Structural and functional analysis of the human POT1-TPP1 telomeric complex

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POT1 and TPP1 are part of the shelterin complex and are essential for telomere length regulation and maintenance. Naturally occurring mutations of the telomeric POT1–TPP1 complex are implicated in familial glioma, melanoma and chronic lymphocytic leukaemia. Here we report the atomic structure of the interacting portion of the human telomeric POT1–TPP1 complex and suggest how several of these mutations contribute to malignant cancer. The POT1 C-terminus (POT1C) forms a bilobal structure consisting of an O8-fold and a holiday junction resolvase domain. TPP1 consists of several loops and helices involved in extensive interactions with POT1C. Biochemical data shows that several of the cancer-associated mutations, partially disrupt the POT1-TPP1 complex, which affects its ability to bind telomeric DNA efficiently. A defective POT1–TPP1 complex leads to longer and fragile telomeres, which in turn promotes genomic instability and cancer.

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Shelterin is a hexameric nucleoprotein complex responsible for maintaining the integrity of the ends of our chromosomes, known as telomeres. Human shelterin consists of TRF1, TRF2, TIN2, RAP1, POT1 and TPP1 (refs 1–3), binds double and single-stranded telomeric DNA and is involved in a wide range of functions4–6. It suppresses DNA damage response by capping and protecting the ends of chromosomes from being recognized as DNA double-strand breaks6,7. It prevents exonuclease degradation by sequestering the telomeric overhang2. It also controls telomere length by regulating access of telomerase to the telomeric overhang2.

POT1 has a wide range of functions at telomeres all of which are geared toward maintaining the integrity of the telomeric overhang. POT1 binds single-stranded, telomeric DNA with high affinity and specificity4,10,11. POT1-DNA binding sequesters the telomeric overhang, thus assisting in telomere capping, downregulation of telomere elongation and ATR dependent DNA damage response4–15. POT1 telomeric DNA binding is mediated by the two N-terminal OB-folds of the protein, while the C-terminal portion of the protein binds TPP1 (refs 10, 16).

During the S-phase of the cell cycle, the human POT1–TPP1 complex recruits telomerase to telomeres9,16,19 via direct contacts of telomerase with the TEL patch located at the N-terminal OB-fold of TPP1 (ref. 20). Binding of POT1 to the telomeric overhang regulates G-quadruplexes and allows for telomerase loading to telomeres for telomere elongation21.

It was recently discovered that POT1 is frequently mutated in chronic lymphocytic leukaemia, familial melanoma and gliom22–25. There are currently 137 naturally occurring mutations of POT1 reported to be associated with human disease (cBioPortal for Cancer Genomics). Many of these mutations localize at the N-terminal OB-folds and disrupt POT1-DNA binding, while others are located at the C-terminus of the protein and were TPP1 binding22–25. POT1 N-terminal mutations primarily disrupt DNA-binding and are associated with chromosomal abnormalities such as irregular telomere length, fragile telomeres and chromosome end-to-end fusions, phenotypes usually associated with telomere uncapping22,24,26,27. However, the precise role of human POT1 C-terminal (POT1C) mutations in human disease is currently unclear.

Here, we investigate the mechanism of POT1–TPP1 assembly and how naturally occurring POT1 mutations contribute to cancer. Our data shows that POT1C consists of two domains, an OB-fold and a holiday junction resolvase (HJR) domain both of which make extensive interactions with TPP1 forming a tight heterodimer. Inspection of the structure reveals that several of these mutations either perturb the POT1C fold and/or disrupt POT1–TPP1 binding. Altering the natural state of the POT1–TPP1 complex affects the integrity of the telomeric overhang, leading to chromosomal abnormalities associated with a dysfunctional telomere capping complex leading to genomics instability and cancer.

**Results**

**Structure of the human POT1–TPP1 complex.** We generated the interacting domains of human POT1 and TPP1 by limited proteolysis and mass spec analysis of the full length POT1–TPP1 complex (Fig. 1a,b). POT1C consists of residues 330–634 and TPP1 255–337 (TPP1(PBD)) (Fig. 1a,b). We solved the structure by the single wavelength anomalous dispersion (SAD) method and a Hg derivative (Table 1). The map showed clear electron density for POT1C residues 332–633 and TPP1(PBD) residues 266–326 (Fig. 1c). POT1C consists of a classic OB-fold and a holiday junction resolvase domain (HJR) (Fig. 1d). The POT1C(OB) is a canonical OB-fold and is structurally most similar to the *Oxytricha nova* Telomere End-Binding Protein (TEBP2, PDB ID: 1OTC–RMSD = 2.2 Å—Fig. 1e). It is worth noting that an overlay of the TEBP alpha and beta dimer (PDB ID: 1OTC, ref. 28) with that of the POT1–TPP1 structure shows no similarities in the organization of the two heterodimers. The organization of the six beta strands of the OB-fold forms a deep and well defined indentation on the surface of the protein, which comprises the canonical binding pocket of an OB-fold (Fig. 1d).

