Dissecting Fission Yeast Shelterin Interactions via MICro-MS Links Disruption of Shelterin Bridge to Tumorigenesis

Graphical Abstract

Highlights
- MICro-MS can identify contact residues in protein interaction interfaces
- MICro-MS allows dissection of Tpz1-centered shelterin interfaces in fission yeast
- A POT1 variant in melanoma weakens shelterin interaction between POT1 and TPP1

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In Brief
Liu et al. develop a strategy that identifies contact sites within a complex without a 3D structure. Using this strategy, they dissect interactions among fission yeast shelterin components and find that failure to maintain an intact human shelterin bridge leads to tumorigenesis.
Dissecting Fission Yeast Shelterin Interactions via MICro-MS Links Disruption of Shelterin Bridge to Tumorigenesis

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SUMMARY

Shelterin, a six-member complex, protects telomeres from nucleolytic attack and regulates their elongation by telomerase. Here, we have developed a strategy, called MICro-MS (Mapping Interfaces via Crosslinking-Mass Spectrometry), that combines crosslinking-mass spectrometry and phylogenetic analysis to identify contact sites within the complex. This strategy allowed identification of separation-of-function mutants of fission yeast Ccq1, Poz1, and Pot1 that selectively disrupt their respective interactions with Tpz1. The various telomere dysregulation phenotypes observed in these mutants further emphasize the critical regulatory roles of Tpz1-centered shelterin interactions in telomere homeostasis. Furthermore, the conservation between fission yeast Tpz1-Pot1 and human TPP1-POT1 interactions led us to map a human melanoma-associated POT1 mutation (A532P) to the TPP1-POT1 interface. Diminished TPP1-POT1 interaction caused by hPOT1-A532P may enable unregulated telomere extension, which, in turn, helps cancer cells to achieve replicative immortality. Therefore, our study reveals a connection between shelterin connectivity and tumorigenicity.

INTRODUCTION

The six-member telomere shelterin complex is vital for eukaryotic cells. It functions to regulate telomere elongation by telomerase, as well as to protect the ends of linear chromosomes from degradation and recognition as DNA damage sites (Artandi and Cooper, 2009; Jain and Cooper, 2010; Palm and de Lange, 2008). In human cells, the shelterin complex consists of double-stranded DNA (dsDNA) binders TRF1 and TRF2, single-stranded DNA (ssDNA) binder POT1, as well as Rap1, Tin2, and TPP1 (de Lange, 2005). The shelterin connects telomeric dsDNA with ssDNA by forming a proteinaceous bridge via protein interactions within the shelterin. Specifically, telomeric dsDNA binders TRF1 and TRF2 recruit Tin2 and Rap1 to the telomere; Tin2 then recruits TPP1-POT1 complex to the telomere (Takai et al., 2011). Since POT1 directly binds to the telomeric ssDNA, where telomere elongation by telomerase happens, TRF1-initiated POT1 loading to the telomere 3’ end via shelterin interactions is believed to directly block telomerase from elongating telomeres (Loayza and De Lange, 2003). Shelterin architecture in fission yeast, Schizosaccharomyces pombe, closely resembles that of mammals (Miyoshi et al., 2008) (Figure 1A). Either its dsDNA binder Taz1 (homolog of hTRF1/2) (Cooper et al., 1997) or its ssDNA binder Pot1 (Baumann and Cech, 2001) can independently recruit other shelterin components, Rap1, Poz1 (hTIN2 homolog), and Ccq1, to telomeres (Miyoshi et al., 2008). Using S. pombe as a model system, we recently discovered that the complete linkage within the shelterin complex, rather than individual shelterin components per se, regulates the extendibility of telomeres by telomerase (Jun et al., 2013). Disruption of this linkage leads to unregulated telomere elongation. These previous studies emphasized a critical role of shelterin complex assembly in telomere length regulation. Indeed, several human POT1 variants were found to predispose to the development of familial melanoma and carriers of some of these mutations have elongated telomeres (Robles-Espinoza et al., 2014; Shi et al., 2014). These mutations may allow cancer cells to achieve replicative immortality, and thus provide the same outcome as previously identified TERT (telomerase reverse transcriptase) promoter mutations (Horn et al., 2013; Huang et al., 2015). Most of melanoma-associated POT1 mutations reside in the highly conserved oligonucleotide and oligosaccharide-binding (OB) domains of POT1, thereby disrupting POT1-telomeric ssDNA interaction (Robles-Espinoza et al., 2014; Shi et al., 2014). In addition, one mutation, Ala532Pro (A532P), was found in the C terminus of POT1, which contains the TPP1-binding domain (Shi et al., 2014). However, the mechanism by which POT1-A532P facilitates melanoma formation is still unknown. To understand the role of shelterin complex in regulating telomere states, we need to have accurate information about protein-protein interfaces between shelterin...
In recent years, crosslinking mass spectrometry (XL-MS) has become an increasingly valuable tool for studying protein-protein interactions and structural interrogation of protein complexes due to technological advancement in mass spectrometry (MS) instrumentation, new development of crosslinking reagents and bioinformatics tools to facilitate MS analysis of crosslinked peptides (Erzberger et al., 2014; Kaake et al., 2014; Kao et al., 2011, 2012; Politis et al., 2014; Walzthoeni et al., 2013; Yang et al., 2012), and an innovative algorithm developed for integrative structural biology utilizing distance restraints obtained from inter-subunit chemical crosslinking (Velázquez-Muriel et al., 2012). XL-MS studies involve protein crosslinking through a chemical linker simultaneously reacting with two amino acids that are in proximity to each other. Digestion of the crosslinked protein complex followed by peptide sequencing leads to the identification of crosslinked peptides, and consequently, proximal residue pairs. Because these crosslinked residues are constrained by the length of the linker used, the distribution of the crosslinked residue pairs helps probe protein-protein proximity. Unlike X-ray crystallography or NMR, XL-MS does not require samples with high concentration and purity. It also captures interactions from dynamic states, thus making it applicable to a broad range of protein complexes. However, XL-MS has proved challenging due to the complex fragmentation pattern of crosslinked peptides, which frequently prevents unambiguous identification of the crosslinked sequences. Recently, a new class of MS-cleavable crosslinker, disuccinimidyl sulfoxide (DSSO) was developed, which contains two symmetric MS-cleavable sites that preferentially cleave prior to the breakage of peptide backbones during collision-induced dissociation (CID) (Kao et al., 2011). In combination with multistage mass spectrometry (MS²) and tailored bioinformatics tools, DSSO-based XL-MS workflow enables easy interpretation and unambiguous identification of crosslinked peptides and has proved effective in elucidating structures of protein complexes (Kao et al., 2011, 2012).

In this study, we have developed a strategy **MICro-MS (Mapping Interfaces via Crosslinking-Mass Spectrometry)** by combining DSSO-based XL-MS workflow, phylogenetic sequence analysis, and in vitro binding assays to identify protein-interaction interfaces without the need of obtaining high-resolution 3D structure of the complex. With this strategy, we comprehensively probed protein-protein proximity in Tpz1-centered complex in fission yeast shelterin and identified separation-of-function mutants of Ccq1, Poz1, or Pot1 that selectively disrupt their respective interactions with Tpz1. The identified mutants further reveal the critical regulatory roles of Tpz1 in telomere maintenance. We also found that POT1-A532P, a melanoma-associated POT1 mutation (Shi et al., 2014), lies in the POT1-TPP1 interface and causes weakened TPP1-POT1 interaction. Just as disrupting POT1-telomeric ssDNA interaction in other POT1 mutants predisposed to melanoma, such as Y89C, Q94E, R273L, and S270N (Robles-Espinoza et al., 2014; Shi et al., 2014), defective TPP1-POT1 interaction by hPOT1-A532P mutation equivalently breaks the complete shelterin linkage between telomeric dsDNA and ssDNA. Therefore, our work links shelterin connectivity to tumorigenesis. In addition, our **MICro-MS strategy of identifying separation-of-function mutants to dissect protein complexes** for...
functional examinations will be generally applicable to other important multi-component protein complexes in the cell.

