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TERT promoter mutations and telomerase activity in urothelial carcinogenesis[\[Au:Edit OK?\]](#)

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Abstract

Telomerase activity imparts eukaryotic cells with unlimited proliferation capacity, one of the cancer hallmarks. Over 90% of human urothelial carcinoma of the bladder (UCB) are positive for telomerase activity. Telomerase activation can occur through several mechanisms. Mutations in the core promoter region of the human telomerase reverse transcriptase gene (*TERT*) cause telomerase reactivation in 60-80% of UCB, whereas the prevalence of these mutations is lower in urothelial cancers of other origins. *TERT* promoter mutations are the most frequent genetic alteration across all stages of UCB, indicating a strong selection pressure during neoplastic transformation. [Au:We avoid formulations referring to the article authors (“we”) in the abstract. I have edited the following text accordingly, OK?] *TERT* [Au:”promoter”?] promoter mutations could arise during regeneration of normal urothelium [Au:I tried to simplify here; is this what you meant?] and due to consequential telomerase reactivation, might be the basis of UCB initiation, which represents a new model for the origination of urothelial carcinogenesis. In the future, *TERT* promoter mutations and telomerase activity might have diagnostic and therapeutic applications in UCB.

[Au:For your information, [H1] and [H3] denote the level of heading and will be removed during the production process. Where indicated, I have edited the headings to conform to the character limits of a Perspective article ([H1] 32 characters incl. spaces; [H3] 70 characters incl. spaces). Please adjust my edits of headings if you have better suggestions.]

[H1]Introduction

Telomerase is a ribonucleoprotein (RNA-containing protein) complex composed of the telomerase reverse transcriptase protein (TERT) and a telomerase RNA component (TERC), which assemble, in a cell cycle-dependent manner, with several accessory factors to form the active holoenzyme¹. Telomerase is a telomere-specific enzyme that extends telomeres and its activity is required to overcome the end replication problem, counteracting the progressive loss of telomeric DNA at the tips of linear chromosomes during DNA replication. In the absence of telomerase activity, telomere shortening would eventually result in the loss of genetic material and genetic catastrophe owing to breakage-fusion-bridge (BFB) cycles. This cycle involves breakage of unstable chromosome structures (e.g. resulting from end-to-end-fusion of telomere-dysfunctional chromosomes) which may be broken during mitosis and initiate repeated rounds of fusion and breakage, leading to genome instability². [Au:Please reference. Edit for flow here OK? Please explain in the text what BFBs are. Thanks.] Telomerase activity is maintained somatically in many animals, including the mouse,³⁻⁷ but is down-regulated during human development and cellular differentiation through transcriptional repression of the catalytic subunit *TERT* gene⁸⁻¹⁰. [Au:Please clarify, is DNA expression repressed or the enzyme itself inhibited?] In human adult non-germ tissues, *TERT* expression and telomerase activity is restricted to the stem or progenitor cell compartment of some tissues and can be activated in some cell types, such as lymphocytes, following proliferative stimulation^{6,7,11-19}. [Au:With “emerges” did you mean “can be activated” as edited?] In the absence of sufficient telomerase activity, telomeres shorten in proliferating cells during aging^{20,21}. Short, dysfunctional telomeres lose capping function and activate DNA-damage responses. When DNA damage checkpoints are intact, telomere shortening limits the proliferative capacity of human cells and serves as a tumour suppressor mechanism by activating cell cycle arrest and senescence (Fig. 1). [Au:Please can you add a brief mention of what activates the DNA damage response. Is it the BFBs?] once the replication limit of ~50 cell divisions (known as the Hayflick limit) is reached²⁰⁻²³. [Au:As far as I am aware, strictly speaking, the term Hayflick limit is used to describe the number of times a cell can divide before excessive telomere shortening and senescence occur. How about reformulating to “A human somatic cell without telomerase activity usually reaches replicative senescence after ~50 cell divisions (the Hayflick limit).”?] These observations are supported by *in vivo* data that show that telomere dysfunction, in combination with a defective p53 pathway, provokes BFB cycles and enhances genomic instability, eventually promoting tumorigenesis²⁴⁻²⁷.

Telomerase activity is found in ~90% of human carcinomas (about 99% in UCB, see below), supporting the idea that activation of telomerase is an essential requirement for the immortalization (unlimited proliferation capacity) of human cells ^{28,29}. [Au:Although you mention it later on in the text, I think it is important to already mention here how frequent telomerase activity is in UCB, especially because you write how frequent ALT in UCB is, so that the reader has a clear comparison, OK?] These observations led to the hypothesis that down-regulation of telomerase activity is a mechanism that protects from tumorigenesis. In line with this hypothesis, telomerase [Au:add “activity” for specificity?] activity is required for malignant conversion of primary human cells ³⁰. Tumours without telomerase maintain their telomere functionality via the alternative lengthening of telomeres mechanism (ALT) ³¹. [Au:Please could you add a short explanation of what ALT involves?] Stabilization of telomere length and functionality by ALT relies on homologous recombination of telomeric DNA between sister chromatids ³². Recurrent mutations in the death-associated protein (DAXX) and the alpha-thalassemia X-linked protein (ATRAX) have been correlated with ALT occurrence and potentially contribute to ALT maintenance ^{33,34}. Of note, there is evidence that telomerase activity suppresses ALT, although the suppressive mechanism is yet to be identified ³⁴⁻⁴⁷. In a comprehensive analysis of 6,110 human tumour specimens from various cancer types, ALT was observed in only ~4% of samples ⁴⁸. Importantly, the prevalence of ALT varied between cancers. Most astrocytomas and sarcomas rely on ALT ⁴⁸⁻⁵⁰, whereas telomerase activity is the primary mechanism for telomere maintenance in most other carcinomas ^{48,51}. [Au:Swapped sentence parts for flow, OK? I also deleted the specific examples, as they did not seem relevant.] In urothelial carcinoma of the bladder (UCB), the prevalence of the ALT mechanism is low (~1%), indicating the importance of telomerase in this type of cancer ⁴⁸.