Interestingly, the HJR is an insertion of the OB-fold and comprises residues P392-L538. The HJR is structurally similar to the *Archaeoglobus fulgidus* resolvase domain (AeHJR, PDBID: 2W1W) with an RMSD 2.7 Å (Fig. 1f). HJR consists of seven antiparallel beta-strands surrounded by five alpha helices (α3, 4, 5, 6, 7) (Fig. 1d,f). Structural comparison of POT1C (HJR) with the AeHJR domain highlighted two distinct differences between these two HJR domains. One difference lies with the organization of the helices present in the HJR domains with four out of the five helices not overlapping (Fig. 1f). Another striking difference between the two HJR domains lies with the DNA binding pocket of the HJR. In AeHJR double stranded DNA binding is mediated primarily by two short antiparallel β-strands located on the surface of the protein and away from the five β-strands that form the core of the domain. In HJR, these two strands have shifted by a 50° rotation and 18 Å translation and are an integral component of the beta-sheet generated by all seven β-strands of this domain (Fig. 1f). The HJR putative DNA binding pocket is occupied by helix α3, the N-terminal helix of HJR. Extensive interactions between the two POT1C domains generates a long, almost cylindrical structure providing an extensive surface area for TPP1 binding. Further stabilization of the bilobal POT1C structure is mediated by a Zn2+ ion coordinated, tetra-cysteine cluster (C382, C385, C503, C506) locate at the interface of the two domains (Fig. 1d).

TPP1(PBD) is an extended coil with four alpha helices distributed throughout the protein. The TPP1 polypeptide spans the entire length of POT1C and makes extensive contacts with both of its domains (Fig. 1d). Interestingly, TPP1(PBD) is organized in the opposite orientation (N—→—C-terminal) to that of POT1C so that the N-terminal portion of TPP1(PBD) (z1) interacts with the HJR while the C-terminal one interacts with the OB-fold. In particular, z1 of TPP1(PBD) makes extensive interactions with the surface of the β-sheet generated by the β-strands of the HJR. It also interacts with helix α5 of POT1C, which is located on the same face of the β-sheet of HJR and in parallel orientation with the β-strands forming the β-sheet (Fig. 1d). The TPP1(PBD) helix z1 is leucine/valine rich and the majority of contacts with the POT1C, HJR are hydrophobic in nature (Fig. 2a). TPP1, Leu271 is buried in the large hydrophobic pocket formed by L445, F438 and F470 of POT1C. The side chain of TPP1, L274 stacks against the side chain of POT1C, F470. TPP1, V272 and A275 are coordinated by the side chains of POT1C, F438 and W424. W424 of POT1C also makes a productive hydrogen bond with E278. L279 is buried in a well-defined hydrophobic pocket formed on the surface of POT1C by residues W424, V436, L466, V434 and the aliphatic portion of the side chain of E462. L281 is also buried within this pocket of POT1 but the contacts are limited to the side chains of W424 and T426.

Contacts between the loop that connects the TPP1(PBD) helices z1 and z2 and POT1C are limited to residues C285 and P288. Both of these residues make minor hydrophobic interactions with the OB-fold of POT1C. The loop is also held in place by a 2.7 Å hydrogen bond between the backbone of this loop and E394 of POT1C.
The TPPI(PBD) helix α2 localizes at the interface of the POT1C, OB-fold and HJR domains, where it is involved in extensive and specific interactions with the protein (Fig. 2b). Of note are the contacts generated by the highly conserved W293 and R297 of TPPI. The large W293 side chain is buried in a deep hydrophobic pocket formed by the POT1 residues F542, V583, V573, L574, F542, V378 and the aliphatic portion of Q580, which comprise part of helices α2, α7 and α8 of the OB-fold. The side chain of POT1 C580 also hydrogen bonds the TPPI α2 backbone and the side chain of H292. R257 of TPPI also localizes at the interface of the OB and HJR of POT1C but unlike W293, its side chain is involved in a network of interactions with POT1C via solvent molecules (Fig. 2d). The only direct contact between R257 and POT1C involves a hydrogen bond with the backbone of V391.

Additional contacts between TPPI and the POT1C OB-fold are extensive and involve helices α3 and α4 and the coils that connect them (Fig. 2c). Both of these helices are docked into the canonical, binding pocket of the OB-fold. More specifically, the TPPI Y306 side chain stacks against P371 of POT1C. The branched side chains of TPPI, V308 and L313, are buried into a deep hydrophobic patch located at the base of the OB-fold’s binding pocket. TPPI, I315 of the linker that connects α3 to α4 coordinates POT1 Y610, F625, and the aliphatic portion of the K608 side chain. Contacts between the C-terminal helix (α4) of TPPI and POT1C are limited to L325 and the side chains of P357 and K608 of POT1C.