RESULTS

Identifying Contact Residues in Protein-Protein Interaction Interfaces by MICro-MS

We set out to utilize chemical crosslinking coupled with mass spectrometry (XL-MS) to identify contact residues located in the interfaces of interacting telomere shelterin components. Since mutations of the contact residues can selectively disrupt defined interactions within shelterin components, this strategy will allow us to identify separation-of-function mutants of shelterin components. Separation-of-function mutants can selectively block specific interactions without disrupting overall protein complex architecture. Thus, they should poise us to elucidate the roles of specific shelterin interactions in telomere maintenance without perturbing their roles in chromosome end protection.

The general workflow is depicted in Figure 1B, in which DSSO crosslinking of purified protein complexes was first carried out to covalently connect residues within certain three-dimensional distance in the protein pairs that are specifically reactive to the crosslinker. Although the crosslinked residues in the protein complex might not be directly involved in mediating protein-protein interactions, they should at least be close to each other surrounding the interface. Therefore, the locations of the crosslinked residues point out where the binding interface is likely to be. The optimal crosslinking conditions were used to yield sufficient amounts of crosslinked products as evaluated by SDS-PAGE. The resulting crosslinked protein complexes were digested either in-solution or in-gel with trypsin and/or chymotrypsin to obtain the maximum coverage of detectable crosslinked peptides for LC-MSⁿ analysis. The mass to charge ratios of DSSO-crosslinked peptides were first measured during MS¹ analysis, and they were then subjected to collision induced dissociation (CID) during MS². Due to the presence of the robust sulf oxide C-S cleavage sites in the linker region, MS³ analysis of a DSSO inter-linked peptide would lead to physical separation of the two crosslinked peptide constituents, thus allowing for MS³ sequencing of single peptide chains and subsequent identification using conventional database searching tools (Kao et al., 2011). Integration of the MS³ data (i.e., MS¹, MS², and MS³) results in simplified and unambiguous identification of DSSO-crosslinked peptides (Kao et al., 2011).

After identifying the crosslinked peptides and localizing lysine residues conjugated by DSSO, we mapped these residues to the phylogenetically compiled sequence alignment. We reasoned that functionally important residues (such as those for enzymatic activity, structural-fold determination, and protein complex assembly) should be evolutionarily conserved, and therefore represent leading candidates that contribute to protein-protein interactions. We introduced mutations to the conserved residues, especially the hydrophobic residues among them (as they constitute the hydrophobic core of protein-protein interactions), within approximately 8 to 15 amino acids from the crosslinked lysines, particularly in the regions where multiple lysine residues were identified as crosslinking points. In vitro GST pull-down and in vivo co-immunoprecipitation assays were subsequently performed to evaluate the degree that the introduced mutations can disrupt the targeted protein-protein interaction. Mutations of the contact residues in the protein-protein interaction interface should efficiently disrupt the protein interaction. We termed this integrated strategy to identify separation-of-function mutants disrupting specific protein interaction interfaces MICro-MS—Mapping Interfaces via Crosslinking-Mass Spectrometry.

Benchmarking MICro-MS with 3D Structure of Proteasome Subunits Rpn8-Rpn11 Complex

Next, we assessed the applicability of MICro-MS to a protein complex with determined high-resolution 3D structure. In 2012, DSSO-based XL-MS strategy was employed to unravel the structural topology of Saccharomyces cerevisiae 19S proteasome regulatory particle (Kao et al., 2012). Five inter-subunit, DSSO-mediated crosslinks were identified between Rpn8 and Rpn11 subunits, suggesting their spatial proximity as well as potential direct protein interaction interfaces between Rpn8 and Rpn11. In 2014, two groups independently solved the crystal structure of heterodimer of the MPN (Mpr1–Pad1–N-terminal) domains of Rpn8 (1–179) and Rpn11 (2–239) (Pathare et al., 2014; Worden et al., 2014). These two subunits primarily interact with each other through α helices forming two distinct interfaces. Interestingly, two pairs of DSSO-crosslinked lysines, Rpn8-K7:Rpn11-K218 and Rpn8-K28:Rpn11-K96, appear to be close to interface A and interface B, respectively, as shown in Figures 2A–2C. The rest three identified lysine-crosslinks locate in the residues beyond the MPN domains and thus are not covered in the crystal structure. From the 3D structure, we calculated distances between the crosslinked lysine pairs to be 19.4 and 18.3 Å, respectively, falling in the range of DSSO effective reaction distance (~20 Å). In Interface A (shown in Figure 2B), the hydrophobic core of the interface is formed by four-helix bundle between x1, x4 of Rpn8 and x1, x4 of Rpn11. Specifically, side chains from residues L15, L16, and L19 in x1 of Rpn8 and residues M212, L216 and L213 in x4 of Rpn11 are the essential constituents of the hydrophobic interface. Remarkably, these interface residues are adjacent (within 15 amino acids) in primary sequence to the crosslinked lysines—K7 in Rpn8 and K218 in Rpn11, respectively. Moreover, they belong to the most phylogenetically conserved residues around the crosslinking sites (Figures S1A and S1B). Interface B (shown in Figure 2C) is located between x2 of Rpn8 and x2 of Rpn11. A cluster of four methionines: M76 and M79 from Rpn8 as well as M91 and M94 from Rpn11 build the core of the interface, which is flanked by salt bridges between Rpn8-R24 and Rpn11-T98 and between Rpn8-D20 and Rpn11-R100. Identified DSSO-crosslinked lysine pair Rpn8-K28:Rpn11-K96 lies at the edge of the interface, very close to the interface residues in primary sequence. Evidently, our benchmarking results demonstrate a strong correlation between DSSO-crosslinked lysine pairs and contact residues in protein-interaction interfaces in the primary sequence. Thus, we are set out to utilize MICro-MS to identify contact residues in interaction interfaces of protein complexes with no existing high-resolution 3D structure.
Dissecting the Ccq1-Tpz1 Interacting Interface via MiCro-MS

Tpz1, the fission yeast homolog of the mammalian shelterin component TPP1 (Houghtaling et al., 2004; Liu et al., 2004; Sexton et al., 2014; Wang et al., 2007; Ye et al., 2004), is a linchpin molecule interacting with three other shelterin components—Ccq1, Poz1 via its C-terminal domain (Tpz1-CTD), and with Pot1 using its N-terminal domain (Tpz1-NTD) (Figure 1A) (Miyoshi et al., 2008). Our previous work identified Tpz1 mutants that selectively disrupt the ability of Tpz1 to interact with each of its interacting partners and revealed an interesting interplay among the positive and negative regulators of telomere length homeostasis (Jun et al., 2013). To achieve a complete dissection of the protein-protein interactions between Tpz1 and its interacting shelterin components, and therefore to define the boundary between positive and negative telomere regulators, we decided to further map the Tpz1-interacting surfaces on Ccq1, Poz1, and Pot1, respectively.