Accumulating evidence shows that telomerase or its components have functions, independent of telomere lengthening, which affect many biological processes, including cell survival and apoptosis, DNA damage repair, mitochondrial function, cell adhesion and migration, and stem cell activity ^{34,52-61}. These alternative functions can be independent of the enzymatic activity of telomerase ⁶² and can involve activation of the WNT- β -catenin signalling pathway by TERT ^{52,59}. [Au:We avoid the use of the ambiguous “may”; is “can” here OK or would “might” be better?] However, the physiological relevance of the latter mechanism has been questioned ^{57,63}. [Au:Please excuse my ignorance but I am not sure how the previous thought (activation of the WNT- β -catenin signalling pathway by TERT) and the processes in the following sentence (NF κ B-dependent gene regulation 48 or ribosomal DNA [Au:OK?] transcription by RNA polymerase 1) are connected. Please could you elaborate in the text, so that also readers who are not familiar with these pathways can follow your discussion? Also, are you indicating that these pathways are relevant in tumorigenesis? If so, please mention specifically in the text, so that the reader is aware why these findings are

mentioned in this Review.] Telomerase activity is required, but the presence of [Au:OK?] TERT alone is insufficient to modulate NFκB-dependent gene regulation⁶⁴ or ribosomal DNA [Au:OK?] transcription by RNA polymerase 1⁶⁵.

Data are emerging that show that telomeres can sense cellular stress conditions and induce cellular senescence to protect from tumorigenesis⁶⁶⁻⁶⁸. [Au:Please reference.] The cellular stress conditions can originate from chromosomal imbalances such as aneuploidy, oxidative damage, or hyperproliferation signals resulting from oncogene activation. [Au:What specifically do you mean by “chromosomal imbalances”. Please specify in the text. Please reference.]^{46,66,69,70} Telomeres are able to form G-quadruplex (G4) structures, four-stranded nucleic acid structures that have been observed in guanine-rich DNA-regions^{50,71-73}. [Au:OK? Please reference. Please explain what G-quadruplex structures are.] G4 structures preferentially form during replication and transcription and could be detected at telomeres⁷⁴. Current understanding is that the G-quadruplex structures impose a challenge to the replication machinery if not resolved properly^{10,75,76}. Human telomeres contain 2,000–3,000 TTAGGG repetitions, which distinguishes telomeres from the rest of the genome and provides the basis for sensing genotoxic and oncogenic cell damage⁷⁷⁻⁸². [Au:I think that we need some discussion of how the TTAGGG repetitions can serve as DNA damage sensors. I understand that this can be a large topic but the basic concept of how this mechanism works needs to be explained to reader. Please add. Thank you.] There is evidence that the activity of specific helicases is required for the progression of the replication machinery to prevent replication fork stalling. Importantly, it was demonstrated that Pif1 helicase requires telomerase to resolve G4-structures at telomeres in *Saccharomyces cerevisiae*⁸³. Based on these observations, it was suggested that in the absence of telomerase, fork progression is impaired at telomeres due to replication fork stalling which finally can lead to DNA-double strand breaks⁷⁷. Importantly, the oncogene-induced senescence (OIS) or aneuploidy-induced senescence (AIS) response is independent of telomere length and can be reduced by telomerase activity (Fig. 2)^{46,68,84,85}. These data open a new perspective on the interplay of telomeres and telomerase in suppressing and promoting tumorigenesis^{43,69}. [Au:By using “new perspective”, it sounds a bit as if a previous view of the interplay of telomeres and telomerase in suppressing and promoting tumorigenesis exists. I am not really sure whether this is what you want to indicate and also what that previous theory was. Please clarify, and if you want to highlight that this is a change from previous paradigms then please summarize the previous paradigm of the interaction in one sentence, so that the reader can quickly grasp the difference. Thank you.] This new idea indicates that in addition to the telomere shortening-induced tumor-protective function as described in Fig. 1, telomeres can act as a barrier to tumor progression under conditions such as oncogene-induced hyperproliferation, genotoxic oxidative damage or the presence of aneuploidy-inducing mutations (Fig. 2). Consequently, suppression of OIS or AIS by telomerase might enable telomerase-positive cells to acquire

tumour-initiating mutations that would normally trigger telomere-mediated senescence in telomerase-negative cells ⁴³. How the activity of telomerase can suppress the telomere-length-independent protective function of telomeres is still unclear.

In this Perspectives, we examine recent data showing a high prevalence of *TERT* promoter mutations in UCB, which occur early during tumorigenesis indicating that the biology of the urothelium provides a selection pressure for this change. [Au:Please state specifically which data.] We consider the implications of telomerase activity for UCB [Au:Might it be better to specify “UCB”?] cell survival in the context of telomere length-dependent and telomere length-independent roles of telomerase. We specifically also discuss the role of OIS and AIS abrogation in tumorigenesis.[Au:OK?] We present a new model for urothelial carcinogenesis that incorporates the role of telomerase activation and the potential of targeting telomerase as a therapeutic approach for UCB.