Several POT1C mutations reduce POT1–TPP1 binding. There are several POT1C mutations (L343F, P446Q, P475L, R477T, A532P, I535F, C591W and Q623H) that are associated with either familial glioma, melanoma or CLL (refs 22–27) (Supplementary Table 1). The majority of these mutations (L343F, P446Q, P475L, R477T and C591W) are somatic and specific to familial glioma and melanoma24. To determine the role of the POT1C disease mutations in POT1–TPP1 binding and complex assembly, we performed Isothermal Titration Calorimetry (ITC) measurements. The experiments were carried out using purified, wild type (WT) TPPI(PBD) (residues 255–337) and WT or mutant POT1C (residues 330–634) (Supplementary Fig. 1), the same constructs we used for the crystallization of the POT1C–TPP1(PBD) complex. The proteins were overexpressed in Escherichia coli and purified to homogeneity using three successive steps of purification as described in the methods section of the manuscript. All TPPI(PBD) and POT1C, WT and mutant proteins, except for I535F, overexpressed stably and in sufficient quantities for the proposed studies (Supplementary Fig. 1).

Considering the extensive network of interactions between the two proteins it is not surprising that the binding constant of TPPI for POT1 is in the nanomolar range (Kd = 120 ± 16 nM—Fig. 3b,f). Of the seven mutant POT1C proteins tested, only P446Q, C591W, and Q623H showed significant loss of TPPI binding (Kds of 289, 870 and 471 nM, respectively—Fig. 3c,g,h,i;
Since the POT1–TPP1 complex is directly linked to telomerase, POT1–TPP1 disruption does not alter telomerase processivity: unclear.

are solvent exposed (R477) and their function is currently and are either involved in the fold of the protein (L343, A532) and R477 are located away from the TPP1 binding sites of POT1 which indirectly affects TPP1 binding. P475 lies in the interior of the HJR and contributes to the fold of this domain. L343, A532 which associates with human disease, affect telomerase processivity. It is worth noting that the TEL patch of TPP1 implicated in telomerase binding is located at the N-terminus of TPP1. To address this question, we performed direct telomerase activity assays using cell extracts overexpressing super telomerase and at assays using cell extracts overexpressing super telomerase and at least 157 nM, respectively—Fig. 5c,d and Supplementary Table 3). P446Q, C591W and Q623H POT1 mutants, which bind TPP1 with 2–7 fold less affinity (Fig. 3c,g,h and i), also show WT telomerase processivity within the margin of error (Fig. 4c–f).

Partial POT1–TPP1 disruption reduces POT1-DNA binding.

POT1 binds the telomeric overhang with high affinity and selectivity, a process enhanced by TPP1 binding. For this reason, we asked if the POT1 disease mutants located in the C-terminal portion of the protein and where TPP1 binds, affect POT1-DNA binding. Fluorescence Polarization (FP) assays using a fluorescently labelled DNA probe consisting of three telomeric repeats (TTAGGG)₃ (18mer) were carried out in the presence of (a) the POT1C and TPP1(PBD) protein complex used for crystal and ITC studies (Figs 1b and 3a) (b) full-length POT1 WT and three mutant (P446Q, C591W, and Q623H) proteins and (c) the E. coli purified, full-length WT and mutant (L343F, P446Q, P475L, R477T, A532P, C591W and Q623H) POT1 and the N-terminally truncated TPP1(87) used in the telomerase direct assays (Fig. 5a).

FP data show that POT1C alone and the POT1C-TPP1(PBD) complex do not bind single-stranded telomeric DNA (Fig. 5a). The full-length WT and mutant (P446Q, C591W and Q623H) POT1 proteins alone all bind single-stranded telomeric DNA with a Kd of 20 nM (Fig. 5b,d; Supplementary Table 3). For the POT1–TPP1 complex our data shows that the WT complex binds the telomeric overhang with approximately 5.8 ± 0.5 nM (Fig. 5c,d and Supplementary Table 3), consistent with what has been reported previously. Interestingly, the P446Q, P475L, C591W and Q623H mutants show a decrease in DNA binding affinity (Kd = 10.3 ± 1.0 nM, 10.7 ± 1.4 nM, 15.6 ± 2.5 and 8.9 ± 1.0 nM respectively—Fig. 5c,d and Supplementary Table 3) in agreement with the ITC data, which shows that these mutant proteins bind TPP1 with 2–7 fold less affinity (Fig. 3c,g,h and i), also show WT telomerase processivity within the margin of error (Fig. 4c–f).

POT1C mutant proteins localize to telomeres. To determine the impact of the POT1C mutations on telomere targeting, we

Table 1 | Data collection, phasing and refinement statistics.

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*Highest resolution shell is shown in parenthesis.