Ccq1 is more than 700 amino acids long, and it is extremely labor intensive and tedious to identify its point mutations that disrupt Ccq1-Tpz1 interaction via mutating every phylogenetically conserved residue in Ccq1. We set out to gather interface information by utilizing MiCro-MS strategy. As Ccq1 (130–439) interacts with Tpz1 (415–458), we co-expressed recombinant Ccq1 (130–439) with Tpz1 (415–458) in E. coli and purified the complex. We then subjected the complex to chemical crosslinking using an optimized concentration of DSSO—5.0 mM. As shown in Figure 3A, the crosslinked Ccq1 (130–439) and Tpz1 (415–458) complex was observed, while the free form of both Ccq1 (130–439) and Tpz1 (415–458) decreased concomitantly. The covalently crosslinked Ccq1 (130–439)/Tpz1 (415–458) shows molecular weight of ~72 kDa. This agrees with the Ccq1-Tpz1 complex forming a dimer of Ccq1-Tpz1 heterodimer, as we observed in gel filtration analysis, in which the protein complex was examined in its native form (Figure S2A). The tryptic digests of crosslinked Ccq1-Tpz1 complexes were then analyzed by LC-MSn. Figure 3B illustrates a representative MSn analysis for unambiguous identification of DSSO crosslinked peptides. As shown, MS3 analysis of a selected peptide (m/z 554.03334+) yielded two peptide fragment pairs, i.e., $\alpha_A/\beta_T$ (m/z 420.7516/678.312+ and $\alpha_T/\beta_A$ (m/z 432.202+/662.832+), which is characteristic to a hetero-inter-linked peptide ($\alpha$-$\beta$) (Kao et al., 2011). Each fragment ($\alpha_A$, $\beta_T$, $\alpha_T$, or $\beta_A$) represents one of the two crosslinked peptide constituents modified with the defined remnants of the linker after its cleavage, thus suggesting this peptide is a DSSO inter-linked peptide. The dominant fragment pair ions ($\alpha_A/\beta_T$) were subsequently subjected to MS3 analysis, in which the series of b (b$_5$−$b_4$) and y (y$_3$−y$_2$) ions detected for the $\alpha_A$ fragment unambiguously identified it as 171IQEKAIR176 of Ccq1, while the series of b (b$_2$−$b_1$) and y (y$_2$−y$_1$) ions detected for $\beta_T$ unambiguously identified the peptide as 434K_TPIP_DYDFM(Ox)K443 of Tpz1. Finally, integration of the MSn data precisely identifies the inter-link between

Identified DSSO-crosslinked lysine pair Rpn8-K28:Rpn11-K96 (linked by an orange dash line) lies at the edge of the interface, very close to the interface residues in primary sequence.

Figure 2. Benchmarking MiCro-MS Using Rpn8-Rpn11 Complex
(A) Crystal structure of Rpn8-Rpn11 heterodimer is presented with Rpn8 colored in blue and Rpn11 colored in green. The two interfaces are designated as Interface A and Interface B. The crosslinked lysines boxed in orange are connected with orange dashed lines; the distances between the lysine pairs are shown next to the lines.
(B) Close-up view showing a DSSO-crosslinked lysine pair (Rpn8-K7:Rpn11-L19) and interface A nearby in Rpn8-Rpn11 complex. The hydrophobic core of the interface is formed by four-helix bundle between K218) and interface A nearby in Rpn8-Rpn11 complex. The hydrophobic interface.
(C) Close-up view showing a DSSO-crosslinked lysine pair (Rpn8-K7:Rpn11-L19) and interface A nearby in Rpn8-Rpn11 complex. Interface B is located between K96) and interface B nearby in Rpn8-Rpn11 complex. The hydrophobic core of the interface (labeled in green), which is flanked by salt bridges between Rpn8-R24 and Rpn11-T98 and between Rpn8-D20 and Rpn11-R100.
Tpz1-K434 and Ccq1-K174, suggesting their close proximity in three-dimensional space. Based on our previous study, Tpz1-L449A is able to disrupt Ccq1-Tpz1 interaction both in vitro and in fission yeast cells. The MS² identification of Tpz1-K434 and Ccq1-K174 crosslinked by DSSO in the Tpz1-Ccq1 complex (Figure 3B) is remarkable, because Tpz1-K434 is only 15 amino acids away from the previously confirmed Ccq1-interacting site on Tpz1 (L449) (Jun et al., 2013). We therefore suspected that Ccq1 residues that mediate Ccq1-Tpz1 interaction should also be around K174 of Ccq1, the residue crosslinked to Tpz1-K434. Next, we introduced point mutations individually to several conserved residues around the K174 region of Ccq1 (Figure S2B), and then the purified recombinant Ccq1 mutants produced in E. coli were subjected to GST pull-down assays to evaluate their binding ability to GST-Tpz1-CTD. As shown in Figure 3C, among nine Ccq1 mutants, Ccq1-L151R and Ccq1-F157A/K174E completely abolished association between Ccq1 and Tpz1. In addition, Ccq1-L171R, Ccq1-K174E, Ccq1-I175R, and Ccq1-F178R all significantly diminished Ccq1-Tpz1 interaction, whereas Ccq1-V152R and Ccq1-L177R still retain wild-type binding to Tpz1. Furthermore, we also confirmed that Ccq1 mutations, which interfere with Ccq1-Tpz1 interaction in vitro, also affect full-length Ccq1-Tpz1 interaction to the similar degree in co-immunoprecipitation assays (Figure 3D); however, they still maintain their interactions with Ccq1 mutants, which interfere with Ccq1-Tpz1 interaction in vitro, also affect full-length Ccq1-Tpz1 interaction to the similar degree in co-immunoprecipitation assays (Figure 3D). After obtaining Ccq1 mutants disrupting Ccq1-Tpz1 interaction, we further asked whether these Ccq1 mutants eliminate the function of Ccq1 as a positive regulator of telomere length. As predicted, telomere length in ccq1-L151R and ccq1-F157A/K174E pombe cells, in which Ccq1-Tpz1 interaction is completely abolished, is stable but ~150 bp shorter than that in the wild-type cells, reminiscent of tpz1-L449A cells (Figures 3E and 3F) (Armstrong et al., 2014; Jun et al., 2013). S. pombe cells bearing ccq1+ mutations that significantly diminish Ccq1-Tpz1 interaction, such as ccq1-L171R, ccq1-K174E, and ccq1-F178R, also have similarly shorter telomeres as tpz1-L449A cells. However, cells bearing ccq1− mutations, ccq1-V152R and ccq1-L177R, which have little effect on
Ccq1-Tpz1 interaction, maintain wild-type telomere length. Furthermore, tpz1-L449A/ccq1-F157A/K174E double-mutant cells have an almost identical telomere phenotype as either ccq1-F157A/K174E or tpz1-L449A cells (Figure S2F), indicating the mutations, either in Tpz1 or in Ccq1, affect the same functionality in telomere maintenance pathway. Much like tpz1-L449A cells, which maintain their short telomeres via homologous recombination pathway involving rad51+ (Jun et al., 2013), ccq1-F157A/K174E cells also require rad51+ to maintain their telomeres as deletion of rad51+ in ccq1-F157A/K174E immediately abrogates the stably maintained short telomeres in ccq1-F157A/K174E cells (Figure S2G). Taken together, utilizing MICro-MS, we were able to identify Ccq1 mutants that are defective in interacting with Tpz1, and we found that these Ccq1 mutants are functionally equivalent to tpz1-L449A in telomere maintenance.

A Poz1 Mutation that Prevents Tpz1 Binding Results in Dramatic Telomere Elongation

Having successfully employed MICro-MS to dissect Ccq1-Tpz1 interaction, we next applied this approach to define the significance of the Poz1-Tpz1 interaction. Using the same procedure as described above for Ccq1-Tpz1 complex, we analyzed DSSO-crosslinked Poz1-Tpz1 complex (Figure 4A) to identify crosslinked peptides and the lysine pairs that make the linkages. Interestingly, Tpz1-K499, a residue adjacent to Tpz1-I501—a previously identified Poz1-interacting residue in Tpz1 (Jun et al., 2013), was found to crosslink to K180, K190, and K192 of Poz1 (Figures 4B, S3A, and S3B). In addition, Tpz1-K500 was also observed to crosslink to Poz1-K190 (Figure S3C). This result suggests that a region around K180 and K192 of Poz1 may contain Tpz1 contact sites (Figure S3D). We therefore introduced mutations individually to three conserved residues around this region, Poz1-W174R, R196E, and W209A, and performed GST pull-down assays to evaluate their Tpz1 binding ability. Among these three Poz1 mutants, only Poz1-R196E completely abrogated Poz1-Tpz1 interaction as shown in Figure 4C. Accordingly, we found that Poz1-R196E also caused loss of Poz1-Tpz1 interaction in the S. pombe cells as detected by co-immunoprecipitation assays (Figure 4D).