[H1]Telomerase in normal urothelium

Under physiological conditions, normal human urothelium is a long-lived, mitotically quiescent tissue with a low cell turnover rate but can develop a strong regenerative response and switch to a proliferative phenotype in response to damage ⁸⁶⁻⁸⁸. In non-proliferating urothelium, telomerase is undetectable, but various reports have shown normal human urothelial (NHU) cells maintained *in vitro* as rapidly proliferating, finite (i.e. non-immortalized) cell lines to express transient, low-level telomerase protein expression and enzyme activity ^{11,13}. [Au:What are the *in vitro* conditions used to achieve proliferation? Please add, so that the reader can distinguish from the immortalized NHU cells mentioned below.] Whether telomerase becomes activated in human bladder urothelium *in situ* in response to physiological stress or regenerative conditions is not known. The regulatory mechanisms underlying the repression of telomerase activity in quiescent human urothelium and its activation during proliferation of NHU cells remain to be revealed. Transient, low-level telomerase expression and activity might be involved in physiological regeneration of the tissue, but stable overexpression of *TERT* or clonal expansion of cells with a mutated *TERT* promoter is required for the immortalization of NHU cells ^{13,41,89}. Of note, immortalization of NHU cells is dependent on the fully functional telomerase holoenzyme, as a C-terminal tagged TERT-HA (TERT protein, fused to a short marker peptide sequence incorporating amino acids 98 to 106 of the human influenza hemagglutinin (HA)_protein) restored enzymatic activity but was incapable of inducing immortalization ⁹⁰. [Au:Please explain what TERT-HA is specifically and how this modification changes the enzyme. Does the TERT-HA actually elongate telomeres? How was enzymatic activity measured if not by telomere elongation?] Telomerase-immortalized NHU cells remain genetically stable under standard

growth conditions *in vitro*^{13,91}. However, when cultured under stress-imposing low-density conditions, genomic alterations that are similar to those seen in primary urothelial tumours[Au:specifically urothelial tumours?] can occur, such as loss of chromosomal region 2q⁹¹. In line with the extratelomeric functions of telomerase, forced expression of *TERT* in NHU cells results in loss of differentiation capacity^{13,90} and gene expression changes including overexpression of the polycomb repressor complex (PRC1 and PRC4) components, BMI1 and SIRT1, and down-regulation of multiple PRC targets and genes associated with differentiation⁹⁰).[Au:Please give some examples of what specifically happens.]

[H1]Telomerase and *TERT* promoter mutations

Telomerase activity, determined by the PCR-based telomerase repeated amplification protocol (TRAP) assay, has been found in 80–100% of UCB, but only in 2–5% of specimens from adjacent normal urothelium⁹²⁻⁹⁴. Similarly, telomerase activity was detectable in 62–100% of urinary samples from patients with UCB⁹²⁻⁹⁴. To date, only one study has implicated specific transcription factors in the regulation of telomerase activity in UCB cells⁹⁵. Ectopic expression of the c-MYC oncoprotein activated *TERT* transcription and telomerase activity, whereas its counterpart MAD1 (mitosis arrest deficiency 1) had a repressive effect. Whether c-MYC and MAD1 affect telomerase activity in urothelial cells under physiological conditions is unknown.

Mutations in the promoter of the human *TERT* gene have been found in several cancer types, including UCB, and are the most common non-coding somatic mutations in cancer^{58,96-101}. Of all UCB samples, 55–83% had *TERT* promoter mutations at one of the two positions responsible for maintaining telomerase activity in cancer cells^{44,102-104}. [Au:Add ref#83 also here?] Nine benign proliferative urothelial lesions (cystitis, nephrogenic adenoma, and inverted papilloma) only had the wild-type *TERT* promoter sequence⁴⁴. [Au:OK? How specifically were these samples matched to the cancer samples?] These observations indicate that urothelial cells with *TERT* promoter mutations arise de novo in a wild-type setting and that the mutation provides a selective advantage to the cell. The evidence further indicates that *TERT* promoter mutations have a tissue-specific role in urothelial tumorigenesis, being reported in different histological variants of primary bladder cancer, including small cell carcinoma. In the case of bladder adenocarcinoma, *TERT* promoter mutations were restricted to non-enteric type rather than enteric-type adenocarcinomas (the latter being typically of urachal or metastatic colorectal derivation)^{39,44}. [Au:Please specify: which type are urachal or metastatic colorectal – nonenteric or enteric?] Nevertheless, other tissue-regulatory factors might contribute to telomerase activity, as >90% of upper tract urothelial cancers

(UTUCs) encompassing renal pelvic carcinomas and ureteric cancers have telomerase activity^{105,106}, but only 43% and 19% of these tumours contain the two common *TERT* promoter mutations, respectively¹⁰⁷. These findings suggest that alternative regulatory mechanisms contribute to establishing telomerase activity [Au:OK?] in UTUC compared with UCB, possibly owing to their different embryological derivations. Similarly, a particularly high prevalence of *TERT* promoter mutations (100%) has been reported for micropapillary urothelial cancer¹⁰⁸.

The two key positions for mutations in the *TERT* promoter are C228T and C250T (located at positions -124 and -146, respectively, relative to the ATG start codon)^{100,109-111}. Mutations in the *TERT* coding region are rare (frequency < 0.5%)¹¹², but *TERT* promoter mutations occur in 60-80% of UCB samples and the C228T and C250T mutations together account for 99% of these mutations. Convincing experimental evidence exists that both these mutations create novel binding sites for the heterotetrameric GA-binding protein (GABP) transcription factor, [Au:Are you referring to a specific subunit or the whole factor "GA-binding protein"?] resulting in increased transcription of *TERT* and activation of telomerase^{97,103,113}.

