TENT4A poly(A) polymerase regulates translesion DNA synthesis and is mutated in endometrial cancer

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Abstract

TENT4A (PAPD7) is a non-canonical poly(A) polymerase, of which little is known. Here we focus on its multilayer regulation of translesion DNA synthesis (TLS), in which DNA lesions are bypassed by error-prone DNA polymerases. We show that TENT4A regulates mRNA stability and/or translation of DNA polymerase η and RAD18 E3 ligase, which guides the polymerase to replication stalling sites, and monoubiquitinates PCNA, thereby enabling recruitment of error-prone DNA polymerases to damaged DNA sites. Remarkably, in addition to the effect on RAD18 mRNA stability via controlling its poly(A) tail, TENT4A indirectly regulates RAD18 via the tumor suppressor CYLD, and via the long non-coding antisense RNA PAXIP1-AS2, which had no known function. Knocking down the expression of TENT4A or CYLD, or overexpression of PAXIP1-AS2 led each to reduced amounts of the RAD18 protein and DNA polymerase η, leading to reduced TLS, highlighting PAXIP1-AS2 as a new TLS regulator. Bioinformatics analysis revealed that TLS error-prone DNA polymerase genes and their TENT4A-related regulators are frequently mutated in endometrial cancer genomes, suggesting that TLS is dysregulated in this cancer.
**Introduction**

Maintaining genome integrity is critical for the proper functions of cells, and if compromised may lead to severe malfunction and a variety of diseases including cancer, immune malfunction, and neuronal disorders (Akbari, Morevati et al., 2015, Errol, Roger et al., 2006, Hanahan & Weinberg, 2011, Li, Woo et al., 2004, Marteijn, Lans et al., 2014, Modrich, 1994). The majority of DNA lesions inflicted on DNA are removed by accurate DNA repair mechanisms that restore the original DNA sequence. However, the high rate at which DNA lesions are formed (estimated to be thousands/cell/day), and the difficulty in locating them in the genome, makes encounters of DNA replication with DNA lesions inevitable in essentially every cell cycle. Such encounters cause arrest of the replication fork, and if not resolved may lead to fork collapse, double-strand breaks (DSBs), and subsequent chromosomal rearrangements or even cell death. DNA damage tolerance (DDT) mechanisms function in these situations to bypass the lesions, without removing them from DNA, a process that preserved the double-stranded continuity of DNA, giving later a chance to accurate excision repair mechanism to remove the lesion (Branzei & Foiani, 2010, Friedberg, 2005, Livneh, Cohen et al., 2016).

Translesion DNA synthesis (TLS), is a DDT mechanism in which specialized low-fidelity DNA polymerases synthesize across the lesion, a process that is inherently mutagenic (Sale, Lehmann et al., 2012). Despite its mutagenic nature, TLS is surprisingly accurate when replicating across several common DNA lesions such as the sunlight-induced cyclobutane pyrimidine dimers (CPD), and the tobacco-smoke induced (+)-trans-BPDE-N2-dG (BP-G) adduct. To explain this, we have previously proposed that the existence of multiple TLS DNA polymerases enables specialization of certain polymerases to their cognate lesions (meaning more effective and more accurate bypass), and when subjected to the appropriate regulation to ensure the activity of the right polymerase at the right lesion and the right time, this will lead to more accurate TLS, and a lower mutation burden (Cohen, Bar et al., 2015, Hendel, Ziv et al., 2008, Livneh, 2006). We also reported, that p53, and its target gene p21 (acting via its interaction with PCNA), are needed to maintain accurate TLS,
partially via their effect on monoubiquitination of PCNA (Avkin, Sevilya et al., 2006). The latter is an important regulatory step, which functions to recruit TLS DNA polymerases to the damaged site in DNA (Hendel, Krijger et al., 2011, Hoege, Pfander et al., 2002, Kannouche, Wing et al., 2004). The molecular deciphering of the regulatory mechanisms of TLS are critical to the understanding of mutation formation, and is the focus of the current study.

*TENT4A* (terminal nucleotidyltransferase 4A; formerly *PAPD7*), is a gene encoding a non-canonical poly(A) RNA polymerase, that we identified in an siRNA-based screen to be required for efficient TLS (Ziv, Zeisel et al., 2014). *TRF4*, its *S. cerevisiae* homolog, was thought to encode a novel DNA polymerase, termed *POLS* (or erroneously, *POLK*), involved in sister chromatid cohesion (Haracska, Johnson et al., 2005, Wang, Castano et al., 2000). However, further studies showed that the *TRF4* and *TRF5* genes encode non-canonical poly(A) RNA polymerases (Haracska et al., 2005). Enzymes of this family are critical in the regulation and quality control of gene expression. There is scarce information about the mammalian *TENT4A*. However, it was reported that its main isoform is 94kDa, considerably larger than the yeast Trf4 and Trf5 proteins (66 and 74 kDa, respectively), and larger than its previously reported size of 62 kDa (Ogami, Cho et al., 2013). Recently *TENT4A* was shown along with its paralog *TENT4B* (*PAPD5*) to synthesize mixed poly(A) tails that contain other inserted nucleotides, primarily guanines, that protect mRNA from deadenylation (Lim, Kim et al., 2018). Still, the functional involvement of *TENT4A* in biological regulatory pathways is largely unknown (Mueller, Lopez et al., 2019).

Focusing on *TENT4A* involvement in TLS, we found that *TENT4A* regulates mRNA stability and/or translation of DNA polymerase η (*polη*; *POLH*), and of the RAD18 E3 ligase. Remarkably, in addition to the effect on RAD18-mRNA stability via controlling its poly(A) tail, *TENT4A* indirectly regulates RAD18 and *polη* via the tumor suppressor CYLD, and via the long non-coding antisense RNA *PAXIP1-AS2*, which had no known function, highlighting it as a novel TLS regulator. We also partially purified *TENT4A* and show that it is a poly(A) RNA polymerase, with no detected DNA polymerase activity. Finally, we report that
bioinformatics analysis of mutations in the genomes of 33 cancer types from the TCGA database revealed that components of TENT4A-regulated TLS are frequently mutated in endometrial cancer, suggesting involvement of dysregulated TLS in the development of this type of cancer.