We generated a yeast strain carrying the poz1-R196E mutation to evaluate its importance for telomere length homeostasis. poz1-R196E mutant cells had dramatically elongated telomeres (Figures 4E and S3E), consistent with the phenotype previously observed for poz1-R196E mutants (Figures 4G and S3G).
observed for tpz1-I501R mutant strain (Jun et al., 2013), which also fails to form the Poz1-Tpz1 complex. Moreover, when poz1-R196E was coupled with tpz1-I501R, the double mutant also showed elongated telomere, similar to poz1-R196E single mutant with no additional telomere elongation (Figure 4F). These results indicate that Poz1-R196 is indeed functionally equivalent to Tpz1-I501 in mediating Tpz1-Poz1 interaction, a key linkage point in the negative regulation force imposed by shelterin complex upon telomerase.

Pot1 Is a Negative Regulator of Telomere Length and Protects Telomeres Redundantly with Tpz1

In S. pombe, Pot1 is the direct single-stranded telomeric DNA binder and is responsible for telomere 3’ end protection by forming a complex with Tpz1 (Miyoshi et al., 2008). Deletion of either protein can result in chromosome circularization, whereas disruption of Tpz1-Pot1 interaction by the Tpz1-I200R mutation conversely leads to telomere elongation (Jun et al., 2013). Pot1 interacts with the N-terminal domain of Tpz1 (Tpz1-NTD), which is distinct from Ccq1 and Poz1 binding regions in the C-terminal domain of Tpz1 (Tpz1-CTD). To achieve the complete dissection of the interfaces in Tpz1-mediated negative regulation of telomere elongation, we aimed to identify regions in Pot1 that mediate its interaction with Tpz1-NTD. As shown in Figure 5A, efficient crosslinking by DSSO was achieved with the purified Pot1–Tpz1-NTD complex. Further MSn analyses revealed that two lysine residues (K180 and K226) in proximity to I200 position of Tpz1 were crosslinked to Pot1 (K403, K535, and K553) (Figures 5B and S4A–S4C). To design separation-of-function mutations in Pot1 for specific disruption of the Pot1-Tpz1 interaction, several conserved residues around the crosslinked lysines were mutated (Figure S4D), and the mutants were tested for their interactions with Tpz1 both in vitro (by GST pull-down) (Figure 5C) and in vivo (by co-immunoprecipitation) (Figure 5D). We found that Pot1-I453R and Pot1-F520A completely disrupt Pot1-Tpz1 interaction, tpz1-I200R serves as the negative control. Cdc2 was shown as the loading control. Input: 1/30 of input WCE. (E) Telomere length analysis southern blots for the indicated pot1 mutant strains from successive re-streaks on agar plates. pot1-I453R and pot1-F520A strains, in which Pot1-Tpz1 interaction is disrupted, have elongated telomeres. (F) Telomere deprotection phenotype was observed in both pot1-F520A/tpz1-I200R and pot1-I453R/tpz1-I200R double mutants.
I200R cells, telomeres in both ssDNA in gel-shift assays (Figure S4G). Reminiscent of this, we found that these Pot1 mutants can bind to telomeric maximum effect on Pot1 or Ccq1 telomere association. In agreement that these mutations only disrupt Pot1-Tpz1 interaction with min-
tutive Pot1 mutant strains (Figures S4E and S4F), demonstrating
localizations of Pot1 and Ccq1 in Pot1-Tpz1 interaction-defec-
ting data. We performed ChIP analyses to evaluate telomeric
binding abilities to GST-hTPP1-NTD. Notably, hPOT1-A532P might disrupt hPOT1-hTPP1 interaction, thereby facilitating tumorigenesis by causing inappropriate telomere elongation, similar to fission yeast Pot1-I453R. To test this possibility, we in vitro translated hPOT1 and its mutants and evaluated their binding abilities to GST-hTPP1-NTD. Notably, hPOT1-A532P, as well as two other mutants in the adjacent regions, hPOT1-V529R and LS38R, has significantly weakened hPOT1-hTPP1 interaction (Figures 6A and 6B). This result emphasizes the importance of the intact “shelterin bridge” in telo-
mere length regulation and explains how mutations in a shelterin component overcome the replicative telomere barrier, and thus lead to tumorigenesis.

**DISCUSSION**

**MiCro-MS Is Effective in Identifying Functional Protein-Protein Contact Residues**

Identifying interfaces between protein components, and gener-
ating separation-of-function mutants thereafter, is a prerequisite for elucidating the functional role of each component in the large multi-protein complex, such as the shelterin complex at the end of our chromosomes. For proteins that have multiple interaction partners, such as Tpz1 in fission yeast shelterin complex, its partners, such as Tpz1 in fission yeast shelterin complex, its protein interaction interface (Figure S4D), consistent with our binding data. We performed ChIP analyses to evaluate telomeric localizations of Pot1 and Ccq1 in Pot1-Tpz1 interaction-defec-
tive Pot1 mutant strains (Figures 6E and S4F), demonstrating that these mutations only disrupt Pot1-Tpz1 interaction with min-
imum effect on Pot1 or Ccq1 telomere association. In agreement with this, we found that these Pot1 mutants can bind to telomeric ssDNA in gel-shift assays (Figure S4G). Reminiscent of tpz1-
l200R cells, telomeres in both pot1-I453R and pot1-F520A
mutant cells are elongated (Figures 5E and S4H) (Jun et al.,
2013), indicating that Pot1 is also a negative regulator of telo-
mere length. However, when we coupled pot1-I453R or pot1-
F520A with tpz1-l200R, the double mutant showed no telomere
signal, with part of the subtelomeric regions disappeared con-
comitantly (Figures 5F and S4I). These results strongly suggest
that there is an alternative pathway that Tpz1 is involved in, in
addition to the Pot1 pathway, to redundantly protect telomere
erosion.

In summary, we have successfully employed MiCro-MS to
comprehensively map Tpz1-centered protein-protein interfaces in fission yeast shelterin. As a result, we successfully identified
mutants of Ccq1, Poz1, and Pot1 that selectively disrupt their respective interactions with Tpz1, the linchpin molecule that is functionally positioned between the positive and negative regu-
lators of telomere elongation.

A Human POT1 Variant in Family Melanoma Patients Has Compromised hPOT1-hTPP1 Interaction

Replicative telomere attrition is one of the key restriction mech-
nisms that form a key barrier to infinite cell proliferation. As a result, a hallmark of cancer cells is their ability to circumvent mul-
tiple regulatory mechanisms that normally restrict cell prolifera-
tion (Hanahan and Weinberg, 2011). The integrity of telomere shelterin structure is essential for telomere length homeostasis,
which, in turn, enables the anti-proliferative barrier set by short telomeres (Gunes and Rudolph, 2013). Indeed, it has been shown that long telomeres can bypass the requirement of telo-
merase activation in the process of tumorigenesis (Taboski et al.,
2012). Recently, two studies have linked 4%-6% of familial melanoma cases to germline variants in the gene encoding hu-
man POT1, the first shelterin component found to be mutated in cancer (Robles-Espinoza et al., 2014; Shi et al., 2014). Among these hPOT1 variants, many alter key residues in the N-terminal OB domains of POT1, causing the inability of hPOT1 to bind to telomeric ssDNA and leading to longer telomeres (Robles-Espino-
zoa et al., 2014; Shi et al., 2014). However, one familial melano-

**Figure 6. A Human Family Melanoma-Associated POT1 Variant Has Compromised hPOT1-hTPP1 Interaction**

(A) In vitro GST pull-down assays testing the binding of hPOT1 mutants to GST-hTPP1-NTD (89–334). hPOT1-A532P (boxed in red) was identified in familiar melanoma patients. hPOT1-V529R and hPOT1-LS38R (boxed in red) also negatively affect the interaction between hPOT1 and GST-hTPP1-NTD. All hPOT1 (WT and mutants) are labeled with 35S and visualized by Bio-Rad Phosphorimager. GST-hTPP1-NTD and GST are visualized by Coomassie blue staining.