[H1] *TERT* mutations as prognostic markers

Several studies have examined potential associations of *TERT* promoter mutations with UCB stage and grade. The studies report *TERT* promoter mutations in 59 to 79% (mean 70%) UCB irrespective of tumour stage or grade^{44,100,102,114,115}. [Au:What specifically were these rates; could you provide a range of values?] One team of researchers assessed disease-specific survival in correlation with *TERT* promoter mutations and *TERT* mRNA levels in two independent cohorts of chemotherapy-naïve patients ($n = 35$; $n = 87$), finding that the abundance of *TERT* mRNA, rather than promoter mutation itself, correlated strongly with reduced disease-specific survival¹⁰⁴. [Au:Please can you mention the number of patients for context.] They further showed that *TERT* promoter mutations correlated with *TERT* mRNA abundance, telomerase activity, and telomere length, compared with telomerase-positive cells without *TERT* promoter mutations. These observations might also explain the results obtained by another group, who analysed urine samples of 230 patients with non muscle invasive bladder cancer (NMIBC) and 25 samples of patients with muscle invasive bladder (MIBC) cancer. They found *TERT* promoter mutations to be most common mutation detected (52% across of all samples) and showed significant associations between *TERT* promoter mutation and progression of NMIBC to MIBC (9 of 110 patients who progressed had *TERT* promoter mutation versus 1 of 109 patients without). When NMIBC were stratified by risk, the presence of *TERT* promoter mutation was highly associated ($p < 0.0001$)⁴⁰. [Au:Please can you mention the number of patients for context?] Also of note, *TERT* promoter mutations

were found to be associated with distant metastases in UTUC ¹⁰⁷, suggesting a selective advantage. In another study, sequencing of 76 urothelial carcinomas demonstrated *TERT* promoter mutations in both low-grade and high-grade UCB, as well as in flat and papillary lesions ¹¹⁶.**[Au:How does this finding relate to the results by Critelli? Did Kinde not see a difference in abundance depending on UCB grade? Why might this discrepancy exist? Please discuss in the text.]** The same mutations were also detectable in urine and were strongly associated with UCB recurrence, providing a potential prognostic**[Au:OK?]** urinary biomarker. The potential to use *TERT* promoter mutations as a urinary biomarker to detect UCB was explored in a prospective study using samples from 475 patients, which showed that *TERT* promoter mutations had the highest sensitivity (81.8%) but also the lowest specificity (83.5%) (among the markers tested: *TERT*, *FGFR3*, *SALL3*, *ONECUT2*, *CCNA1*, *BCL2*, *EOMES*, and *VIM*) to detect UCB ¹¹⁷.**[Au:Please mention which the other tested markers were for context.]**

[H1]TERT promoter mutations and cancer initiation[Au:Change to conform to character limit OK?]

Mutations that occur in adult stem cells are believed to have the largest effect on the mutational load of tissues, owing to their capacity to give rise to all descendant cells in a tissue ^{118,119}. Stem cell tracing experiments support this idea by showing that mutations that occur in stem cells are efficient cancer drivers, whereas the same mutations in differentiated cells fail to initiate cancer ¹²⁰. This view is supported by reports showing the accumulation of mutations in adult stem cells during life ³⁷. However, stem cells are rare and considered to divide infrequently, giving rise to a rapidly-expanding supra-basal compartment of highly proliferating transit-amplifying cells. In the past 5 years, an alternative cancer cell origin theory has emerged in favour of the transit-amplifying cell population. ¹²¹⁻¹²³.**[Au:We avoid the use of “recently” as it can be interpreted differently by different readers; “In the past 5 years” instead OK? Also, please explain in the text what transit-amplifying cells are.]** This concept takes into account that mutation rates are increased in replicating cells ¹²¹ and that most heritable mutations occur in the transit-amplifying cells that constitute the majority of the stem or progenitor cell pool, rather than in the small number of rarely-dividing adult stem cells (Fig. 3).

The presence and nature of stem cells in human bladder urothelium is still under debate, but studies in animal models indicate that subpopulations of bladder urothelial cells (either P63-positive, SHH-positive, KRT5-positive, or KRT14-positive subpopulations)**[Au:For specificity: Do you indicate three subpopulations (P63+, SHH+, and KRT5+/KRT14+) or four subpopulations (P63+, SHH+, KRT5+, and KRT14+) here?]** confer self-renewal ability and are

considered to constitute the stem or progenitor cell population of the bladder epithelium¹²⁴⁻¹²⁷. In one study in mice, KRT14-positive cells, which are a subpopulation of KRT5-positive cells, gave rise to all cell types of the urothelium during injury-induced regeneration and were regarded as the cells of origin of urothelial cancer¹²⁸. By contrast, studies with mouse and pig urothelial cells suggest that both the basal and intermediate cells can contribute to the regenerative potential of the bladder urothelium, indicating that a stringent requirement for a stem cell population does not apply to bladder urothelium^{88,129}. These studies might be helpful in understanding the contribution of different cell types to bladder regeneration and cancer in different species but, unlike in human somatic tissues, telomerase is constitutively expressed in all mouse tissues^{5,6}. **[Au:Please reference again.]** Thus, irrespective of the stem cell debate, the common occurrence of *TERT* promoter mutations that result in constitutive telomerase activity demonstrates the critical importance of **this tumour suppressor in human UCB.** **[Au:Please can you reformulate here and specify what exactly the tumour suppressive block is in this context?]**