Supplementary Table 2). P475L displayed a marginal decrease in TPP1 binding (164 ± 32 nM) but was not statistically significant (P = 0.22). In contrast, L343F, R477T and A532P displayed almost WT TPP1 binding affinity (Kds 114, 123 and 117 nM, respectively—Fig. 3b,e,f,i and Supplementary Table 2). Consistent with what has been previously reported, the POT1 mutants L343F, P475L, R477T, A532P and I535F showed WT telomerase processivity (Fig. 4c–f), in agreement with the ITC data, which shows that these mutant proteins bind TPP1 with WT binding affinity (Fig. 3a,b,d–f and i). Unexpectedly, the P446Q, C591W and Q623H POT1 mutants, which bind TPP1 with 2–7 fold less affinity (Fig. 3c,g,h and i), also show WT telomerase processivity within the margin of error (Fig. 4c–f).

POT1–TPP1 complex do not alter telomerase processivity.

Since the POT1–TPP1 complex is directly linked to telomerase processivity, we asked whether the POT1C mutations, associated with human disease, affect telomerase processivity. It is worth noting that the TEL patch of TPP1 implicated in telomerase binding is located at the N-terminus of TPP1. To address this question, we performed direct telomerase activity assays using cell extracts overexpressing super telomerase and at saturated levels of full length, WT or mutant POT1–TPP1. HEK293T cells extracts overexpressing human super-telomerase (the super-telomerase plasmids were a gift of the Lingner lab) were prepared as described by Lingner et al. We also overexpressed and co-purified the full length POT1 and an N-terminal truncation of TPP1 consisting of residues 87–544 (TPP1(87)) (Fig. 1a). The two proteins were overexpressed separately in E. coli and the cells co-cracked to allow stable POT1–TPP1 complex formation prior to purification. We found that independent purification of the two proteins resulted in partial TPP1 degradation suggesting that TPP1 is not stable alone (Fig. 4a). We were unable to purify sufficient amounts of the full-length I535F POT1 mutant for this assay. In addition, we prepared HEK293T lysates overexpressing human super-telomerase, full-length POT1 and TPP1 as described by Nandakumar et al. We examined the stability and levels of WT and mutant POT1 and TPP1 proteins overexpressed in transfected HEK293T cells using western blot analysis. Western blot analysis shows that all proteins (WT and mutant POT1 and TPP1) express at levels similar to the WT protein (Fig. 4b).
co-expressed YFP-tagged POT1 with mCherry-tagged TRF2 in HEK293T cells and used confocal imaging to examine their localization. HEK293T cells were transiently transfected with Cherry-TRF2 and YFP-POT1 and fixed 24 h later. Western blot analysis of whole-cell lysates of transfected HEK293T cells showed overexpression of the WT and mutant YFP-POT1 proteins (Fig. 6a). All of the POT1 mutants co-localized with Cherry-TRF2, similar to wild type, which indicates that the mutations do not prevent POT1 telomere targeting (Fig. 6b).

**Partial POT1–TPP1 disruption leads to longer telomeres.** To determine if the POT1 cancer mutations maintain telomere length homeostasis, we carried out southern blot analysis of WT and mutant POT1 transfected HEK293T cells. Stable HEK293T cell lines expressing Flag-POT1 were prepared using lentiviral and mutant POT1 transfected HEK293T cells. Stable HEK293T lines using western blot analysis, Fig. 7b) using fluorescence in situ hybridization (FISH). The endogenous POT1 in these cell lines was reduced by infection with shPOT1 (Fig. 7a). We prepared metaphase spreads of these cell lines by fixing the chromosomes to microscope slides with formaldehyde, and hybridizing telomeres with a 5’ TelC-Tamra peptide nucleic acid (PNA) probe. Chromosomal DNA was stained with DAPI prior to imaging.

We counted the frequency of chromosome fusions, fragile telomeres, and telomere free ends 50 population doublings after infection. HEK293T cells carrying the empty vector showed an average of 2–3% of chromosomes with chromosome fusions, fragile and missing telomeres, similar to that of the cell line overexpressing WT POT1. Elevated levels of fusions were observed for the L343F (7%) and I535F (5%) POT1 mutants, respectively (Fig. 8a,b). Interestingly, all POT1 mutants showed a significant increase of fragile telomeres (L343F 9%; P446Q, R477T respectively (Fig. 7c–f). The POT1 mutants P446Q, C591W and Q623H show 3–5 Kb increase in telomere length is observed for P446Q, C591W and Q623H respectively (Fig. 7c–f), while L343F, P475L, R477T, A532P and I535F show 3–5 Kb increase in telomere length (Fig. 7c–f).

Partial POT1–TPP1 disruption leads to fragile telomeres. To better understand the role of the POT1 cancer-associated mutations in telomere maintenance, we examined the phenotype of HEK293T cells infected with WT or mutant POT1 (same as those used for Southern blot analysis, Fig. 7b) using fluorescence in situ hybridization (FISH). The endogenous POT1 in these cell lines was reduced by infection with shPOT1 (Fig. 7a). We prepared metaphase spreads of these cell lines by fixing the chromosomes to microscope slides with formaldehyde, and hybridizing telomeres with a 5’ TelC-Tamra peptide nucleic acid (PNA) probe. Chromosomal DNA was stained with DAPI prior to imaging.