**Results**

**TENT4A is needed for effective TLS**

A screen performed in our lab, identified TENT4A (PAPD7; POLS) as a novel TLS gene (Ziv et al., 2014). To further confirm its effect on TLS, we used the gap-lesion plasmid-based assay, with DNA constructs each containing a single defined lesion (Hendel et al., 2011, Shachar, Ziv et al., 2009, Ziv et al., 2014), and two cell lines: The human osteosarcoma U2OS cell line and the MCF-7 breast cancer cell line. As can be seen in Fig 1A, B and C, Appendix Fig S1, and Appendix Tables S1 and S4, knocking down the expression of TENT4A in these cells, but not of TENT4B, led to a decrease in TLS across four types of DNA lesions: a BP-G adduct, a major lesion caused by tobacco smoke; a thymine-thymine cyclobutane pyrimidine dimer (TT-CPD) and a thymine-thymine 6-4 photoproduct (TT-6-4PP), the most common, and the second most common type of UV light-induced DNA damage, respectively; and a cisplatin guanine-guanine (cisPt-GG), a major intrastrand crosslink formed by the chemotherapeutic drug cisplatin. DNA sequence analysis showed that the error frequency of the residual TLS under TENT4A knockdown did not substantially change (Appendix Tables S2, S3, S5 and S6). The broad DNA damage spectrum of the effect suggests that TENT4A regulation of TLS is not directed to a specific TLS DNA polymerase, but instead functions in a rather more general TLS regulatory step that exerts a global effect.
Purified TENT4A is a non-canonical poly(A) RNA polymerase, which incorporates also other ribonucleotides

We partially purified overexpressed full-length human TENT4A as an 8xHis-MBP-tagged protein, (Appendix Fig S2), and assayed its potential activities as a poly(A) RNA polymerase and DNA polymerase. In parallel, we purified a TENT4A variant that carries the D277A and D279A mutations (Appendix Fig S2), which were expected to inactivate the coordination of the divalent metal ion as a co-substrate. As can be seen in Fig 1D, TENT4A used ATP in the presence of Mg" to extend the oligo (A)_{20} substrate. In contrast, the TENT4A mutant was inactive. A similar activity was observed with the oligo 5’ r(AGAGUUUGAUCCUGGCUCGA)-3’ (Fig 1E). Unlike E. coli poly(A) RNA polymerase, which was used as a positive control, and performed extensive extension of the substrate, TENT4A added a limited number of AMP residues, which is typical of a non-canonical poly(A) RNA polymerase activity (Fig 1D and E). The polyadenylation activity of TENT4A was stimulated when Mn" was used instead of Mg"", whereas the mutant TENT4A remained unaffected (Fig 1D and E). TENT4A preferentially polymerizes AMP residues, although it can use other rNTPs to some extent (Fig 1F), consistent with a previous report (Lim et al., 2018). Of note, the shorter form of TENT4A, previously believed to be the full-length enzyme, had negligible poly(A) RNA polymerase activity (not shown). We also examined the DNA polymerase activity of the recombinant tagged TENT4A, and found no activity (Fig 1G). We conclude that TENT4A is a poly(A) RNA polymerase, and as far as we can tell, it has no detectable DNA polymerase activity.

Effect of TENT4A on mRNA of genes involved in TLS

Poly(A) RNA polymerases are typically involved in regulating mRNA via 3’-polyadenylation, and therefore to start addressing the mechanism by which TENT4A regulates TLS, we examined whether the mRNA of TLS-related genes bind TENT4A. To that end, we used RNA immunoprecipitation-qPCR (RIP-qPCR). We overexpressed FLAG-TENT4A, then immunoprecipitated the protein using anti-FLAG antibody, and extracted the RNA from
protein-RNA complexes. The amount of specific mRNA was determined by preparing cDNA, and performing qPCR using gene specific primers. As can be seen in Fig 2A, while the mRNAs of POLH, POLK and POLI showed no significant binding, the mRNAs of REV3L encoding the catalytic subunit of DNA polymerase ζ, and of REV1, encoding a TLS scaffold protein/polymerase, as well as RAD18 and CYLD, showed significant preferential binding. RAD18 is the E3 ligase that monoubiquitinates PCNA (Watanabe, Tateishi et al., 2004), a central signalling event in TLS, and CYLD is a deubiquitinase involved in cancer and required for efficient TLS, as we have previously reported (Ziv et al., 2014).

To examine whether TENT4A affects the length of the poly(A) tail of these genes, we used ePAT (extension poly(A) test; (Janicke, Vancuylenberg et al., 2012)). We examined the TLS regulators RAD18 and CYLD, which bound TENT4A, and also POLH, which did not bind TENT4A. As can be seen in Fig 2, knocking down the expression of TENT4A caused a shift to shorter amplified tail fragments of RAD18, and similar results were obtained for the tail fragments of CYLD (Fig 2B and C). Interestingly, knocking down the expression of TENT4A caused a shift to shorter amplified tail fragments of POLH too, despite its lack of binding to TENT4A, suggesting indirect regulation. Knocking down both TENT4A and TENT4B caused a similar shift (Fig 2B, C and D).

Because the length of the poly(A) tail may affect mRNA stability, we examined the effect of TENT4A on the stability of the mRNA of these TLS-related genes by measuring their half-life in the presence of the transcription inhibitor Actinomycin D. As can be seen in Fig 2E, knocking down the expression of TENT4A, but not TENT4B, caused a decrease of about 33% in the half-life of RAD18 mRNA, whereas knocking down the expression of both caused a slightly stronger decrease of 43%. The half-life of CYLD mRNA was decreased by 53% when the expression of TENT4A was knocked-down, with a smaller effect of TENT4B knockdown (Fig 2F). Interestingly, for POLH mRNA, knocking down the expression of TENT4A had a decrease in half-life (29%), slightly lower than the effect of TENT4B knockdown (39% decrease) (Fig 2G). The stability of a control POLD3 mRNA was essentially unaffected by knocking down either TENT4A, TENT4B or both (Fig 2H), as was
the stable RNA18S rRNA (Appendix Fig S3). These results suggest that TENT4A directly regulates the stability of RAD18 and CYLD mRNA, and indirectly the stability of POLH mRNA via the length of their poly(A) tails.

**TENT4A affects the amounts of RAD18, CYLD and POLH proteins via different mechanisms**

Since effects on mRNA are often, but not always, manifested in the levels of the encoded proteins, we next analysed whether TENT4A affects also the protein products of the RAD18, POLH and CYLD genes. As can be seen in Fig 3A, upon knocking down the expression of TENT4A, there was a decrease in the amount of RAD18, observed in both unirradiated and UV-irradiated cells. Knocking down TENT4A expression led also to a decrease in the amounts of the CYLD protein (Fig 3B) and polh (POLH gene product (Fig 3B).

To explore whether this reduction in the amounts of the RAD18, CYLD and POLH proteins was a result of reduced translation efficiency, we performed polysome profiling under condition in which TENT4A expression was knocked-down. As can be seen in Fig 3C, there was an increase in the 80S monosome and a decrease in the polysomal fractions when TENT4A expression was knocked-down, indicating an inhibition of translation efficiency. Analysis of individual genes revealed a shift from the heavy to the light fractions of CYLD and POLH mRNA (Fig 3E and F), but not of RAD18 mRNA (Fig 3D). Translation of the control gene POLD3 (Fig 3H) was unaffected by knocking down the expression of TENT4A. Thus, the reduction in CYLD and POLH protein amount following TENT4A knockdown is due to both their reduced mRNA stability and its reduced translation efficiency, but the decreased amount of RAD18 protein following TENT4A knockdown is due to reduced mRNA stability, as the translation efficiency was unaffected.

**Regulation of PCNA monoubiquitination by TENT4A**

RAD18 is the key E3 ligase that monoubiquitinates PCNA, and we therefore examined whether the TENT4A effect of reducing the amounts of RAD18 is also manifested in the
level of mUb-PCNA. As can be seen in Fig 4, upon TENT4A knockdown the amount of mUb-PCNA was mildly decreased in UV-irradiated cells (Fig 4, lane 10), and strongly decreased in unirradiated cells (Fig 4, lane 2). The amount of mUb-PCNA was calculated as the mUb-PCNA/PCNA ratio, and as the amount (independent of PCNA) normalized to the loading control GAPDH, generally showing a similar behaviour upon TENT4A knockdown.