(B) Quantification of the binding between GST-hTPP1-NTD and hPOT1 mutants from (A). The interaction between GST-hTPP1-NTD and hPOT1-WT is set to 1.
of the protein complex. Thus, only a limited number of protein complexes are suitable for these high-resolution techniques. Here, utilizing XL-MS with a recently developed MS-cleavable linker DSSO, coupled with phylogenetic and biochemical analyses, we present an integrated strategy (Figure 1B) that successfully identified contact residues in Tpz1-centered protein-protein interfaces of fission yeast shelterin. The DSSO-based XL-MS workflow greatly facilitates the identification of crosslinked peptides, which, in turn, provides essential information about regions that are very likely to mediate protein-protein interactions. Phylogenetic analysis further narrows down the candidate residues to be mutated in the regions close to crosslinked residues, and, thereafter, GST pull-down assays identify residues directly involved in and contribute to the protein-protein interaction. This strategy can be directly applied to other protein complexes due to its simplicity, sensitivity, efficiency, accuracy, and speed. In addition, recent studies suggested the existence of evolutionarily co-varied residues as good candidates for contact residues across protein interaction interface because compensatory mutations occur in the interfaces to maintain interaction stability during evolution (Marks et al., 2011; Ovchinnikov et al., 2014; Weigt et al., 2009). Therefore, evolutionary covariation analysis can be combined with MiCro-MS to more accurately identify interface residues by prioritizing candidate residues to mutate.

For MiCro-MS, chemical crosslinking sets up the foundation for the overall outcome of this strategy. The linker length of the chemical crosslinker is crucial for linking residues pairs around the protein-interaction interface. DSSO has a linker length of 10.1 Å, resulting in potential linkages between lysines within ~20 Å. This is a distance that has been determined to be optimal for specifically crosslinking lysines across interfaces. When lysines are scarce in one of the interacting proteins or both, other crosslinking reagents targeting other amino acid residues such as cysteines or acidic residues (Lettnier et al., 2014) can be utilized similarly to determine protein interaction contacts. Interfaces that are small can be potentially probed by crosslinkers with shorter linker length such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride), which targets acidic residues (D and E), with zero linker length. Incorporating CID-inducible cleavage group into chemical crosslinkers with different linker lengths and/or reactive groups targeting various side chains apparently has urgent need.

For structurally heterogeneous samples, crosslinking mass spectrometry has the advantage of probing proximal regions of interacting proteins in different conformations. Unlike most other biophysical techniques, in which sample heterogeneity interferes with the generation of characteristic signals, for MiCro-MS, different protein interactions existing in different conformations can be identified as distinct crosslinking pairs with crosslinked peptide counts proportional to its population in the sample. Moreover, capturing stable interactions in general is much easier than identifying transient ones, but stability is not a requirement; this is another aspect of MiCro-MS that allows the stabilization of transient complexes through chemical crosslinking for subsequent analysis to identify residues close to the interfaces. Finally, methods such as yeast two-hybrid and peptide arrays are known to identify binary interactions and have been used extensively to identify domains that mediate protein interaction. They can also be utilized to identifying protein-protein interacting surfaces residues when coupled with alanine scanning of the whole or selected region of the protein pairs. In addition, once protein domains responsible for interaction are identified via yeast two hybrid, they can be subject to MiCro-MS, which provides leading candidate residues for both methods to test, significantly reducing the number of residues to mutate. It is worth mentioning that MiCro-MS allows the study of each individual interface in multi-protein complexes concurrently.

**Shelterin Connectivity, Telomere Length Regulation, and Familial Melanoma**

Our recent study of fission yeast shelterin revealed that the complete linkage within shelterin, connecting double-stranded and single-stranded telomeric DNA, governs the telomerase-nonextendable state of the telomere (Jun et al., 2013). In this study, taking advantage of XL-MS with a MS-cleavable linker strategy, we achieved complete dissection of Tpz1-centered shelterin component interactions (Tpz1-Ccq1, Tpz1-Poz1, Tpz1-Pot1) and obtained separation-of-function mutants that facilitate the investigation of their roles in telomerase regulation, telomere protection, and potentially other cellular functions such as telomere silencing (Tadeo et al., 2013) and meiosis. These mutants emphasize the importance of shelterin connectivity in negatively regulating telomerase as we concluded from Tpz1 separation-of-function mutants. This is because any mutation that disrupts the shelterin connectivity from dsDNA to ssDNA, no matter in Tpz1 or in the Tpz1 interacting partners, uniformly leads to over-elongated telomeres. They also make exploring the regulation of telomere length homeostasis by shelterin components, especially its activator Ccq1 (Webb and Zakian, 2012), possible.

In addition, our study also has clinical significance. Since fission yeast and human have homologous shelterin components and architecture, the shelterin interaction interface information obtained from fission yeast can be easily applied to the human shelterin. This information will not only help design separation-of-function mutants of human shelterin components, it will also offer mechanistic insights to a constellation of genetic diseases caused by impaired telomere maintenance due to defects in telomere shelterin. Human POT1 is the first member of shelterin components whose loss-of-function variants were found to predispose to human cancers, such as familial melanoma (Robles-Espinoza et al., 2014; Shi et al., 2014) and chronic lymphocytic (Ramsay et al., 2013; Speedy et al., 2014). Many identified POT1 mutations occur in the N-terminal OB-fold domain that directly binds to the telomeric ssDNA. All four well-studied OB-fold domain mutants of hPOT1 identified in familial melanoma patients, e.g., POT1-Y89C, POT1-Q94E, POT1-R273L, and POT1-S270N, fail to bind to telomeric ssDNA and cause cells to have elongated telomeres (Robles-Espinoza et al., 2014; Shi et al., 2014). This can be mechanistically explained by the loss of complete linkage connecting telomeric dsDNA to ssDNA via shelterin, due to the inability of the mutants to bind to ssDNA, and thereby keeping the telomere constitutively in the extendible state. With sufficiently long telomeres reserve, tumorigenesis will not be impeded by the lack of telomerase in the cell, and cancer cells thus can automatically overcome the anti-proliferative
barrier set by short telomeres (Taboski et al., 2012). Long telomeres help cells gain replicative time in order to accumulate other mutations (loss of function for tumor suppressor and gain of function for tumor activator). Exploiting homology between fission yeast and human shelterins, we find that another POT1 mutation (POT1-AS32P), found in familial melanoma patients, affects POT1-TPP1 interaction. Based on what we learned from fission yeast, disrupting any point in the proteinaceous shelterin bridge between telomeric dsDNA and ssDNA would lead to elongated telomeres. Therefore, in that sense, defect in POT1-TPP1 interaction is equivalent to that in POT1-ssDNA interaction, both acting to downregulate the negative force of telomere elongation. Recently, in addition to POT1, point mutations clustered in the POT1-interacting domain of TPP1 were found in melanoma patients (Aoude et al., 2015), presumably disrupting POT1-TPP1 interaction just as POT1-AS32P does. Interestingly, gain-of-function mutations in the hTERT promoter, which increase gene expression of hTERT, were recently found to be driver alterations in melanoma and other cancers (Horn et al., 2013; Huang et al., 2013). Upregulation of positive regulators of telomere elongation (such as hTERT) is equivalent to downregulation of the negative regulators (such as the shelterin bridge) (as shown in Figure 7) in leading to the deregulated telomere over-elongation, both helping the cancer cells to achieve replicative immortality, a key barrier to tumorigenesis.