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The cancer phenotypes associated with POT1C naturally occurring mutations are diverse (familial melanoma, glioma and CLL (refs 22–25,27), Supplementary Table 1), which points to the complex nature and function of the telomeric complex POT1–TPP1 at telomeres. N-terminal, POT1 disease mutations disrupt POT1-DNA binding and release the telomeric overhang, which results in persistent telomere replication by telomerase22–25. There is no evidence currently that supports POT1C or POT1C-TPP1(PBD), DNA binding (Fig. 5a). However, POT1C interacts with TPP1, and POT1 mutations that disrupt the complex would be expected to influence its functions, which include localization to...
telomeres, stimulating telomerase processivity, and enhanced POT1 DNA binding activity.4,5,9 Moreover, the fact that a POT1 mutation confers a telomere instability phenotype, over a long period of time might provide the genetic variation that is required for clonal evolution. This would be even more apparent in tumours with a high-tumour burden, such as CLL, where the total number of cells will be in the 10–100 s of billions range. Thus, a combination of the long time it takes for these tumours to evolve and the tumour burden means that even subtle defects in telomere stability could have a dramatic effect on long-term clinical outcome.

Our data on several of the referenced disease associated POT1 mutations supports the above hypothesis. Cell imaging shows that all reported POT1 mutant proteins localize to telomeres (Fig. 6). Despite the fact that all POT1 mutant proteins localize to the telomeric overhang, FP assays show that only the mutants (P446Q, C591W and Q623H) that partially disrupt the POT1–TPP1 complex display significantly lower affinity for telomeric DNA (Figs 3 and 5c,d). Reduced affinity of the POT1–TPP1 complex for the telomeric overhang will result in persistent telomere elongation by telomerase.30 Persistent telomerase action at the end of chromosomes will generate longer telomeres than those observed for the WT POT1–TPP1 complex (Fig. 7c,d). This observation is consistent with the Southern blot analysis presented here, which shows that HEK293T cells transfected with the POT1C mutants have longer telomeres than the cells transfected with WT POT1. This observation is particularly distinct for the POT1 mutants that partially disrupt the POT1–TPP1 complex with ~4.5, 10 and 12.5 Kb increase in telomere length for P446Q, C591W and Q623H respectively (Fig. 7). It has also been well established that unregulated telomere length results in chromosomal abnormalities associated with telomere signal free ends and fragile telomeres.31–33 Consistent with this hypothesis POT1 mutations that disrupt the POT1–TPP1 complex show elevated levels of missing and fragile telomeres (Fig. 8). This defect is particularly prominent when it comes to fragile telomeres with an average of 10% of fragile telomeres for all POT1 mutants when compared to ~3% for the empty vector and WT POT1. Interestingly, none of the disease mutations appear to affect telomerase processivity (Fig. 4c–f).
We show that several of the referenced POT1 mutations (P446Q, C591W and Q623H) partially disrupt the POT1–TPP1 complex (Fig. 3c,d,g–i). This observation is in agreement with the Liu et al., which shows that the 417–445 and 616–628 of POT1 peptides make direct contacts with TPP1. P446Q is critical for TPP1 binding as it comprises part of the loop that connects the POT1C, OB-fold, and the solvent accessible side chain coordinates the backbone of helix \( \alpha \)8, which spans the entire length of the side of the OB-fold and plays a critical role in the organization of this region of POT1C (Fig. 2e). Moreover, the N-terminal portion of helix \( \alpha \)8 makes extensive interactions with \( \alpha \)2 of TPP1 and in particular the side chain of W293. Displacement of the POT1 helix \( \alpha \)8 would lead to re-organization of this region of POT1C(OB), thus affecting TPP1 binding. The POT1–TPP1 structure also shows that Q623 directly engages TPP1 (Fig. 2e). The Q623 residue is located at the heart of the canonical binding pocket of the POT1C OB-fold, and the solvent accessible side chain coordinates the backbone of helix \( \alpha \)3 of TPP1. More specifically, the NE2 of Q623 is 18 Å away. A532 is part of the \( \alpha \)7 of the HJR domain and like R477, is located on the opposite side of the HJR domain where helix \( \alpha \)3 and its connecting loops of TPP1 with V543, Y558, M560, Y610 and F625 of the POT1C OB-fold, thus affecting POT1–TPP1 binding.