PCNA monoubiquitination in untreated cells was reported to be carried out by the DTL (CTD2) E3 ligase (Terai, Abbas et al., 2010), and we therefore examined the effect of TENT4A knockdown on DTL. As can be seen in Fig 4 lanes 2 and 10, knocking down the expression of TENT4A caused a strong reduction in the amount of the DTL protein, in both unirradiated and UV-irradiated cells. In parallel, also the amount of the RAD18 protein was decreased, consistent with the results shown above. We then examined whether RAD18 and DTL affect each other. Interestingly, when the expression of DTL was knocked-down, the amount of the RAD18 protein was also reduced in both unirradiated (Fig 4, lane 4) and UV-irradiated cells (Fig 4, lane 12). On the other hand, when the expression of RAD18 alone was knocked-down, the amount of DTL was increased, in both unirradiated (Fig 4, lane 3) and UV-irradiated cells (Fig 4, lane 11). When both TENT4A and RAD18 were knocked-down, DTL was still decreased, but to an extent lesser than with TENT4A knockdown alone, indicating an additive effect (Fig 4, lanes 5 and 13). On the other hand, the TENT4A and DTL double knockdown had a similar effect to the single gene knockdown, indicating the two are epistatic (Fig 4, lanes 6 and 14).

How do these effects on RAD18 and DTL translate into levels of mUb-PCNA? In UV-irradiated cells RAD18 knockdown caused a strong reduction in mUb-PCNA, despite the increase in DTL, consistent with RAD18 being the major E3 ligase that monoubiquitinates PCNA in UV-irradiated cells, with a similar effect observed with the TENT4A and RAD18 double knockdown (Fig 4, lanes 11 and 13). Interestingly, knocking down DTL caused a mild decrease in mUb-PCNA in UV-irradiated cells (Fig 4, lane 12), likely because under these conditions RAD18 was also decreased. In unirradiated cells, knocking down the expression of DTL caused a strong decrease in mUb-PCNA, consistent with previous work (Terai et al.,...
but also knocking down RAD18 caused a decrease in mUb-PCNA (Fig 4, lane 3), despite a slight increase in the amount of DTL. Similar effects were observed when both TENT4A and DTL were knocked-down (Fig 4, lane 6). When both TENT4A and RAD18 were knocked-down, mUb-PCNA was strongly reduced (Fig 4, lane 5), as under these conditions the two E3 ligases were reduced.

**TENT4A-regulated CYLD regulates RAD18, DTL and POLH**

CYLD, previously identified by us in a screen as a TLS regulator, acts downstream to TENT4A, and is regulated by TENT4A at the mRNA stability and translation levels as shown above. To examine its own effect on TLS, we knocked-down CYLD expression, and examined levels of RAD18, mUb-PCNA and POLH. As can be seen in Fig 5A, knocking down the expression of CYLD caused a strong 3-5 fold decrease in the total amount of RAD18, clearly visible in both unirradiated cells and UV-irradiated cells. Similarly, the POLH protein decreased about 3-fold (Fig 5A). It thus appears that in addition to its direct effect on RAD18, TENT4A has an indirect effect on both RAD18 and POLH proteins, mediated via its target gene CYLD.

An analysis of mUb-PCNA in cells in which CYLD was knocked-down revealed that despite the reduction in RAD18 protein, UV-induced mUb-PCNA levels were little affected (Fig 5A). Assuming that DTL might be backing up RAD18 under these conditions, we knocked-down the expression of CYLD, RAD18 or DTL individually, or in combinations of two or three genes. As can be seen in Fig 5B, knocking down CYLD caused a strong decrease of both RAD18 and DTL, in both unirradiated (Fig 5B, lane 2) and UV irradiated cells (Fig 5B, lane 10). Of note, a decrease in RAD18 protein was not always paralleled by a decrease in mUb-PCNA (Fig. 5B).
A *TENT4A*-regulated long non-coding antisense RNA to the *PAXIP1* (*PTIP*) gene regulates RAD18, POLH and mUb-PCNA

In an effort to further explore *TENT4A*-regulated genes which affect TLS, an RNA-seq analysis was performed, comparing genes in control cells to the same cells in which *TENT4A* was knocked-down (the results of the RNA-seq analysis will be published elsewhere). This analysis revealed that in cells in which *TENT4A* expression was knocked-down, *PAXIP1-AS2*, a long non-coding RNA, antisense to the *PAXIP1* gene, was upregulated. Because *PAXIP1* was reported to promote PCNA monoubiquitination (Gohler, Munoz et al., 2008), we studied the involvement in TLS of *PAXIP1-AS2*, for which no biological function was yet assigned.

Knocking down the expression of *TENT4A* caused a significant increase in the amount of the *PAXIP1-AS2* transcript (Fig 6A), as expected from the RNA-seq analysis. This effect was not observed when *TENT4B* was knocked-down, suggesting that it is specific to *TENT4A* (Fig 6A). We next overexpressed *PAXIP1-AS2* and examined its effect on TLS. As can be seen in Fig 6B and Appendix Tables S7 and S8, TLS across cisPt-GG was strongly suppressed by overexpressing *PAXIP1-AS2*. This effect was not mediated via the amount or stability of *PAXIP1* mRNA, which remained essentially unchanged upon overexpression of the antisense RNA (Fig 6C and D), however, the amount of *PAXIP1* protein was diminished (Fig 6E). Directly knocking down the expression of *PAXIP1* also inhibited TLS across a TT-CPD lesion (Fig 6F and Appendix Tables S9 and S10), consistent with the effect of the antisense RNA.

We next examined the effects of *PAXIP1* and *PAXIP1-AS2* on POLH, RAD18 and mUb-PCNA. As can be seen in Fig 6G and H, *PAXIP1-AS2* overexpression, and similarly knockdown of *PAXIP1*, each caused a decrease in the amount of POLH, whereas the effect on RAD18 and mUb-PCNA was marginal.

As described above, TENT4A did not bind POLH mRNA, yet it affected its stability, raising the possibility that the effect was mediated via *PAXIP1*. Knocking down the expression of *PAXIP1* reduced the half-life of POLH mRNA to an extent similar to knocking-
down the expression of TENT4A (Fig 7A). Knocking down both TENT4A and PAXIP1 caused a decrease of POLH mRNA stability similar to that of each gene alone, suggesting that the two are epistatic, and that possibly the effect of TENT4A on POLH mRNA is mediated largely via PAXIP1. Western blot analysis revealed that the decrease in polH amount was more pronounced under PAXIP1 than TENT4A knockdown (Fig 7C). Thus, while the effects of the two seem to be epistatic at the mRNA level, additional regulation affects the polH protein level by mechanisms which are yet to be explored, e.g., via a positive regulation by PAXIP1, or a branch of negative regulation by TENT4A. A similar epistatic analysis was performed with TENT4A and PAXIP1 for RAD18 mRNA, which binds TENT4A. Knocking down the expression of TENT4A had a bigger effect than PAXIP1 of decreasing the half-life of RAD18 mRNA, while knocking down the two had an intermediate effect (Fig 7B). A similar effect of observed also at the protein level, in both unirradiated, and UV-irradiated cells (Fig 7C). Thus, the direct effect of TENT4A on RAD18 mRNA appears to be dominant over the PAXIP1 axis.