In a series of experiments performed on cell fractions enriched from isolated UCB and normal urothelium, one team of researchers located *TERT* promoter mutations (primarily the C228T mutation) in basal cells (defined as CD44⁺ CK5⁺ CK20⁻) from UCB but not from normal urothelium (normal bladder basal cells; NBBC)¹³⁰. **[Au:Deletion of "lin-" OK, as seemed not required to define cell population?]** Furthermore, **these *TERT* promoter mutations** were enriched in basal compared with non-basal bladder cancer cells. Importantly, restoring the C228T mutation to the wild-type sequence abolished the tumour-forming ability of bladder cancer cells in mouse xenograft experiments. Conversely, basal urothelial cells isolated from **[Au:human?]** non-cancerous adjacent **human** bladder tissue expressed wild-type *TERT* and only developed xenograft tumours in nude mice when the C228T mutation was introduced at the genomic level, suggesting that *TERT* promoter mutation is a crucial event in the malignant transformation of human urothelium¹³⁰. Pending formal demonstration, these data suggest that tumour-adjacent normal bladder urothelial cells have accumulated cancer-initiating genetic mutations that are not sufficient for malignant transformation by themselves but require activation of telomerase as the tumour-promoting step. **[Au:Please excuse if I missed it but does any evidence exist that the tumour-adjacent normal urothelial cells have other mutations already? The reader might wonder whether the *TERT* mutation could not also be sufficient for malignant transformation by itself. Please explain the dependency on other pre-existing mutations in the text. Thank you.]** Of note, it was demonstrated that telomerase is not an oncogene per se (Harley, 2002). One further observation from **the above mentioned** study was that **human** **[Au:human?]** NBBCs had no telomerase activity¹³⁰. This feature differentiates NBBCs from intestinal stem cells and

haematopoietic stem cells, which have a constitutive telomerase activity.**[Au:Please add the values for comparison and reference.]** Consistent with the reports showing transient activation of telomerase in normal urothelial cells in culture ^{11,13},**[Au:Please reference again.]** it seems plausible that NBBCs might be able to up-regulate telomerase**[Au:add “expression”?] expression** transiently in response to regenerative signals.

The implications of these data in the context of cancer-initiating cells require further clarification.**[Au:We avoid open questions in our articles. Reformulated sentence OK?] [Au:”activity” or “expression”; please add.]** The presence or absence of telomerase activity in resting NBBCs has to be demonstrated unequivocally, but current data indicate that these cells are telomerase-negative ¹³⁰. In the absence of telomerase, harmful cancer-initiating mutations, such as oncogene activation (leading to OIS) or aneuploidy-inducing mutations (leading to AIS), would interfere with cell fitness and would induce premature senescence as a tumour suppressor mechanism (Fig. 2).**[Au:Are you referring to OIS and AIS here, which are mediated through the sensing ability of the telomeres specifically? I think the reader needs to be reminded of this connection here. In addition, please do ensure that you describe in more detail how this sensing works at the beginning of the article where I have asked for it. Thank you.]** We therefore suggest that, in UCB, the first tumour-initiating mutation is likely to be a telomerase re-activating mutation (for example, in the *TERT* promoter) in cells that have been activated for proliferation (Fig. 3).**[Au:I think to make this point, you need to ensure that you sufficiently discuss earlier in the text the “activated for proliferation” mutations that already exist in cells. Please add above where I have asked for it.]** Telomerase activity could then facilitate the survival of cells with secondary cancer-promoting mutations**[Au:Do you mean that these cancer-promoting mutations occur after the telomerase activating mutations? Are these then different from the “activated for proliferation” mutations mentioned in the preceding sentence? Please clarify.]** and prevent entry into cellular senescence. The fact that *TERT* promoter mutations occur preferentially or exclusively in tissues that have low or absent telomerase activity in their non-proliferating cells, such as bladder urothelium ^{98,99}.**[Au:Please reference again.]** supports this hypothesis. In fact, high rates of *TERT* promoter mutations have been observed in liver cancer, and the liver is another mitotically quiescent highly regenerative tissue in which the identity of stem cells has not been clarified.**[Au>Edit for clarity OK? Is this also from ref#6, please reference.]** Human hepatocytes are telomerase-negative in the resting state but can up-regulate telomerase activity following stimulatory signals during regeneration ⁷.

In a study published in 2015, researchers introduced the common *TERT* promoter mutations into human embryonic stem cells (hESCs) and demonstrated that these mutations prevented down-regulation of the *TERT* promoter and telomerase activity during differentiation of these

mutant hESCs but not wild-type hESCs¹³¹. These data indicate that *TERT* promoter mutations that occur in stem or progenitor cells prevent differentiation and the shortening of telomeres. **[Au:You seem to only mention that telomerase activity is maintained but do not mention any readouts that relate to differentiation or specifically to telomere shortening. Were the researchers testing for these effects? Please provide further evidence to back up your statement.]** By contrast, the absence of *TERT* promoter mutations in cancers arising from highly proliferative tissues with a stem cell compartment^{38,132}. **[Au:Please add examples and reference.]** might be explained by the inherently high telomerase activity of their stem cells³⁵. This hypothesis assumes that, if the stem cells are the origin of *TERT* promoter mutations, the mutation rate of the *TERT* promoter would be expected to occur at a similar rate in all tissues.

[Au:I feel like this final summary of the proposed mechanisms would benefit immensely from an accompanying Figure. It seems that several different events occur in different sequence in different models. Please could you draft a schematic that summarizes this model?]