Unlike P446Q, C591W and Q623H, the L343F, P475L, R477T and A532P POT1C mutants displayed WT, TPP1 and DNA binding affinity (Figs 3 and 5). L343F comprises part of the loop that connects the POT1C, OB-fold, \( \beta \)-strand \( \beta \)1 to the \( \alpha \)-helix \( \alpha \)1. L343 is important for the structural organization of this loop and does not make direct contacts with TPP1 (Fig. 2a). Similarly, R477T is part of the \( \beta \)8 of HJR and like R477, is located at the opposite face of this domain and where helix \( \alpha \)1 of TPP1 binds (Fig. 2d). Unlike L343, R477 is solvent exposed and does not make any contacts with TPP1; in fact, the nearest TPP1 contact point is \( \sim \)18 Å away. A532 is part of \( \alpha \)7 of POT1(HJR) and like R477, is located on the opposite side of the HJR domain where helix \( \alpha \)1 of TPP1 binds. I535 is located in \( \alpha \)7 of the HJR domain with the hydrophobic side chain buried into the core of this domain. Introducing the larger phenylaline side chain would disrupt the core of the HJR domain and therefore lead to the destabilization of the protein (Fig. 2d). Interestingly, isolation of the A532P and in particular of I535F POT1C and flPOT1 mutant proteins resulted in lower levels of proteins compared to WT protein (Fig. 2d). Isolation of the A532P and I535F POT1C mutant proteins likely affects the stability of these mutant POT1 proteins.

Isolation of the I535F protein did not produce adequate amounts of this polypeptide for TPP1 and DNA binding assays. However, these mutant proteins appear to be at WT levels in cell based

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**Figure 5 | POT1–TPP1 telomeric DNA binding assays.** (a) FP assays of POT1C and the POT1C–TPP1(PBD) complex with a single-stranded DNA probe consisting of 3 telomeric repeats (18mer). (b) FP assays of the WT and mutant (those that partially disrupt the POT1–TPP1 complex P446Q, C591W and Q623H) fIPOT1 with the 18mer DNA probe. (c) FP assays of the WT and mutant fIPOT1– TPPI(87) complex with the 18mer. (d) Bar graph showing the differences in Kd (nM) between the WT and mutant fIPOT1 and fIPOT1–TPPI(87) complex. The values are the average of three independent measurements and a two-tailed Student's t-test was performed with respect to WT POT1–TPP1(PBD) complex: *\( P < 0.05 \), **\( P < 0.01 \).
assays allowing us to study their effect in telomerase activity, processivity, telomere localization and telomere defects including telomere length, and chromosomal abnormalities (Figs 4, 6–8). Southern blot analysis showed telomere length changes of \( \sim 3-5 \) Kb—Fig. 7d,e. There is also a significant increase in telomere fusions, fragile and missing telomeres in the telomeric FISH staining for the I535F mutant (Fig. 8).

Taken together our data show that it is a confluence of factors that contribute to malignant effects associated with POT1C mutants. Some of these mutations (P446Q, C591W and Q623H) partially disrupt the POT1–TPP1 complex and the DNA binding affinity of POT1, which in turn affects the ability of the complex to regulate telomerase access to telomeres efficiently. Deregulation of telomerase activity at the chromosomal terminus would result in constitutive telomeric elongation and increased proliferative potential23. In part, POT1 regulates telomere length34–37, by regulating access of telomerase to telomeres, an effect modulated by TPP1 binding9,21,38,39. Partial disruption of the POT1–TPP1 complex could therefore lead to persistent access of telomerase to the telomeric overhang and result in longer, fragile telomeres.

Surprisingly, a set of the reported disease mutations (L343F, A532P and R477T) do not appear to have a significant effect in the assembly of the POT1–TPP1 complex as indicated by our structural and biochemical data (Figs 2–7). It is however worth noting that all of these mutations show a significant increase in fragile telomeres with defects in telomere length, missing telomeres and chromosome fusions. The precise role of these mutations in POT1–TPP1 function are currently unclear and further studies are required to better understand their role in cancer.

Germline variants in POT1 have been detected in familial melanoma and glioma22–25. Somatic mutations have been identified in POT1 and has been identified as a susceptibility locus for CLL (ref. 40). Somatic mutations in POT1 have also been identified in CLL B-cell clones and whilst the impact of these mutations on telomere length has not been established they are associated with increased chromosomal instability22. It is worth noting that POT1 mutations drive carcinogenesis in melanoma, glioma and CLL patients usually in combination with an array of other defective genes, such as CDKN2A, CDK4, BAPI, Notch1, SF3B1, TP53 and ATM, which have already been identified to predispose patients to these malignant diseases. Stratification of CLL patients based on the telomere length of their CLL B-cell clones, reveals that those with short telomeres, within the length ranges in which telomere fusion can be detected, exhibit an extremely poor prognosis41. This is presumed to arise as a consequence of telomere driven genome instability, clonal evolution and tumour progression27. While the majority of patients with long telomeres exhibit a better prognosis and a more stable genome, a subset of patients still progress with their disease. It will thus be of interest to examine the relationship between somatic POT1 mutation, telomere length and chromosomal instability and how this impacts on disease progression.

