RIF1 | This study |
| EFSY9       | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::natMX/CDC13 pif1-m2/PIF1 | This study |
| EFSY31      | MATa MATa ade2-1/ADE2 can1-100/can1-100 his3-11,15/URS3-1,110 leu2-3,112/trp1-1::TRP1 ura3-1/RAD5 RADS cdc13-2::natMX/CDC13 pif1-m2/PIF1 tef1::URA3/TEL1 | This study |
In summary, these findings indicate that mutation of \textit{PIF1} allows sufficient telomerase recruitment to avoid replicative senescence caused by disruption of the Cdc13\_EBM\_C–Est1 interaction alone, or double disruption of both the Cdc13\_EBM\_N–Est1 and yKu–TLC1 interactions. However, suppression is not possible when both the Cdc13\_EBM\_N–Est1 and yKu–TLC1 interactions are abolished. Disruption of both the Cdc13\_EBM\_N–Est1 and Cdc13\_EBM\_C–Est1 interactions can be suppressed by mutation of \textit{PIF1} (Figure 2E), suggesting that the Cdc13\_EBM\_N–Est1 interaction plays a more minor role, likely in support of the Cdc13\_EBM\_C–Est1 interaction. Our findings suggest that Pif1 inhibits telomerase regardless of how telomerase is recruited: mutation of \textit{PIF1} in \textit{cdc13-2} cells allows increased telomerase recruitment via the yKu–TLC1 pathway, while mutation of \textit{PIF1} in \textit{cdc13-F237A tlc1D48} cells allows increased telomerase recruitment via the Cdc13\_EBM\_C–Est1 pathway.

\textbf{Mutation of \textit{PIF1} cannot suppress the replicative senescence of est1Δ}

The \textit{cdc13-2} mutation greatly reduces the recruitment of Est1 to telomeres (Chan \textit{et al.} 2008), and the expression of Cdc13–Est2 fusion protein allows cells to stably maintain their telomeres in the absence of Est1 (Evans and Lundblad 1999). Therefore, it was possible that \textit{pif1-m2} suppresses the replicative senescence caused by \textit{cdc13-2} by somehow bypassing the need for Est1 for telomerase-mediated telomere extension. To test this idea, we sporulated an \textit{EST1/est1D PIF1/pif1-m2} heterozygous diploid and monitored growth of the haploid meiotic progeny (Figure 2F). We find that \textit{est1D pif1-m2} double mutants senesce like \textit{est1D} single mutants, indicating that mutation of \textit{PIF1} cannot bypass the need for Est1.

\textbf{Tel1 acts through the Cdc13\_EBM\_C motif to regulate telomere length}

Because the \textit{cdc13-2} mutation normally results in a complete defect in telomerase-mediated telomere extension, it has not been possible to perform classical genetic epistasis experiments to determine which telomere length regulators act through the Cdc13\_EBM\_C–Est1 pathway. The viability and non-senescent of \textit{cdc13-2 pif1-m2} strains give us the opportunity to do so. The Rap1-interacting factors, Rif1 and Rif2, negatively regulate telomerase (Hardy \textit{et al.} 1992; Wotton and Shore 1997), while the Tel1 kinase is a positive regulator (Greenwell \textit{et al.} 1995). We measured the telomere length of haploid strains propagated for over 100 population doublings after being generated from the sporulation of heterozygous diploids (Figure 3A). We find that \textit{cdc13-2 pif1-m2} cells have short telomeres, which is in contrast to the more wild-type, but heterogeneous, length telomeres shown in Figure 1. The difference is most likely due to different genetic backgrounds (strains in Figure 1 are of the S288C background, with an additional deletion of \textit{RAD52}, while all other strains used in
this study are of the W303 background; Table 1), but not due to cdc13-2 being expressed from a high-copy plasmid in Figure 1, because overexpression of neither CDC13 nor cdc13-2 affects telomere length (Figure 3B). While deletion of RIF1 elongates cdc13-2 pif1-m2 telomeres, both cdc13-2 pif1-m2 rif2Δ and cdc13-2 pif1-m2 tel1Δ triple mutants have very similar telomere lengths compared with cdc13-2 pif1-m2, indicating that Rif2 and Tel1 function upstream and in the same pathway as the Cdc13 EBM-C motif (Figure 3). Our results are consistent with previous observations showing that tel1Δ is epistatic to rif2Δ in terms of

**Figure 2** Telomeres are maintained by the yKu-TLC1 pathway in cdc13-2 pif1-m2 cells. Senescence was monitored in liquid culture by serial passaging of haploid meiotic progeny derived from the sporulation of VSY20 (A), VSY7 (B), EFSY142 (C), EFSY73 (D), FRY867 (E), CAY2 (F), and MCY815 (G). Average cell density ±SEM of 3–9 independent isolates per genotype (except n = 2 for cdc13-F237A, E252K) is plotted.
telomere length, while the relationship between Tel1 and Rif1 is more complex and telomere-specific (Craven and Petes 1999; Sholes et al. 2021).

Tel1 often functions in concert with a related kinase, Mec1. Mutation of both MEC1 and TEL1 results in an est phenotype (Ritchie et al. 1999). Because Tel1 promotes telomerase activity through the Cdc13 EBM-C–Est1 interaction, we examined whether the same is true for Mec1. If so, the replicative senescence of mec1 tel1 double mutants, like cdc13-2, should also be suppressed by pif1-m2. We sporulated a MEC1/mec1-21 TEL1/tel1Δ PIF1/pif1-m2 diploid strain and monitored the growth of the mec1-21 tel1Δ and mec1-21 tel1Δ pif1-m2 haploid meiotic progeny. Both strains exhibited a similar rate of senescence (Figure 2G), indicating that pif1-m2 cannot suppress the est phenotype of a mec1 tel1 double mutant, and that Mec1 functions in a different pathway than Tel1 to promote telomerase activity, as previously proposed (Ritchie et al. 1999; Keener et al. 2019).

In summary, our findings provide new insight into how telomerase is recruited to telomeres in S. cerevisiae. Further work is needed to determine how the Cdc13 EBM-C motif functions, what its relationship is with the Cdc13 EBM-N motif, and the role of Tel1 in promoting telomerase recruitment.

Data availability
Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

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**Conflicts of interest**

The authors declare that there is no conflict of interest.

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