**TENT4A and TLS genes are frequently mutated in endometrial cancer**

The importance of TLS in tolerating DNA damage and affecting genetic stability prompted us to examine whether mutations in genes of the TLS pathway are over-represented in particular cancer types. To that end we used the TCGA database, and first analysed, for each cancer type, the percentage of the samples that contained mutations in at least one of the genes that we defined as a TLS-related genes group, containing TENT4A, CYLD, NPM1, TENT4B, PAXIP1, PCNA, POLH, POLI, POLK, PRIMPOL, RAD18, REV1, REV3L and USP1. As can be seen in Appendix Table S11, there is a big variation in the percent of samples with mutations in the TLS genes group, ranging from about 1-2% (e.g., Thyroid Carcinoma; THCA) up to 37% for Uterine Corpus Endometrial Carcinoma (UCEC; endometrial cancer). While as expected, cancer types with a higher overall number of mutations tend to exhibit a higher percentage of samples with mutations in TLS-related genes.
genes (e.g., Skin Cutaneous Melanoma, SKCM; Colon Adenocarcinoma, COAD; Lung Squamous Cell Carcinoma; LUSC; Appendix Table S11), endometrial cancer stands out with the highest occurrence of samples with mutations in TLS genes group, despite a higher median overall number of mutations in several other cancers (e.g., Bladder Cancer, BLCA; COAD; Lung Adenocarcinoma, LUAD; Appendix Table S11).

To probe the statistical significance of the high percentage of samples with mutations in the TLS genes group, we estimated the probability that this high percentage was obtained by chance. To that end we chose a control gene set of 14 random genes (the size of the TLS-related gene group), and examined in each cancer type the percentage of samples with mutations in at least one gene of this control gene set. This was repeated 1000 times and used to plot the chance distribution and calculate the probability of the TLS genes group (Appendix Fig S4, Appendix Table S11). Appendix Fig S4 shows also the average number of samples in each distribution, as well as the fraction of samples with TLS-related genes group in each cancer type. The fraction of the 1000 random sets which yielded a number of samples with mutations higher than the number of TLS mutations, represents the probability that the fraction of TLS-related genes group was obtained by chance, and was termed P-total value. Only three cancer types passed this statistical test with P-total<0.05, namely Acute Myeloid Leukemia (LAML), Thymoma (THYM) and UCEC (Appendix Table S11, Table 1A). For these three we tested the statistical significance of the difference between the number of samples with TLS mutations and the average number of samples with random mutation sets, using the Chi-squared test. Only LAML and UCEC passed this additional test with P<0.05 (Table 1A). Analysis of the fraction of samples with mutations in each TLS gene (Table 1B), shows that UCEC has indeed a considerable percentage of mutations in each of the TLS-related genes, including TENT4A (Table 1B), whereas in the LAML, the majority of samples contained mutations in a single gene, namely NPM1 (Table 1B).
Discussion

DNA damage tolerance by TLS must be carefully regulated to balance between the beneficial effect of enabling overcoming replication obstacles, with minimal adverse effect of genetic stability. Indeed, no less than 17 genes were previously identified in our lab as new regulators of TLS (Ziv, Zeisel et al., 2014), amongst them TENT4A (PAPD7). While initially this gene was proposed to encode in yeast a DNA polymerase, it was subsequently shown to encode a poly(A) RNA polymerase in both yeast (Haracska et al., 2005) and human cells (Lim et al., 2018), with no detectable DNA polymerase activity, as confirmed in this study.

Very little was known on biological processes that TENT4A regulates, and therefore the finding that it is involved in TLS prompted us to further explore the molecular mechanisms that underlie this involvement. Interestingly, the TENT4A-dependent regulation of TLS uncovered in the current study, spans several major components of the TLS machinery, i.e., RAD18, DTL, POLH and mUb-PCNA, in a complex interrelationship using both direct and indirect mechanisms (Fig 8). Interestingly, the CYLD tumor suppressor gene, previously identified as a TLS regulator (Ziv, Zeisel et al., 2014) turned out to be acting downstream to TENT4A, and directly regulated by it at the mRNA stability and translation levels, and PAXIP1-AS2 was identified as a new significant TENT4A-regulated TLS regulator. Of note, PAXIP1, which PAXIP1-AS2 regulates, is a paired box (PAX) gene with six BRCT domains, which is involved in development (Callen, Faryabi et al., 2012, Schwab, Smith et al., 2013) and responses to DNA damage (Mijic, Zellweger et al., 2017, Wang, Aroumougame et al., 2014). It is a suggested lung cancer tumor suppressor (Wu, Tian et al., 2018), and linked to breast and ovarian cancer, and response to chemotherapy (Jhuraney, Woods et al., 2016).

While TENTA directly regulates RAD18 and CYLD via binding their mRNAs and controlling the length of their poly(A) tails and stability, consistent with its activity as non-canonical poly(A) RNA polymerase, POLH mRNA regulation appears to be indirect, since no binding of POLH mRNA to TENT4A protein was observed. It is likely mediated via the PAXIP1 protein (see below) which showed an effect similar and epistatic to TENT4A.
Interestingly, *TENT4A* knockdown had also a global effect on translation, as indicated by the shift in ribosomes profile, and specifically manifested in the inhibited translation of the *POLH* and *CYLD* genes, but not *RAD18*.

Knocking down the expression of *TENT4A* or its downstream effector *CYLD* reduced the level of *RAD18*, and was therefore expected to be manifested in a decrease in mUb-PCNA. Unexpectedly, such a decrease was not necessarily observed, leading to the unravelling of an interesting relationship between *TENT4A*, *RAD18* and *DTL*. First, in addition to *RAD18*, *DTL* is also regulated by *TENT4A*, whose knockdown strongly decreased the level of *DTL* protein. Second, knocking down the expression of *DTL*, caused a decrease in the amount of the *RAD18* protein, in both unirradiated and UV-irradiated cells. This is not due to cross targeting of the siRNAs, since they are gene specific, raising the possibility that *DTL* acts upstream to *RAD18*. Because knocking down the expression of *CYLD* also reduced the amount of *DTL*, it is possible that the *CYLD* branch regulated by *TENT4A* operates in the order of *TENT4A* => *CYLD* => *DTL* => *RAD18*.

Interestingly, knocking down the expression of *RAD18* led to an increase in the amount of *DTL* protein, as if to compensate for the absence of this activity, possibly acting via a feedback loop. The fact that a reduction in the amount of *RAD18* is not always manifested in a significant decrease in mUb-PCNA, may have been due to the remaining amounts of the *RAD18*, which were sufficient to carry out significant PCNA monoubiquitination, and/or there might exist a third, as yet unknown E3 ligase which monoubiquitinates PCNA.