**Methods**

**Protein expression and purification.** Human POT1C, comprising residues 330–634, was identified via limited proteolysis and cloned into a pET28b vector containing a N-terminal hexa histidine—pMocr fusion tag, cleavable by TEV protease. The TPP1(PBD) construct was designed to contain residues 255–337 and was cloned into a pET28b vector containing a N-terminal hexa histidine tag cleavable by TEV protease. Both POT1C and TPP1(PBD) were overexpressed in *E. coli*.
ScrabXpress T7 lac competent cells (Scrab Genomics) at 18 and 30 °C for 4h, respectively, using 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Gold Biotechnology). The cells were harvested by centrifugation and lysed in a buffer containing 25 mM Tris–HCl, pH 7.5, 1.0 M KC1, 1.0 M Urea, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine (Ni Buffer A) via sonication. The proteins were purified over a Ni-nitrilotriacetic acid (Ni-NTA - MCLab) column, buffer exchanged while on the Ni-NTA column with 25 mM Tris–HCl, pH 7.5, 0.2 M KC1 and 5% glycerol (Ni Buffer C). The complex was eluted with 300 mM imidazole onto a tandem HS(poros)—HQ(poros) columns (Applied Biosystems) equilibrated with Ni Buffer C. The HS–HQ columns were then detached and the POT1C-TPP1(PBD) complex was eluted from the HQ column with a salt gradient of 0.2 M KC1 to 1.0 M KC1. The fusion tags were cleaved by TEV overnight at 4 °C. The POT1–TPP1 complex was then buffer exchanged to remove the maltose and hexahistidine-MBP (maltose binding protein) cleavable by TEV protease overnight at 4 °C using 1 mM IPTG. The cells were harvested by centrifugation and lysed in Ni buffer A via sonication. The proteins were first purified over a Ni-NTA column and then eluted onto an amylose column (New England Biotech) to further purify the POT1–TPP1 complex and remove excess POT1 before eluting with a buffer containing 25 mM Tris–HCl, pH 7.5, 0.5 M KC1, 5% glycerol, 1 mM DTT and 30 mM maltose. The fusion tags were cleaved with TEV protease overnight at 4 °C. The POT1–TPP1 complex was then buffer exchanged to remove the maltose and passed over an orthogonal Ni-NTA and amylose column to remove any residue fusion tags and TEV protease from the samples. The purified full-length POT1–TPP1 complex was then concentrated and ran on an SDS–PAGE gel with known concentrations of BSA standard and quantified using ImageQuant TL (GE Healthcare) to determine the concentration of the complex.

Protein crystallization and structure determination. POT1C-TPP1(PBD), crystal screening produced a crystal hit under sitting-drop vapour diffusion at room temperature in a crystallization buffer containing 2.4 M KC1, 50 mM K/Na Tartrate, 20 mM BaCl2, and 0.1 M Sodium Citrate, pH 5.5. (A longer construct of POT1C consisting of residues 325–634 produced a different crystal form that belonged to the P1 space group and diffracted to 3 Å at best.) The new crystal form belongs to the P41,22 (sq91, 1 copy in asymmetric unit) and diffracted to 2.1 Å. The native data set (containing Zn) was collected from 2 crystals at 1.03317 Å wave-length at BL12-2 SSRL to 2.1 Å resolution. The radiation damage was slowed with a careful absorbed dose estimate, allowing high-multiplicity and accumulating a significant anomalous signal from the present Zn and Sulfurs in the protein. Both the native and derivative data were processed with XDS (autoxds script at SSRL) with a zero dose correction.

The structure was solved using a single methyl mercury (methyl) derivative using the SAD approach as implemented in SHELX/CDE using the graphical interface of the HKL2MAP software. SHELDX identified five well defined Hg sites and SHELEX extended and optimized the initial phases to 2.1 Å and generated a preliminary structure of 330 residues with excellent contrast (0.943) and connectivity (0.843) resulting in FOM of 0.605. Subsequently the model was traced using 10 cycles of BUCCANEER. The resulting model was almost complete—358 sequenced residues. The remaining model was improved in COOT and refined.
Error bars are indicating s.d. An average of 500 chromosomes were counted in each experiment.

Figure 8 | Fluorescence in situ hybridization data. (a) Telomeric FISH metaphase spreads of cells expressing WT and mutant (L343F, P446Q, P475L, R477T, A532P, I535F, C591W and Q623H) hPOT1 proteins after 50 population doublings (Red, TelC-Tamra; blue, DAPI). Endogenous POT1 levels were reduced with shPOT1. Telomere fusions, fragile telomeres and missing telomeres are indicated by red, pink and green arrows respectively. Scale bar is 5 μm. (b-d). Quantification of telomere fusions, fragile, and missing telomeres from the metaphase spreads of (a). Bars indicate the per cent of metaphase events. Error bars are indicating s.d. An average of 500 chromosomes were counted in each experiment.

Fluorescence polarization (FP) assays. We performed FP DNA binding assays using an Envision Xcite Multilabel Plate Reader (PerkinElmer). 20 μl binding reactions were carried out in a buffer containing 20 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM TCEP, protein concentration was measured using a Bradford Assay. TPP1 at a concentration of 100 μM was injected into a cell containing 10 μM POT1C until saturation was reached. For the ITC experiment, the cell of the calorimeter was kept at 25 °C, and the volume of each injection was 2.47 μl with a total of 16 injections. Analysis of the ITC data used the Origin analysis software (GE Healthcare) to obtain binding constants and ratios.