In summary, we hypothesize that *TERT* promoter mutations occur in telomerase-negative cells that are activated for proliferation “on demand”. It is known that injury and physiological stimuli can induce telomerase activity in human tissues¹³³⁻¹³⁶. It was also shown that human *TERT* promoter is activated in response to liver injury in a mouse model carrying a human *TERT* promoter fragment in front of the lacZ reporter gene⁷. In the bladder urothelium, it seems plausible to assume that *TERT* promoter mutations occur in proliferating cells that have transiently activated telomerase in response to injury, which is in line with the alternative model of transit-amplifying cells as the cancer-initiating cells with the highest mutation rate (Fig. 3). **[Au:Please excuse if I missed it, but did you discuss evidence that telomerase is transiently activated in response to injury in bladder cells? If so please briefly reiterate here and reference.]** Most notably, no *TERT* promoter mutations could be found in an carcinogen-induced bladder cancer model that otherwise shows a mutational signature similar to human bladder cancer (e.g. Trp53) and recapitulates the molecular alterations of human muscle invasive bladder cancer¹³⁷. The lack of *TERT* promoter mutations in this mouse model can be explained by the constitutive *TERT* gene expression (and constitutive telomerase activity) in mouse tissues as described above³⁻⁷. In light of the novel findings that telomerase can suppress senescence induction caused by genome instability (AIS) or oxidative stress (OIS), **[Au:Please reference. Is this OIS and AIS? Please excuse if I missed it, but I am not sure that you explain these specific findings in detail. Please expand the description of the studies that demonstrate these mechanisms.]** we extend this model to suggest that *TERT* promoter mutations are likely to be the first tumour-initiating mutations in tissues that lack telomerase activity in their non-proliferating cells. Interestingly, the lack of *TERT* promoter mutations in primary bladder adenocarcinoma suggests that this entity might

have a different origin of cancer¹³⁸. This idea might also support the conclusion regarding the cell type of origin for UTUC,**[Au:OK?]** in which a low rate of *TERT* promoter mutations is observed despite activation of telomerase in the vast majority of UTUCs¹⁰⁵⁻¹⁰⁷. In urothelial tumours (of any origin) that lack *TERT* promoter mutations, whether telomerase reactivation is a late event caused by telomere dysfunction initiated genome instability or whether telomerase reactivation occurs early during cellular transformation, potentially promoting tumorigenesis by telomere-length-independent mechanisms, remains to be elucidated. Of note, telomerase reactivation is not the primary tumour-initiating event in these tumour types but might result from loss of negative regulatory factors that suppress *TERT* promoter activity **[Au:“activity”?]** in normal cells and/or from an aberrant**[Au:as in “increased”?]** increased expression of factors that positively regulate *TERT* gene expression⁴³.

[H1] Opportunities in diagnosis and therapy

The high frequency of specific *TERT* gene promoter mutations across all UCB grades and stages and its absence from adjacent histologically normal urothelium indicates an important function in both neoplastic transformation and maintenance of UCB^{44,100,102,114,115}. **[Au:Please reference again.]** This finding has led several groups to examine whether detection of *TERT* promoter mutations could be a urinary marker for bladder cancer detection¹⁰². A non-invasive liquid biopsy approach is highly attractive¹³⁹, as it might replace costly long-term cystoscopic surveillance for recurrence of low-grade non-invasive UCB and function as a screening tool for detecting UCB (particularly early detection of MIBC) in high-risk groups^{117,140}. *TERT* promoter mutation detected in urine might not only be indicative of UCB but also of UTUC or renal cell carcinoma, although *TERT* promoter mutation rates in these tumour types are lower than in UCB^{107,141}. **[Au:Comparator added OK?]** One retrospective study has indicated that urinary *TERT* promoter mutation detection is not prognostic of UCB clinical outcome¹⁰², but it might be a suitable marker for monitoring of disease recurrence¹⁰⁴. **[Au:Please move ref of study showing no prognostication to before the comma.]** However, large prospective studies are awaited to determine the value of any clinical test on the basis of *TERT* promoter mutation detection¹⁴².

The presence of telomerase activity in most human cancers, along with mouse model data indicating its requirement for tumour progression, has inspired the development of telomerase inhibitors and their testing in preclinical studies^{19,143-148}. UCB is a good candidate for telomerase-based therapies, as most UCBs are telomerase-positive. The first generation of inhibitors targeted telomerase activity itself, for example by stabilising the G-quadruplex⁴⁵. The first of these inhibitors to enter phase II trials, imetelstat, showed promising clinical

activity against myeloproliferative neoplasms rather than solid tumours¹⁴⁹⁻¹⁵¹. [Au: I am not sure that this study tested imetelstat also in solid tumours, which you currently seem to indicate. Please clarify. Was imetelstat tested in different tumour types in another study?] This finding indicates the need to identify novel targets on the basis of the specific molecular understanding of telomeric and cancer-associated extra-telomeric functions of telomerase in UCB³⁴.

Alternative targeting of telomerase activity has been investigated with the nucleoside analogue 6-thio-2'-deoxyguanosine. This compound is a telomerase substrate precursor that becomes incorporated into telomeric DNA by telomerase during DNA replication, resulting in rapid telomere deprotection and apoptosis in telomerase-positive cancer cell lines originating from colon, (HCT116), from lung (A549) or liver (HCC15) while telomerase negative primary human fibroblasts (BJ) or telomerase-negative but ALT-positive immortal human fibroblast (AV13) were resistant to the treatment^{152,153}. [Au: Which cells (tumour type) specifically? Also, please explain a bit more what telomere deprotection is. Thank you.] The protein components of the telomere-protective shelterin complex, a complex of six protein components (telomeric repeat-binding factor 1 and 2 (TERF1 and TERF2, also known as TRF1 and TRF2), TRF2-interacting protein 1 (TRF2IP, also known as RAP1), TRF1-interacting nuclear factor 2 (TINF2, also known as TIN2), protection of telomeres protein 1 (POT1), and tripeptidyl-peptidase 1 (TPP1)) that specifically associate with telomeres, also offer alternative targets. These shelterin components protect telomere length and structure, partly by regulating telomerase activity and access to the telomeric DNA¹⁵⁴. In cells with dysfunctional telomeres, chromosome ends are no longer protected efficiently by the shelterin components. These unprotected telomeres resemble chromosome breaks and activate the DNA-damage response¹⁵⁵⁻¹⁵⁷. Furthermore, they are prone to degradation by exonucleases and lose their capping function to protect from end-to-end fusions or loss of genetic material. This process of telomere-uncapping and de-protection can be induced by progressive telomere shortening in the absence of sufficient telomerase activity as well as by mutant or genetically-modified shelterin proteins, resulting in activation of p53-dependent senescence and/or apoptotic responses¹⁵⁸. *PINX1* is of particular relevance in UCB, as it encodes a shelterin-recruited telomerase-regulatory protein and displays single-nucleotide polymorphisms that are associated with significantly reduced bladder cancer risk¹⁵⁹⁻¹⁶¹. [Au: Journal style is to use "significant" only in a statistical context. Please add the P value or change to e.g. "substantially" OK?] Hence, specific targeting of shelterin components or telomerase-associated proteins could be a useful strategy to modulate telomerase activity for UCB therapy.