**Figure 7. Model of Telomere Length Dysregulation-Related Tumorigenesis**

Upregulation of positive regulators of telomere elongation (such as hTERT) is equivalent to downregulation of the negative regulators (such as the shelterin bridge) in leading to the deregulated telomere over-elongation, both helping the cancer cells to achieve replicative immortality, a key barrier to tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Gene Tagging, and Mutagenesis**

Fission yeast strains used in this study are listed in Table S1. Single mutant strains were constructed by one-step gene replacement of the entire open reading frame (ORF) with the selectable marker. Double- and triple-mutant strains were produced by mating, sporulation, dissection, and selection followed by PCR verification of genotypes. Genes were fused to specific epitope tags at the C terminus by homologous recombination; the pFA6A plasmid modules were used as templates for PCR (Bahler et al., 1998; Sato et al., 2005). Point mutations were made by site-directed mutagenesis PCR using the high-fidelity polymerase Pfu (Agilent). All mutations were confirmed by DNA sequencing (Eton).

**Protein Expression and Purification**

Plasmids for recombinant protein expression in E. coli were transformed into Rosetta-BL21 (DE3) cells. Protein expression was induced with 0.4 mM isopropyl-D-1-thiogalactopyranoside (IPTG) for 4 hr at 30°C and 0.2 mM IPTG overnight at 16°C. Cells were disrupted by sonication in lysis buffer (25 mM Tris-HCl [pH 8.0], 350 mM NaCl, 15 mM imidazole, 5 mM 1-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine). The supernatant was incubated with Ni-NTA (QIAGEN) resin for 1 hr. After washing, the bound protein was eluted from the beads with elution buffer containing 300 mM imidazole. Some proteins were further purified with additional ion-exchange and/or gel filtration steps.

**GST Pull-Down Assay**

15 μg GST fusion protein in 30 μl GST pull-down buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 10 mM i-α-ME, 0.1% Tween 20) was incubated with 20 μl glutathione Sepharose resin (QIAGEN) for 1 hr. Then the target protein (20 μg in 120 μl GST pull-down buffer) was added to the resin. After 1 hr incubation with rotation, the resin was washed three times, and the protein was eluted with 15 μl 2 × SDS loading buffer at 95°C for 5 min. The eluted proteins were resolved by 10% SDS-PAGE and visualized by Coomassie blue staining.

**Crosslinking Mass Spectrometry Analysis**

45 μl purified protein complex (4.5 mg/ml for Tpz1-Ccq1; 4 mg/ml for Tpz1-Poz1; 1.25 mg/ml for Tpz1-Pot1) was mixed with 5 μl DSSO (dissolved in DMSO) to the final concentration as indicated in results. Crosslinking was performed for 30 min and quenched with 2 μl 1 M Tris-HCl (pH 8.0) for 15 min. The cysteine residues were reduced with 4 mM TCEP and alkylated with 20 mM iodoacetamide in dark, followed by terminating alklylation reaction with 20 mM cysteine for 30 min. The crosslinked proteins were digested overnight at 37°C with trypsin (2%, w/w) and chymotrypsin (5%, w/w), separately. Crosslinked peptides were analyzed by LC-MS2 utilizing an LTQ-Orbitrap XL MS (Thermo Fisher Scientific) coupled online with an Easy-nLC 1000 (Thermo Fisher Scientific) as described (Kao et al., 2011). Each MS2 experiment consists of one MS scan in FT mode (350–1,400 m/z, resolution of 60,000 at m/z 400) followed by two data-dependent MS2 scans in FT mode (resolution of 7,500) with normalized collision energy at 20% on the top two MS peaks with charges 3+ or up, and the three MS2 scans in the LTQ with normalized collision energy at 35% on the top three peaks from each MS2.

**Identification of DSSO Crosslinked Peptides by LC-MS**

MS3 data were subjected to a developmental version of Protein Prospector (v.5.10.10) for database searching, using Batch-Tag with mass tolerances for parent ions and fragment ions set as ±0.2 ppm and 0.6 Da, respectively. Trypsin was set as the enzyme with five maximum missed cleavages allowed. A maximum of five variable modifications were also allowed, including protein N-terminal acetylation, methionine oxidation, N-terminal conversion of glutamine to pyroglutamic acid, asparagine deamidation, and cysteine carbamidomethylation. In addition, three defined modifications on uncleaved lysines and free protein N termini were also selected: alkene (A: C3H2O, +54 Da); sulfenic acid (S: C7H14O2S, +104 Da), and unsaturated thiol (T: C2H3OS, +86 Da) modifications, due to remnant moieties of DSSO. Initial acceptance criteria for peptide identification required a reported expectation value ≤0.1.

Integration of MS2 data was carried out using the in-house program LinkHunter, a revised version of the previously written LinkFinder program, to validate and summarize crosslinked peptides as previously described (Kao et al., 2011, 2012).

**Co-Immunoprecipitation**

The indicated strains were grown in YEAD and harvested at OD600 = 0.6–0.8. Then the cell pellets were cryogenically disrupted with FastPrep MP with two pulses (60 s) of bead-beating in ice-cold lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 15 mM EGTA, 15 mM MgCl2, 0.1% NP40, 0.5 mM Na2VO4, 1 mM NaF, 2 mM PMSF, 2 mM benzamidine). Complete proteinase inhibitor
[Roche]). The crude extracts were clarified by centrifuge for 10 min and adjusted to 15 mg/ml. Anti-Flag M2 affinity gel (Sigma) or anti-Myc resin (9E10, Santa Cruz Biotechnology) was equilibrated with same lysis buffer, and immunoprecipitation was performed for 3 hr at 4°C. Then, the proteins were eluted with 30 μl 0.1 M glycine (pH 2.0) at room temperature for 10 min with gentle shaking and neutralized by Tris-HCl (pH 8.0) immediately after elution. Eluted proteins were resolved by 8% SDS-PAGE and then subjected to western blotting. Western blot analysis was performed using monoclonal anti-Flag (M2-F1804, from Sigma), monoclonal anti-Myc (9E10, from Covance), monoclonal anti-HA (F-7, from Santa Cruz), anti-Ccq1 rabbit serum, and anti-FlagM2 (y100.4, from Abcam). 20 μg whole-cell extract was used for input control.

Telomere Length Analysis

S. pombe cells grown in 5 ml YEAYU overnight were harvested for genomic DNA extraction. EcoRI-digested genomic DNA was separated on 1% agarose gel and then transferred to N+ membrane (GE Healthcare) via capillary blot. The extraction. EcoRI-digested genomic DNA was separated on 1% agarose gel and then transferred to N+ membrane (GE Healthcare) via capillary blot. The telomeric probe was prepared as previously described (Jun et al., 2013). The template of pol1-1 was amplified with 5' primer (GGTGCAGAAACAGGTCGCAAG) and 3’ primer (CTTACACTGAAAGGATGCGG), and the pol1-1 probe was generated by High Prime (Roche). Southern blots were imaged using a Bio-Rad phosphorimager.

In Vitro Translation Coupled with GST Pull-Down Assay

hPoT1 in a pET28 vector was mutared by site-directed mutagenesis PCR. WT and all mutants were in vitro expressed using TnT coupled reticulocyte lysate kit (Promega) following the manual. Briefly, a 25-μl reaction containing 20 μl reticulocyte lysate, 0.5 μg plasmid, 1 μl 1Mm methionine, and 1 μl [35S]methionine was incubated at 30°C for 90 min. 22 μl of each in vitro translation reaction was directly applied to GST pull-down assay as previously described, but with 7 μg of GST fusion protein. The pull-downs were resolved by 8% SDS-PAGE and imaged with a Bio-Rad phosphorimager.

Electrophoretic Mobility Shift Assay

20 nM 5’ end labeled ssDNA (GTTAGCAGTTACGGTTACG) was mixed with Pol1 proteins of specified concentrations in 20 μl reaction buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM DTT, 2 mM MgCl2, 10% glycerol). The mixtures were then incubated at 4°C for 30 min. The protein-ssDNA complex were resolved by 7% non-denaturing polyacrylamide gel and imaged with a Bio-Rad phosphorimager.