Cell culture. The WT and mutant full-length human POT1 genes were cloned in the pLU-EF1A-iBlast (pLU) vector with an N-terminal 1xFlag tag and 4 μg of vector was used to transfect HEK 293T cells using lipofectamine 2000 (Invitrogen) to test expression. HEK293T cells were cultured in growth medium containing Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) and 100 units ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Sigma). All human cells were cultured at 37 °C with 5% CO2 and harvested 48 h after transfection.

Western blot. For the western blot analysis of HEK293T cells co-transfected with hTERT, hTER, POT1 and TPP1, standard immunoblot protocols were used with the following antibody dilutions: anti-human TERT (ab120550, Abbeza, 1:1,000, dilution), anti-human POT1 antibody (ab21283, Abcam, 1:1,000 dilution), anti-human ACD (SARB2100024, Sigma, 1:1,000 dilution), anti-Actin antibody conjugated to HRP (A3854, Sigma, 1:1,000 dilution), and secondary HRP-conjugated anti-sheep IgG (sc-2924, Santa Cruz, 1:1,000) and anti-rabbit IgG (A0545, Sigma, 1:1,000 dilution) were used to detect the human TERT and human POT1 and TPP1 antibody, respectively.

For western blot analysis of HEK293T cells stably expressing shPOT1 and WT and mutant POT1 proteins, standard immunoblot protocols were used with the following antibody dilutions: anti-human POT1 antibody (P0096, Sigma, 1:1,000 dilution), anti-GAPDH antibody conjugated to HRP (2118S, Cell Signaling Technology, Danvers, MA, 1:5,000 dilution), and secondary HRP-conjugated anti-rabbit IgG antibody (A0545, Sigma, 1:1,000 dilution) was used to detect the human POT1 antibody.
conjugated anti-rabbit IgG antibody (A0545, Sigma, 1:1,000 dilution) was used to detect the human POT1 antibody.

Detection of antibody signal was done with chemiluminescence activated using detection software (Scientific Volume Imaging, The Netherlands). The files were then presented as maximum projection images using Leica LAS software.

**Southern blot.** For telomere length analysis we carried out Southern blots on HEK293T cells stably expressing WT and mutant 1xFlag-POT1 and an shRNA targeting the 3'UTR of the endogenous POT1. Genomic DNA from cells at various passages was purified using a QIAamp DNA mini kit (QIAGEN) and 10 μg of genomic DNA was then digested with XhoI + MboI restriction enzymes. 5'SP-labelled GeneRuler 1 kb Plus DNA ladder (Thermo) and 2 μg of digested DNA was then fractionated in a 0.7% agarose gel, denatured and transferred onto a GeneScreen Phoshyridization membrane (Perkin Elmer) overnight. The membrane was washed with PBS-Twice and hybridized at 42 °C with 5' end-labelled 32P-(TTAGGG) 18 oligonucleotide probe. Following this, the blot was washed briefly with PBSTwice, then rewashed with PBSTwice for 15 min each and then washed with wash buffer (0.2 M NaHPO4, pH 7.2, 1 mM EDA, and 2% SDS) at 37°C. The membrane was exposed to a phosphor storage plate overnight and visualized by Typhoon 9410 Image (GE Healthcare). Telomere length was calculated using the software TeloTool (MATLAB).^{85}

**Reverse transcription PCR and quantitative PCR analyses.** To test the effectiveness of the shRNA targeting the 3'UTR of endogenous POT1, we collected RNA from cells treated with shRNA by lysing pelleted cells in TRIzol (Life Technologies) and purified RNA using the RNeasy Mini Kit (Qiagen). The RNA was eluted in DEPC water. The total RNA quality and quantity were measured using Nanodrop and Agilent 2100 Bioanalyzer, respectively. Two micrograms of RNA was reverse transcribed to cDNA using SuperScript III reverse transcriptase (Life Technologies) and oligo-dT as a primer. The cDNA was then diluted 1:10 before use in qPCR.

**Direct telomerase activity assays.** Telomerase preparations were carried out as previously described by Cristofari et al.^{9,44}. Briefly, super telomerase extracts were prepared by lysing HEK293T cells overexpressing human hTERT and hTER (pcDNA6-hTERT and pBS-U1-hTER plasmids) in IP buffer (75 mM KCl for 30 min at 37°C). The extracts were then centrifuged to remove debris and washed with buffer (10 mM Tris pH 7.5, 0.5% Odyessy blocking buffer (LiCor) according to the manufacturer's instructions. Slides were stained with DAPI and imaged using a Nikon 80i upright microscope using a × 100 objective image.

**Data availability.** The atomic coordinates and structure factors for the POT1-TPP1 complex described here have been deposited in the Protein Data Bank under the accession code 5UN7. The data that support the findings of this study are available from the corresponding author.

**References.**