As expected from its sequence complementarity, we found that *PAXIP1-AS2* is indeed a negative regulator of *PAXIP1* (Fig 6E). However, somewhat unexpectedly, this regulatory effect was not mediated via an effect on the amount or stability of *PAXIP1* mRNA (Fig 6C and D). It was previously reported that antisense long non-coding RNA can control translation (Carrieri, Cimatti et al., 2012), and a similar effect may explain the effect of *PAXIP1-AS2*. 
**PAXIP1-AS2** overexpression, **PAXIP1** or CYLD knockdown each reduced the levels of POLH, and CYLD knockdown also reduced the level of RAD18, causing a significant decrease in TLS, despite little change in the level of mUb-PCNA. This decreased TLS was likely caused by the decreased level of POLH, which performs actual TLS reactions, with possible participation of RAD18, which in addition to the monoubiquitination of PCNA has a non-catalytic function in TLS, involving an interaction with POLH and guiding it to replication stalling sites (Durando, Tateishi et al., 2013, Huang, Zhou et al., 2018, Watanabe et al., 2004). The phenomenon, in which TLS was reduced but mUb-PCNA remained essentially unchanged, indicates that the formation of mUb-PCNA cannot be used as a surrogate for TLS, as is sometimes done, and may lead to erroneous conclusions.

TLS is involved in a variety of biological processes, most notably DNA damage tolerance (Livne et al., 2016) and the generation of somatic hypermutation in the immune system (Casali, Pal et al., 2006). Dysregulation of TLS can cause an altered load of point mutations, or if TLS is inhibited it can lead to an increase in chromosomal aberrations due to the activity of alternative recombinational and repair events (Wittschieben, Reshmi et al., 2006). Of the 33 cancer types examined for occurrence of mutations in TLS-related genes, endometrial cancer is the only one which exhibits TLS mutations in a considerable fraction of samples, spread over all 14 genes, including **TENT4A** (Table 1), suggest that dysregulated or downregulated TLS is involved in endometrial carcinogenesis (Cancer Genome Atlas Research, Kandoth et al., 2013, Suhaimi, Ab Mutalib et al., 2016). It is yet unclear why this should be specific to endometrial cancer, however, dysregulation or inactivation of discrete DNA repair mechanisms is generally cancer type-specific, for reasons which are not fully understood (Akbari et al., 2015, Errol et al., 2006, Hanahan & Weinberg, 2011, Li et al., 2004, Marteijn et al., 2014, Modrich, 1994). Of note, the loss of TLS might sensitize cancer cells to DNA damaging chemotherapy, providing potential prognostic and therapeutic value (Gallo & Brown, 2019, Zafar & Eoff, 2017). To the best of our knowledge, this is the first report on such extensive somatic mutations in genes of the TLS pathway in sporadic cancers.
Materials and Methods

Cell cultures and transfections

Osteosarcoma U2OS, breast cancer MCF-7, human embryonic kidney HEK 293T and 293FT epithelial cells were cultured in DMEM (Gibco) supplemented with 2 mM L-Alanyl-L-Glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 1 mM sodium pyruvate (Biological Industries), and 10% fetal bovine serum (HyClone). XPA (XP12RO) human fibroblasts derived from xeroderma pigmentosum patients were a gift from A. R. Lehmann (University of Sussex, Brighton, U.K.). XP12RO cells were cultured in MEM Eagle (Biological Industries) supplemented with 2 mM L-Alanyl-L-Glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin (Biological Industries), and 15% fetal bovine serum (HyClone). The cells were incubated at 37°C in a humidified incubator with 5% atmosphere and periodically examined for mycoplasma contaminations by EZ-PCR test kit (Biological Industries). For plasmid transfection, cells were transfected with Lipofectamine 2000 (Invitrogen) or JetPRIME reagent (Polyplus) according to the manufacturer’s instructions. For siRNA transfection, the siGENOME SMART pool siRNA oligonucleotides, siGENOME Non-Targeting Control siRNA #5 (D-001210-05) and ON-TARGETplus SMART pool siRNA oligonucleotides and ON-TARGETplus NON-targeting pool (D-001810) (Dharmacon) were transfected with 25 or 50 nM siRNA by Lipofectamine RNAimax (Invitrogen) for 48 to 72 h following the manufacturer’s protocol. For combinatorial knockdown, an equal amount of siRNAs against each gene was mixed to have a final combined concentration of 100 nM for the double knockdown and 150 nM for the triple knockdown, unless otherwise stated. siRNAs used in this study are listed in Appendix Table S12.

Generation of lentiviral stable cell lines

Lentiviral pLKO.1-puro empty vector control plasmid and human TENT4A shRNA pLKO.1-puro plasmid (clone TRCN0000053036, target sequence CCAACAATCAGACCAGGTDTA) were obtained from Sigma (Mission shRNA library). Lentiviruses were produced in 293FT

17
cells, by co-transfecting pLKO-derived plasmids and the second-generation packaging plasmids using JetPRIME. Lentiviral particles were harvested 48 h post-transfection. The resulting lentiviral particles were used to infect the U2OS cells. 48 h post-infection, 2 μg/ml of puromycin (InvivoGen) was added to select for infected cells. After two weeks of selection, individual colonies were isolated and tested for knockdown of TENT4A by quantitative real-time PCR (qPCR).

**TLS assay in cultured mammalian cells**

The TLS assay was described earlier (Diamant, Hendel et al., 2012, Shachar et al., 2009, Ziv, Diamant et al., 2012). The TLS gap-plasmid transfection was performed by co-transfecting with a mixture containing 50 ng of a lesions plasmid (KanR), 50 ng of a gapped plasmid without a lesion (CmR), and 1900 ng of the carrier plasmid pUC18, using Lipofectamine 2000. The cells were incubated for 4 h (for the TTCPD gap-lesion plasmid), 20 h (TT 6-4PP), 16 h (BP-G), or 6 h (cisPt-GG) to allow TLS. The plasmids were extracted using alkaline lysis conditions followed by renaturation, such that only covalently closed plasmids remained nondenatured. A fraction of the purified DNA was used to transform a TLS-defective *E. coli* recA strain JM109, which was then plated on LB-kan and LB-cm plates. The efficiency of gap repair was calculated by dividing the number of transformants obtained from the gap-lesion plasmid (number of colonies on LB-kan plates) by the number of corresponding transformants obtained with the control gapped plasmid GP20-cm (number of colonies on LB-cm plates). To obtain precise TLS extents, the plasmid repair extents were multiplied by the fraction of TLS events out of all plasmid repair events, based on the DNA sequence analysis of the plasmids from KanR colonies. To determine the DNA sequence changes that have occurred during plasmid repair, sequence analysis was carried using the TempliPhi DNA Sequencing Template Amplification Kit and the BigDye Terminator v1.1 Cycle Sequencing Kit. Reactions were analyzed by capillary electrophoresis on an ABI 3130XL Genetic Analyzer from Applied Biosystems.
RNA isolation and qPCR

Total RNA was isolated using RNeasy plus mini kit (Qiagen) according to the manufacturer’s instructions, including treatment with RNase-free DNase I (Qiagen). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using qPCRBIO SyGreen Blue Mix (PCR Biosystems) and run on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Primer sequences used were predesigned KiCqStart SYBR® Green primers (Sigma-Aldrich) and are included in Appendix Table S13. The expression of the indicated transcript was normalized to endogenous reference control GAPDH according to ΔΔCt method using the DataAssist software v3.0 (Applied Biosystems).

mRNA stability assay

The expression of TENT4A, TENT4B, PAXIP1 or TENT4A plus TENT4B, or TENT4A plus PAXIP1 was knocked-down for 48 h in MCF-7 cells. To measure mRNA stability, 5 µg/ml Actinomycin D (Sigma-Aldrich) was added to the growth medium to inhibit transcription, cells were harvested at the indicated time points and mRNA expression was measured by qPCR. The half-life of the mRNA was calculated by the one-phase-decay equation using GraphPad Prism 8 software.