Chromatin Immunoprecipitation

Fresh S. pombe cells in liquid culture were fixed with an 11% formaldehyde solution (11% formaldehyde, 100 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA, 50 mM Tris-HCl [pH 8.0]) for 20 min, followed by termination with 125 mM glycine for 5 min. Cell pellets were disrupted in 400 μl of lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, complete protease inhibitor [Roche], 1 mM PMSF, 1 mM benzamidine, 1 mM Na2VO4, 1 mM NaF) with FastPrep MP. After three pulses (1 min) of beads-beating, at least 90% cells were broken. Cell extracts were sonicated three times for 30 s in 18 cycles using a Bio-ruptor. Clarified cell extracts were incubated with anti-Myc resin (9E10, Santa Cruz) or anti-Ccq1 rabbit serum followed by protein G-agarose (Roche) for 3 hr at 4°C. Then, the beads were washed twice with lysis buffer, lysis buffer with 500 mM NaCl, wash buffer, and 1 × TE buffer sequentially. Each sample was added with 100 μl of 10% Chelex100 resin and boiled for 15 min, followed by 20 μg proteinase K treatment for 30 min at 55°C. The recovered DNA were denatured with 0.4 M NaOH and transferred to a Hybond-XL membrane by using a slot module. The blots were hybridized with telomeric probe; the same blot was then re-probed with RNA probe after stripping.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.043.

AUTHOR CONTRIBUTIONS

J.L. performed biochemical and molecular genetics experiments. C.Y. performed LC-MS analyses with supervision from L.H. X.H. performed ChiP experiments. J.-K.K. prepared some recombinant proteins, participated in benchmarking of MiCro-MS, and made protein structure figures. J.C.B. participated in the initial stage of the project and made some Ccq1 mutant constructs/strains. H.-I.J. aided in telomere length analysis. S.D.R. provided chemical reagent DSSO. F.Q. conceived the project. F.Q., L.H., J.L., and C.Y. designed the experimental plans. F.Q. and J.L. wrote the manuscript with L.H. and C.Y. writing the mass spectrometry part.

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Dissecting Fission Yeast Shelterin Interactions via MICro-MS Links Disruption of Shelterin Bridge to Tumorigenesis

Jinqiang Liu, Clinton Yu, Xichan Hu, Jin-Kwang Kim, Jan C. Bierma, Hyun-Ik Jun, Scott D. Rychnovsky, Lan Huang, and Feng Qiao
Figure S3

**A**

MS+ analysis identifies a linkage between residue K180 of Poz1 and K499 of Tpz1.

**B**

MS+ analysis identifies a linkage between residue K190 of Poz1 and K499 of Tpz1.

**C**

MS+ analysis identifies a linkage between residue K190 of Poz1 and K500 of Tpz1.

**D**

**E**
Figure S4

A

MS² analysis identifies a linkage between residue K403 of Pot1 and K226 of Tpz1.

B

C

D

Pot1

Pot1-TID

S. pombe 373 374 375
S. japonicus 432 433 434
S. octosporus 524 525 526
S. cryophilus 453 454 455
O. nova 441 442 443

Tpz1

I200R I453R

TEBBe (Tpz1)

F520A

TEBPα (Pot1)

TEBPα (Pot1)

ssDNA
Figure S4

**E**

Pot1-ChIP

|          | WCE | ChIP |
|----------|-----|------|
| no-tag   |     |      |
| pot1-wt  |     |      |
| pot1-H53R|     |      |
| pot1-W519R|   |      |
| pot1-F520A|   |      |

Telomere probe

rDNA probe

**F**

Ccq1-ChIP

|          | WCE | ChIP |
|----------|-----|------|
| ccq1/+/pot1-wt | |      |
| ccq1-wt/pot1-wt | |      |
| ccq1-wt/pot1-I483R| |      |
| ccq1-wt/pot1-W519R| |      |
| ccq1-wt/pot1-F520A| |      |

Telomere probe

rDNA probe

**G**

| Conc. (mM) | Free ssDNA | Pot1-ssDNA Complex |
|------------|------------|--------------------|
| 0          |            |                    |
| 0.05       |            |                    |
| 0.1         |            |                    |
| 0.2         |            |                    |
| 0.5         |            |                    |
| 1.0         |            |                    |
| 5.0         |            |                    |
| 10.0        |            |                    |

**H**

|          | WT | pot1-FL200R | ptr1-200R |
|----------|----|-------------|------------|

WT

pot1-FL200R

ptr1-200R

**I**

Amplification

1kb 10kb 20kb Continuers

|        | I | II | III | IV |
|--------|---|----|-----|----|
| WT     |   |    |     |    |
| pot1-FL200R | | | | |
| ptr1-200R   | | | | |

Supplementary Figure Legends

Figure S1. (Related to Figure 2)
Sequence alignments of Rpn8 (A) and Rpn11 (B) from fungi to human. Identical residues are highlighted in black, and chemically similar residues are highlighted in gray. The crosslinked lysine residues are highlighted in blue and green for Rpn8 and Rpn11, respectively. Crosslinked peptides are shown on the top with crosslinked lysines indicated. The residues colored in blue or green with residue numbers above are the contact residues observed in the interface from the crystal structure from Rpn8 and Rpn11, respectively. In sequence alignment of Rpn11 (B), Zn$^{2+}$-coordinating residues are colored in blue and residues involved in catalysis are in red.

Figure S2. (Related to Figure 3)
(A) Gel filtration chromatography showing Tpz1-CTD and Ccq1-NTD complex forms a dimer of the Tpz1-CTD/Ccq1-NTD heterodimer.
(B) Sequence alignment of Ccq1 from four different fission yeast species. The crosslinked lysine is highlighted in dark blue with corresponding Tpz1 fragment on the top. The targeted residues for mutation are colored in dark blue and were mutated to the amino acids indicated above them.
(C) Co-immunoprecipitation (co-IP) assays evaluating the binding between Clr3 and Ccq1 mutants. The mutants colored in dark blue are Tpz1-binding deficient. Cdc2 was shown as the loading control. Input: 1/30 of input WCE (whole cell extract).
(D) Localization of Ccq1 mutants to telomere is monitored by ChIP assay. Slot-blot was used to visualize telomeric association of Ccq1 in each genetic background. Telomeric enrichment of Ccq1 was expressed as immunoprecipitate (IP)/whole-cell extract (WCE) from the telomere DNA probe hybridization. The same membrane was stripped and then hybridized with the rDNA probe. Error bars in the quantitation of the slot blot analysis represent standard deviations of two individual repeats.
(E) ccq1-wt-I3myc has wild-type telomere length. Therefore, C-terminal tagging of ccq1$^+$ does not interfere with telomere maintenance.
(F) ccq1-F157A/K174E tpz1-L449A double-mutant cells have identical telomere phenotype as either ccq1-F157A/K174E or tpz1-L449A cells.
(G) Telomere maintenance in ccq1-F157A/K174E cells is not telomerase dependent, but HR dependent.
Figure S3. (Related to Figure 4)

(A) MS\(^n\) analysis of a DSSO crosslinked peptide representing an interaction between Poz1 and Tpz1.
(a) MS\(^2\) spectrum of a quadruply-charged crosslinked peptide α-β (m/z 739.5940\(^4+\)) in which two characteristic peptide fragment pairs were detected: α_1/β_0 (m/z 662.30\(^5+\)/758.02\(^3+\)) and α_0/β_1 (m/z 630.33\(^4+\)/769.01\(^3+\)) with defined modifications. Respective MS3 spectra of (b) α_1 (m/z 662.30\(^5+\)) and (c) β_0 (m/z 758.02\(^3+\)) fragment ions unambiguously identified their sequences as DWK_TK of Tpz1 and FLQDWGSHNEKAEMEALQR of Poz1. This identifies a linkage between residue K180 of Poz1 and K499 of Tpz1.