In addition, evidence of non-canonical, extra-telomeric functions of telomerase and their utility as treatment targets is accumulating. As an example, eribulin, an anticancer drug that interferes with microtubule elongation, inhibits the RNA-dependent RNA-polymerase activity of telomerase¹⁶². Further understanding of the non-canonical roles of telomerase has potential for developing new cancer-specific therapeutics.

As the prevalence of the ALT mechanism is low or absent in UCB, telomerase targeting can be considered a promising first-line strategy. However, secondary activation of the ALT mechanism is a potential drawback of any telomerase-inhibiting therapy. Studies in a lymphoma-prone mouse model have shown that deletion of telomerase in tumours provoked activation of ALT in cancer cells as an adaptive response¹⁶³. [Au:Which tumours specifically?] Whether ALT activation is of concern in [Au:“human”?] human UCB is not yet clear, but research on the potential telomere-length-independent functions of telomerase gives reason to hope that telomerase inhibition might result in a telomere-length-independent senescence response that protects from tumorigenesis without activating the ALT mechanism. In the meantime, ALT itself remains a potential target. [Au:I've changed “proleptic” to “potential”, as I'm not sure all readers would understand. Is this the meaning you had in mind?]

[H1]Conclusions

The high prevalence of telomerase activity in UCB and identification of two hot-spot mutations within the *TERT* promoter region as the most frequent genetic alteration across all disease stages indicate a very strong selection pressure for telomerase activation during neoplastic transformation. Three conclusions can be drawn from our current understanding of telomerase function in the initiation and maintenance of UCB and the prospects for developing cancer-specific therapies. First, *TERT* promoter mutations resulting in telomerase overactivity occur in telomerase-negative cells that reside in tissues without a well-defined telomerase-positive stem cell compartment. Notably, a potential transient function of telomerase in the regenerative response of urothelium remains an open question. Secondly, the observations that *TERT* promoter mutations are detectable in all stages of UCB, but not overtly normal urothelium, indicate that telomerase activation provides a survival advantage during the initial stages of urothelial tumorigenesis. This notion is in line with the non-canonical functions of telomerase in the abrogation of AIS and/or suppression of OIS. Thus, in the absence of telomerase, harmful cancer-initiating mutations, such as oncogene activation or aneuploidy-inducing mutations, would interfere with cell fitness and induce premature senescence as a tumour suppressor mechanism through sensing by telomeres. [Au:through sensing by telomeres, or also alternative mechanism? Please clarify.] By contrast, urothelial cells that acquire telomerase-activating *TERT* promoter mutations

obtain a survival advantage. This insight might lead to new strategies for cancer therapy. Finally, on the basis of the alternative cancer cell origin theory which suggests the transit-amplifying cells as the cell of origin of cancer, as described above^{122,123}, [Au:Instead of mentioning the researcher names, please refer to (explain) this model as you have done in the main text above, so that the reader immediately knows what you are referring to.] *TERT* mutations should occur in proliferating rather than in mitotically quiescent urothelial cells. These cells with *TERT* promoter mutations [Au:“with *TERT* mutations”?] are probably the cancer-initiating cells, which then accumulate additional oncogenic mutations during UCB progression. Consequently, detection of *TERT* promoter mutations in body fluids might be an early marker of UCB and potentially for early cancer therapy by targeting telomerase.

Competing interests statement

The authors declare no competing interests.