RNA-Immunoprecipitation (RNA-IP) and extension poly(A) test (ePAT)

RNA-IP to assay TENT4A/RNA interactions was performed as described (Keene, Komisarow et al., 2006), and the ePAT assay was performed as described with some modifications (Janicke et al., 2012), and the two are presented in the Appendix Materials and Methods and Appendix Tables S13 and S14.

UV-irradiation, Whole cell extracts, and Western blotting

When indicated, cells were rinsed in Hanks’ buffer and irradiated in Hanks’ buffer with UV-C using a low-pressure mercury lamp (TUV 15W G15T8, Philips) at a dose rate of 0.2 J/m²/s. UV dose rate was measured using an UVX Radiometer (UVP) equipped with a 254-nm
detector. After irradiation, Hanks’ buffer was removed and the cells were incubated in fresh growth medium for additional time before harvest.

Cells were resuspended in CellLytic M lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail, phosphatase inhibitor 2 and 3 cocktail (Sigma-Aldrich), 2.5 mM MgCl₂, and 50 units/ml Benzonase (Merck), incubated on ice for 30 min followed by centrifugation. The supernatant was collected and the protein concentration was determined using the Bradford protein assay (Bio-Rad). The extracts were fractionated on a 4-20% ExpressPlus™ PAGE gel (Genscript) using SDS-MOPS buffer and transferred onto nitrocellulose membrane (BioTrace™ NT Nitrocellulose transfer membrane, Pall Corporation), followed by blocking of membranes with Odyssey Blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. The membranes were incubated with primary antibodies diluted in Odyssey Blocking Buffer–0.1% Tween-20 overnight at 4 °C. The membranes were washed 3x10 min with Tris-buffered saline with 0.1 % Tween-20 (TBST) and further incubated for 1 h at room temperature in the IRDYE-680 or 800 conjugated secondary antibodies diluted in Odyssey Blocking Buffer–0.1% Tween-20. Membranes were washed 3x10 min TBST and finally rinse with TBS to remove residual Tween-20. The membranes were imaged on a LI-COR Odyssey Fc Imager (LI-COR Biosciences) and the bands were quantified by ImageStudio v 5.2.5 (LI-COR Biosciences) software.

**Antibodies**

Commercially available antibodies used were as follows: rabbit anti-RAD18 (Cell Signaling, 9040S, dilution 1:2000); rabbit anti-ubiquityl-PCNA (Lys164) (Cell Signaling, 13439S, dilution 1:2000); rabbit anti-GAPDH (Cell Signaling, 5174S, dilution 1:10000); mouse anti-GAPDH (Milipore, MAB374, dilution 1:10000); rabbit anti-POLH (Cell Signaling, 13848S, dilution 1:1000); rabbit anti-CYLD (Cell Signaling, 8462S, dilution 1:1000); mouse anti-PCNA (PC-10) (Santa Cruz, sc-56, dilution 1:1000); mouse anti-FLAG M2 (Sigma-Aldrich, F1804, dilution 1:1000); rabbit anti-PTIP (PAXIP1) (Bethyl, A300-370A, dilution 1:2000); rabbit anti-DTL (Bethyl, A300-948A, dilution 1:1000); rabbit anti-TRF4 (H-172) (TENT4A) (Santa Cruz, A300-455A, dilution 1:1000).
sc-98490, dilution 1:500); anti-MBP monoclonal antibody, HRP conjugated (New England Biolabs, E8038S, dilution 1:10000); IRDye 680RD goat anti-mouse IgG (LI-COR, 926-68070, dilution 1:10000); IRDye 800CW goat anti-mouse IgG (LI-COR, 926-32210, dilution 1:10000); IRDye 680RD goat anti-rabbit IgG (LI-COR, 926-68071, dilution 1:10000); IRDye 800CW goat anti-rabbit IgG (LI-COR, 926-32211, dilution 1:10000).

TCGA database mutation analysis
The R package ‘TCGAbiolinks’ (Colaprico, Silva et al., 2016) was used to download all the MAF (Mutation Annotation Format) files related to the 33 cancer types from TCGA database. Loading and summarizing the MAF files for each cancer was done using the R package ‘maftools’ (Mayakonda, Lin et al., 2018). For the analysis of each cancer type, MAF files from all four pipeline analysis available in the TCGA database ("muse", "varscan2", "somaticsniper", "mutect2") were merged using the ‘merge_mafs’ function from ‘maftools’. The variant classifications, "Frame_Shift_Del", "Frame_Shift_Ins", "Splice_Site", "Translation_Start_Site", "Nonsense_Mutation", "Nonstop_Mutation", In_Frame_Del", "In_Frame_Ins", "Missense_Mutation","3'UTR", "5'UTR", "3'Flank", "Targeted_Region", "Silent", "Intron","RNA", "IGR", "Splice_Region", "5'Flank", "lincRNA", "De_novo_Start_InFrame", "De_novo_Start_OutOfFrame", "Start_Codon_Ins", "Start_Codon_SNP", "Stop_Codon_Del", were used in the analysis of mutations. Mutations frequencies were calculated as the number of mutated samples divided by the total number of samples for each gene per cancer.

Statistical analysis
The statistical results were obtained from at least three independent biological replicates unless otherwise stated. All results were presented as mean ± SEM. P values were obtained via the Student’s t-test (two-tailed), unless otherwise stated, using GraphPad Prism 8.0 software. *P < 0.05, **P < 0.01, ***P < 0.001.
See Appendix for additional Materials and Methods.

**Data availability**

This study includes no data deposited in external repositories

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**Author contribution**

UmSw designed, performed and analysed most experiments, and participated in writing the manuscript. UrSe performed polysome profiling experiments, analysed their data, and commented on the manuscript. ASP and RR performed the mutational analysis based on the TCGA data base. GF performed the RNAseq analysis and commented on the manuscript. CE, TC and NS provided oligonucleotides and commented on the manuscript. TPE, participated in designing experiments, analysis of the data, and writing the manuscript. RD provided advice, contributed to analysis of the results and commented on the manuscript. ZL conceived, devised and supervised the study, and wrote the manuscript.