(B) MS\(^n\) analysis of a DSSO crosslinked peptide representing an interaction between Poz1 and Tpz1.
(a) MS\(^2\) analysis of a triply-charged parent ion α-β (m/z 449.2354\(^3+\)) in which two characteristic peptide fragment pairs were detected: α_1/β_0 (m/z 333.70\(^2+\)/662.30\(^1+\)) and α_0/β_1 (m/z 349.69\(^2+\)/630.33\(^1+\)), unique to DSSO inter-linked peptides; MS\(^3\) spectra of (b) α_1 (m/z 333.70\(^2+\)) and (c) β_0 (m/z 662.30\(^1+\)) fragment ions detected in (a), which unambiguously identify their sequences as LSK_AHK of Poz1 and DWK_TK of Tpz1, respectively. This identifies a linkage between residue K190 of Poz1 and K499 of Tpz1. Note: K_A: alkene modified lysine; K_T: unsaturated thiol modified lysine.

(C) MS\(^n\) analysis of a DSSO crosslinked peptide representing an interaction between Poz1 and Tpz1.
(a) MS\(^2\) spectrum of a triply-charged parent ion α-β (m/z 530.9461\(^3+\)), in which a characteristic peptide fragment pair was detected: α_1/β_0 (m/z 438.23\(^2+\)/698.37\(^1+\)), unique to DSSO inter-linked peptides; MS\(^3\) spectra of (b) α_1 (m/z 438.23\(^2+\)) and (c) β_0 (m/z 698.37\(^1+\)) fragment ions detected in (a), which unambiguously identified their sequences as K_AIEEFR of Tpz1 and LSK_THK of Poz1, respectively, indicating a linkage between residue K190 of Poz1 and K500 of Tpz1. Note: K_A: alkene modified lysine; K_T: unsaturated thiol modified lysine.

(D) Sequence alignment of Poz1 from four different fission yeast species. The crosslinked lysines are highlighted in dark blue with corresponding Tpz1 peptide fragments on the top. The targeted residues for mutation are colored in green and were mutated to the amino acids indicated above them.

(E) poz1\(^+\)-I3myc has wild-type telomere length. Therefore, C-terminal tagging of poz1\(^+\) does not interfere with telomere maintenance.

Figure S4. (Related to Figure 5)

(A) MS\(^n\) analysis of a DSSO crosslinked representing an interaction between Pot1 and Tpz1. (a) MS\(^2\) spectrum of a triply-charged parent ion α-β (m/z 818.7060\(^3+\)), in which a characteristic peptide fragment pair was detected: α_1/β_0 (m/z 520.26\(^2+\)/1108.65\(^1+\)), unique to DSSO inter-linked peptides; MS\(^3\) spectra of (b) α_1 (m/z 520.26\(^2+\)) and (c) β_0 (m/z 1108.65\(^1+\)) fragment ions detected in (a), which
unambiguously identified their sequences as KₐMAQGLHNS of Tpz1 and LTTISTILHAPLQNLLKPR of Pot1, respectively, indicating a linkage between residue K403 of Pot1 and K226 of Tpz1. Note: Kₐ: alkene modified lysine; Kₜ: unsaturated thiol modified lysine.

(B) MSⁿ analysis of a DSSO crosslinked peptide representing an interaction between Pot1 and Tpz1. (a) MS² spectrum of a quadruply-charged crosslinked parent ion α-β (m/z 869.196⁴⁺), in which two characteristic peptide fragment pairs were detected: αₐ/βₜ (m/z 520.26²⁺/1209.62²⁺) and αₚ/βₜ (m/z 536.24²⁺/1193.64²⁺), unique to DSSO interlinked peptides. MS³ spectra of (b) αₐ (m/z 520.26²⁺) and (c) βₜ (m/z 1209.62²⁺) fragment ions unambiguously identified their sequences as KₐMAQGLHNS of Tpz1 and EYIPVIGNTKTDHQSLTFLQK of Pot1, respectively, indicating a linkage between residues K535 of Pot1 and K226 of Tpz1.

(C) MSⁿ analysis of a DSSO crosslinked peptide representing an interaction between Pot1 and Tpz1. (a) MS² spectrum of the triply-charged parent ion α-β (m/z 621.9794³⁺) in which three characteristic peptide fragments were detected: αₐ/βₜ (m/z 520.26²⁺/807.41¹⁺) and αₚ (m/z 536.24²⁺), unique to DSSO interlinked peptides; MS³ spectra of (b) αₚ (m/z 520.26²⁺) and (c) βₜ (m/z 807.41¹⁺) fragment ions detected in (a), which unambiguously identified their sequences as KₐMAQGLHNS of Tpz1 and GFGTKIV of Pot1, respectively. This demonstrates a linkage between residue K553 of Pot1 and K226 of Tpz1. Note: Kₐ: alkene modified lysine; Kₜ: unsaturated thiol modified lysine.

(D) Left: Sequence alignment of Pot1 from four different fission yeast species. The crosslinked lysines are highlighted in light brown with the corresponding Tpz1 fragment (colored in red) shown on the top. The crosslinked lysines all fall in the Tpz1-interacting region, which is indicated by two arrows colored in light brown. Middle: An enlarged view of the Tpz1-interacting region of Pot1. The selected residues of Pot1 for mutation are colored in light brown and mutated to the amino acids indicated above them. Right: Structural representation of Oxytricha nova TEBPa/TEBPβ complex, an ortholog of Tpz1/Pot1 complex. The loop region of TEBPβ that mediates protein-protein interaction between TEBP-α and -β is colored green and the location of a previously identified Tpz1 mutant I200R that disrupts Tpz1-Pot1 interaction is indicated. The locations two Pot1 mutants (I453R and F520A) identified in this study, based on sequence alignment, are also indicated by arrows. The structural elements in which I453R and F520A reside in are color yellow and dark red, respectively.

(E) Telomere localization of Pot1 mutants is monitored by ChIP assay. Slot-blot was used to quantitate telomeric association of Pot1 in each genetic background. Error bars in the quantitation of the slot-blot represent standard deviations of two individual repeats.

(F) Telomere localization of Ccq1 to telomere is monitored by ChIP assay in Pot1 mutant strains. Slot-blot was used to visualize telomere association of Ccq1 in each Pot1 mutant background. Error bars in
the quantitation of the slot-blot analysis represent standard deviations of two individual repeats.

(G) Gel-shift experiments evaluating the binding ability of Pot1 mutants to a $^{32}$P-labeled telomeric ssDNA. The position of unbound telomeric ssDNA in the gel is indicated by an arrow. The Tpz1-binding defective mutants are colored in light brown. Pot1ΔNTD is a Pot1 truncation variant without N-terminal OB1-OB2 domains, serving as a negative control.

(H) pot1-wt-stop-13myc has wild-type telomere length. Therefore, selection cassette insertion does not affect telomere maintenance.

(I) Upper: Schematic diagram of chromosome showing sub-telomeric regions. Greek numbers indicate the locations where the PCR primers are designed. Lower: pot1-F520A/tpz1-I200R and pot1-I453R/tpz1-I200R mutants lost sub-telomeric I and II regions. The PCR products (from I to IV) are amplified from corresponded regions on chromosomes (upper diagram). For pombe cells carrying circular chromosomes due to telomere deprotection, both sub-telomeric regions I and II are eroded from chromosome ends.

**Figure S5. (Related to Figure 6)**

Sequence alignment of Pot1 from fission yeast to different vertebrates. The S. pombe Pot1 and human POT1 are highlighted in grey. Tpz1-interacting region is indicated by two arrows. In the sequence alignment, the Tpz1-binding defective mutant of S. pombe Pot1-I453R (colored in light brown) resides in the corresponding region of human POT1-A532P identified in familial melanoma patients (highlighted in red box).