**Conflict of interest**

All authors declare no competing interests.
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monoubiquitinates PCNA to promote translesion DNA synthesis. Mol Cell 37: 143-9


**Figure Legends**

**Figure 1.** Involvement of *TENT4A* in TLS, and its poly (A) RNA polymerase enzyme activity.  

(A) TLS across a TT-6-4PP and BP-G in U2OS cells. *TENT4A* expression was knocked-down using lentivirus-mediated shRNA combined with siRNA against *TENT4A*. The results are presented as the mean ± SEM of three independent experiments (see Appendix Table S1 for details). Statistical analysis was performed using the two-tailed Student’s *t*-test (***P < 0.01). (B) and (C) TLS across a cisPt-GG and TT-CPD, respectively, in MCF-7 cells in which the expression of *TENT4A* and/or *TENT4B* genes was knocked-down using specific siRNAs. The results are presented as the mean ± SEM of six independent experiments for cisPt-GG and three independent experiments for TT-CPD (see Appendix Table S4 for details). Statistical analysis was performed using the two tailed Student’s *t*-test (****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05).  

(D) Poly(A) RNA polymerase activity measured by the extension of an oligo (A)\textsubscript{20} substrate. Poly(A) polymerase assays were performed with the indicated amount of partially purified recombinant human 8xHis-MBP tagged *TENT4A* (lanes 1-3 and 9-11) or the *TENT4A* mutant D277A,D279A (lanes 4-6 and 12-14), using 5’\textsuperscript{32}P-labeled oligo(A)\textsubscript{20} and 1 mM ATP in presence of 5 mM MgCl\textsubscript{2} (lanes 1-6) or 1 mM MnCl\textsubscript{2} (lanes 9-14). For positive and negative controls, parallel reactions were also carried out with 0.5 units of *E. coli* poly(A) polymerase (lanes 8 and 16) or no added protein (lanes 7 and 15), respectively. The 5’\textsuperscript{32}P-labeled RNA oligo sequence is shown below the image. Reaction products were resolved on a 15% polyacrylamide gel containing 8 M urea and analyzed by PhosphorImaging.  

(E) Poly(A) RNA polymerase assays with an oligo RNA. The assays were performed as described in panel (D), except that the oligo RNA shown underneath the gel image was used, as well as the four NTPs (1 mM each).  

(F) Ribonucleotide specificity of *TENT4A*. The \textsuperscript{32}P-labeled RNA oligo(A)\textsubscript{20} substrate was incubated with the *TENT4A* protein (lanes 1-5) or its mutant (lanes 6-10) in the presence of each of the four NTPs (1 mM each).  

(G) Assays of *TENT4A* DNA polymerase activity. Assays were performed with the indicated amount of partially purified recombinant human 8xHis-MBP tagged *TENT4A* (lanes 2-4 and 10-12) and mutant (lanes 5-7 and 13-15), using
0.5 pmol of $^{32}$P-labeled 15/60-nt primer/template in the presence of the four dNTPs (100 µM each), 5 mM MgCl$_2$ (lanes 2-7) or 1 mM MnCl$_2$ (lanes 10-15). For positive and negative controls, parallel reactions were carried out with the Klenow fragment of Pol I (lanes 1 and 9), or no added protein (lanes 8 and 16), respectively. The sequence of the primer/template DNA substrate is shown below the image.

Figure 2. Effect of TENT4A on binding and stability of TLS-related mRNAs. (A) FLAG-tagged TENT4A expressed in HEK293T cells was immunoprecipitated with an anti-FLAG antibody, and RNA bound to it was analyzed by qPCR. The results are shown as fold-change relative to empty vector. The error bars indicate SEM (two independent experiments). (B), (C), and (D) Representative gel images of RAD18, CYLD, and POLH poly(A) tail are shown, each with the poly(A) tail densitometric trace underneath, using Image J. The position of TVN is indicated by an oligonucleotide that predominantly recognizes A$_{12}$, serving as an internal control. (E), (F), (G), (H), TENT4A and/or TENT4B expression was knocked-down in MCF-7 cells for 48 h, after which the cells were treated with 5 µg/ml Actinomycin D for up to 8 h. The expression levels of RAD18, CYLD, POLH, and POLD3 genes respectively, were each determined by qPCR relative to the expression level at time 0. Half-life was calculated by using one phase decay and significance of the differences was calculated using Student’s t-test (one-tailed); Data are presented as mean ± SEM from three independent experiments. ns: $P>0.05$.

Figure 3. Effect of TENT4A on levels and translation of TLS proteins. (A) MCF-7 cells were transfected with TENT4A-targeted siRNA (siTENT4A) or non-targeting control siRNA (siControl). At 65 h post-transfection, the cells were UV-irradiated at 20 J/m$^2$ and harvested 1 or 6 h post-irradiation. Whole cell extracts were fractionated by SDS-PAGE followed by western blot with indicated antibodies. Protein levels were normalized to GAPDH, and presented each relative to its level in unirradiated siControl-treated cells. (B) Effect of cell treatment (72 h) with siTENT4A or siControl on the levels of the CYLD and POLH proteins in
MCF-7 cells. Whole cell extracts were fractionated by SDS-PAGE and analyzed by western blot with indicated antibodies. Protein levels were normalized to GAPDH, and presented each relative to its level in siControl-treated cells. (C) Polysome profile analysis in MCF-7 cells in which TENT4A expression was knocked-down. Absorbance trace of polysome fractionation on a sucrose gradient. (D) qPCR analysis of mRNA levels of TLS-related genes in each fraction were expressed as a percentage of total levels summed over all fractions. The results are presented as the mean ± SEM of two independent experiments. (E), (F) and (G) Fraction of polysome-associated mRNA of the genes CYLD, POLH and GAPDH, respectively. The values were taken from (D). (H), Fraction of polysome-associated mRNA of the POLD3 control gene. The results are presented as the mean ± SEM of two independent experiments. Statistical significance was determined using Student’s t-test (one-tailed); *P < 0.05, ns: P > 0.05.

**Figure 4.** Interrelationship among TENT4A, RAD18 and DTL in determining amounts of RAD18 and DTL and the extent of PCNA monoubiquitination.

MCF-7 cells were transfected with siRNA against TENT4A, RAD18, and DTL or their double or triple combinations. At 65 h post-transfection, the cells were UV-irradiated at 20 J/m² and harvested 6 h post-irradiation. Whole cell extracts were fractionated by SDS-PAGE followed by western blot with indicated antibodies. Protein levels were normalized to GAPDH and presented each relative to its level in unirradiated or UV-irradiated siControl-treated cells.

**Figure 5.** Effect of CYLD on RAD18, DTL, POLH and PCNA monoubiquitination.

(A) MCF-7 cells were transfected with CYLD-targeted siRNA (siCYLD) or siControl. At 65 h post-transfection, the cells were irradiated at 20 J/m² UV and harvested 1 or 6 h post-irradiation. (B) MCF-7 cells were transfected with siRNA against CYLD, RAD18, and DTL or their double or triple combinations. At 65 h post-transfection, the cells were UV-irradiated at 20 J/m² and harvested 6 h post-irradiation. Whole cell extracts were fractionated by SDS-PAGE and analyzed by western blot with indicated antibodies. Protein levels were
normalized to GAPDH and presented each relative to its level in unirradiated or UV-irradiated siControl-treated cells.

**Figure 6.** Involvement of PAXIP1-AS2 and PAXIP1 in TLS. (A) Expression of TENT4A and/or TENT4B was knocked-down for 48 h, after which the level of PAXIP1-AS2 transcript was measured by qPCR, normalized to GAPDH, and compared to cells treated with siControl. The results are presented as the mean ± SEM of three independent experiments. (B) TLS across a cisPt-GG in MCF-7 cells in which PAXIP1-AS2 antisense RNA was overexpressed (See Appendix Tables S7 and S8). The results are presented as the mean ± SEM of two independent experiments. Statistical analysis was performed using the two-tailed Student’s t-test (**P < 0.01). (C) PAXIP1-AS2 antisense RNA was overexpressed in XP12RO or MCF-7 cells for 48 h, after which the amount of PAXIP1 mRNA was measured by qPCR, normalized to GAPDH and presented relative to cells transfected with an empty vector (EV). The results are presented as the mean ± SEM of three independent experiments. (D) PAXIP1-AS2 antisense transcript was overexpressed for 48 h in MCF-7 cells, after which PAXIP1 mRNA levels were determined as described in Fig 2. GAPDH was used as normalized control. The results are presented as the mean ± SEM of two independent experiments. (E) Effect of overexpression of PAXIP1-AS2 antisense RNA on PAXIP1 protein. Cells were harvested after 48 h and whole cell extracts were analyzed by SDS–PAGE followed by western blot with indicated antibodies. (F) TLS across a TT-CPD lesion in MCF-7 cells in which the expression of PAXIP1 was knocked-down for 68 h. TT-CPD TLS assay was performed for 4 h. See Appendix Tables S9 and S10 for details. The results are presented as the mean ± SEM of two independent experiments. Statistical analysis was performed using the two-tailed Student’s t-test (*P < 0.05). (G) Effect of overexpression of PAXIP-AS2 on TLS-related proteins in UV-irradiated cells. PAXIP1-AS2 was overexpressed in XP12RO cells for 48 h after which cells were UV-irradiated at 10 J/m² UV and harvested 1 or 6 h post-irradiation. Whole cell extracts were analyzed by SDS–PAGE followed by western blot with indicated antibodies. Protein levels are presented.
relative to those in extracts of unirradiated cells transfected with empty vector. (H) Effect of PAXIP1-knockdown on TLS proteins. MCF-7 cells were knocked-down by siRNA against PAXIP1 for 72 h, after which cells were UV-irradiated at 20 J/m² UV and harvested 1 or 6 h post-irradiation. Whole cell extracts were analyzed by SDS–PAGE and detected with indicated antibodies. Amounts are presented relative to those in extracts of unirradiated cells treated with siControl.

**Figure 7.** Effect of TENT4A and PAXIP1 on stability of TLS-related mRNAs and level of TLS-related proteins. (A) and (B) TENT4A and/or PAXIP1 expression was knocked-down in MCF-7 cells for 48 h, after which the cells were treated with 5 µg/ml Actinomycin D for up to 8 h. The expression levels of POLH and RAD18 were each determined by qPCR relative to the expression level at time 0. Half-life was calculated by using one phase decay and significance of the differences was calculated using Student’s t-test (one-tailed); Data are presented as mean ± SEM from three independent experiments. ns: P>0.05. (C) MCF-7 cells were transfected with TENT4A and/or PAXIP1 or non-targeting control siRNA for a final combined concentration of 100 nM. At 65 h post-transfection, the cells were UV-irradiated at 20 J/m² and harvested 1 or 6 h post-irradiation. Whole cell extracts were fractionated by SDS–PAGE followed by western blot with indicated antibodies. Amounts are presented relative to those in extracts of unirradiated cells treated with siControl and the values are shown in the corresponding position of the blots.

**Figure 8.** A scheme representing the three-branches of regulation of TLS by TENT4A. See text for details.
Table 1. Analysis of the frequency of TLS-related gene mutations in cancer genomes

(A) Number of samples with mutations in TLS-related vs. random genes in top ranking cancers

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Samples, n</th>
<th>Samples with mutations, n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLS-related genes</td>
<td>Random genes, Ave</td>
<td>Chi-squared</td>
</tr>
<tr>
<td>LAML</td>
<td>144</td>
<td>21</td>
<td>5.03</td>
</tr>
<tr>
<td>THYM</td>
<td>123</td>
<td>9</td>
<td>3.30</td>
</tr>
<tr>
<td>UCEC</td>
<td>530</td>
<td>196</td>
<td>153.92</td>
</tr>
</tbody>
</table>

(B) Fraction of cancer samples with mutations in TLS-related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Samples with mutations</th>
<th>Gene</th>
<th>Samples with mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYLD</td>
<td>1%</td>
<td>PRIMPOL</td>
<td>1%</td>
</tr>
<tr>
<td>NPM1</td>
<td>13%</td>
<td>RAD18</td>
<td>0%</td>
</tr>
<tr>
<td>PAXIP1</td>
<td>1%</td>
<td>REV1</td>
<td>0%</td>
</tr>
<tr>
<td>PCNA</td>
<td>0%</td>
<td>REV3L</td>
<td>2%</td>
</tr>
<tr>
<td>POLH</td>
<td>0%</td>
<td>TENT4A</td>
<td>0%</td>
</tr>
<tr>
<td>POLI</td>
<td>1%</td>
<td>TENT4B</td>
<td>1%</td>
</tr>
<tr>
<td>POLK</td>
<td>0%</td>
<td>USP1</td>
<td>0%</td>
</tr>
</tbody>
</table>

Note:

a All mutations included.
b Cancer type according to TCGA cancer acronyms. LAML, Acute Myeloid Leukemia; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma.
c Number of samples for which sequence information is available in the TCGA database.
d Number of samples with a mutation in at least one of 14 TLS-related genes listed in Table 1B.
e Number of samples with a mutation in at least one of 14 random genes; Average of 1000 random sets.
f Fraction of the 1000 random runs which yielded a number of samples ≥ fraction of TLS genes.
g P-value of the difference between the TLS-related genes and the average random genes (f and e).

First the average number of samples with mutations in random genes was rounded, and then the statistical significance of the difference between the two groups (TLS and random) was estimated using the chi-squared test. For example, for LAML the groups are: 21 samples with TLS mutations and 123 without mutations in these genes, compared to 5 samples with mutations in a set of random genes and 139 samples without mutations in these genes.
Figure 1
Swain et al 2020
Figure 2
Swain et al 2020
Figure 4
Swain et al 2020
Figure 5
Swain et al 2020
Figure 6
Swain et al 2020
Figure 7
Swain et al 2020
TENT4A (PAPD7)*

**TENT4A knockdown**

- CYLD mRNA destabilized
- CYLD DUB* protein

- DTL E3 ligase protein
- RAD18 E3 ligase* protein

- mUb-PCNA

- DNA polymerase η*
- Other TLS polymerases?

- Reduced DNA damage tolerance via TLS

* Mutated in uterine corpus endometrial cancer

Figure 8
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