References

- 1 Vogan, J. M. & Collins, K. Dynamics of Human Telomerase Holoenzyme Assembly and Subunit Exchange across the Cell Cycle. *J Biol Chem* **290**, 21320-21335, doi:10.1074/jbc.M115.659359 (2015).
- 2 Bailey, S. M. & Murnane, J. P. Telomeres, chromosome instability and cancer. *Nucleic Acids Res* **34**, 2408-2417, doi:10.1093/nar/gkl303 (2006).
- 3 Chadeneau, C., Hay, K., Hirte, H. W., Gallinger, S. & Bacchetti, S. Telomerase Activity Associated with Acquisition of Malignancy in Human Colorectal-Cancer. *Cancer Res* **55**, 2533-2536 (1995).
- 4 Greenberg, R. A., Allsopp, R. C., Chin, L., Morin, G. B. & DePinho, R. A. Expression of mouse telomerase reverse transcriptase during development, differentiation and proliferation. *Oncogene* **16**, 1723-1730, doi:DOI 10.1038/sj.onc.1201933 (1998).
- 5 Prowse, K. R. & Greider, C. W. Developmental and Tissue-Specific Regulation of Mouse Telomerase and Telomere Length. *P Natl Acad Sci USA* **92**, 4818-4822, doi:DOI 10.1073/pnas.92.11.4818 (1995).
- 6 Ritz, J. M. *et al.* A novel transgenic mouse model reveals humanlike regulation of an 8-kbp human TERT gene promoter fragment in normal and tumor tissues. *Cancer Res* **65**, 1187-1196, doi:10.1158/0008-5472.CAN-04-3046 (2005).
- 7 Sirma, H. *et al.* The Promoter of Human Telomerase Reverse Transcriptase Is Activated During Liver Regeneration and Hepatocyte Proliferation. *Gastroenterology* **141**, 326-U434, doi:10.1053/j.gastro.2011.03.047 (2011).
- 8 Gunes, C., Lichtsteiner, S., Vasserot, A. P. & Englert, C. Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1. *Cancer Res* **60**, 2116-2121 (2000).
- 9 Kilian, A. *et al.* Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet* **6**, 2011-2019, doi:DOI 10.1093/hmg/6.12.2011 (1997).
- 10 Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W. & Shay, J. W. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* **18**, 173-179 (1996).
- 11 Belair, C. D., Yeager, T. R., Lopez, P. M. & Reznikoff, C. A. Telomerase activity: A biomarker of cell proliferation, not malignant transformation. *P Natl Acad Sci USA* **94**, 13677-13682, doi:DOI 10.1073/pnas.94.25.13677 (1997).
- 12 Chiu, C. P. *et al.* Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells* **14**, 239-248 (1996).
- 13 Georgopoulos, N. T. *et al.* Immortalisation of Normal Human Urothelial Cells Compromises Differentiation Capacity. *Eur Urol* **60**, 141-149, doi:10.1016/j.eururo.2011.02.022 (2011).
- 14 Hiyama, E. *et al.* Telomerase activity in human intestine. *Int J Oncol* **9**, 453-458 (1996).
- 15 Hiyama, K. *et al.* Activation of Telomerase in Human-Lymphocytes and Hematopoietic Progenitor Cells. *J Immunol* **155**, 3711-3715 (1995).
- 16 Morrison, S. J., Prowse, K. R., Ho, P. & Weissman, I. L. Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity* **5**, 207-216, doi:Doi 10.1016/S1074-7613(00)80316-7 (1996).
- 17 Ramirez, R. D., Wright, W. E., Shay, J. W. & Taylor, R. S. Telomerase activity concentrates in the mitotically active segments of human hair follicles. *J Invest Dermatol* **108**, 113-117, doi:DOI 10.1111/1523-1747.ep12285654 (1997).
- 18 Ravindranath, N., Dalal, R., Solomon, B., Djakiew, D. & Dym, M. Loss of telomerase activity during male germ cell differentiation. *Endocrinology* **138**, 4026-4029, doi:DOI 10.1210/en.138.9.4026 (1997).
- 19 Weise, J. M. & Gunes, C. Telomeres and telomerase. A survey about methods and recent advances in cancer diagnostic and therapy. *Histol Histopathol* **21**, 1249-1261 (2006).
- 20 Harley, C. B., Futcher, A. B. & Greider, C. W. Telomeres Shorten during Aging of Human Fibroblasts. *Nature* **345**, 458-460, doi:DOI 10.1038/345458a0 (1990).
- 21 Shay, J. W., Pereirasmith, O. M. & Wright, W. E. A Role for Both Rb and P53 in the Regulation of Human Cellular Senescence. *Exp Cell Res* **196**, 33-39, doi:Doi 10.1016/0014-4827(91)90453-2 (1991).
- 22 Allsopp, R. C. *et al.* Telomere Length Predicts Replicative Capacity of Human Fibroblasts. *P Natl Acad Sci USA* **89**, 10114-10118, doi:DOI 10.1073/pnas.89.21.10114 (1992).
- 23 Chang, E. & Harley, C. B. Telomere Length and Replicative Aging in Human Vascular Tissues. *P Natl Acad Sci USA* **92**, 11190-11194, doi:DOI 10.1073/pnas.92.24.11190 (1995).
- 24 Artandi, S. E. *et al.* Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* **406**, 641-645 (2000).

- 25 Chin, L. *et al.* p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* **97**, 527-538, doi:Doi 10.1016/S0092-8674(00)80762-X (1999).
- 26 Hackett, J. A., Feldser, D. M. & Greider, C. W. Telomere dysfunction increases mutation rate and genomic instability. *Cell* **106**, 275-286, doi:Doi 10.1016/S0092-8674(01)00457-3 (2001).
- 27 O'Hagan, R. C. *et al.* Telomere dysfunction provokes regional amplification and deletion in cancer genomes. *Cancer Cell* **2**, 149-155, doi:Doi 10.1016/S1535-6108(02)00094-6 (2002).
- 28 Kim, N. W. *et al.* Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. *Science* **266**, 2011-2015, doi:DOI 10.1126/science.7605428 (1994).
- 29 Meyerson, M. *et al.* hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90**, 785-795, doi:Doi 10.1016/S0092-8674(00)80538-3 (1997).
- 30 Hahn, W. C. *et al.* Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464-468, doi:10.1038/22780 (1999).
- 31 Bryan, T. M., Englezou, A., DallaPozza, L., Dunham, M. A. & Reddel, R. R. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* **3**, 1271-1274, doi:DOI 10.1038/nm1197-1271 (1997).
- 32 Londono-Vallejo, J. A., Der-Sarkissian, H., Cazes, L., Bacchetti, S. & Reddel, R. R. Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. *Cancer Res* **64**, 2324-2327, doi:Doi 10.1158/0008-5472.Can-03-4035 (2004).
- 33 Heaphy, C. M. *et al.* Altered Telomeres in Tumors with ATRX and DAXX Mutations. *Science* **333**, 425-425, doi:10.1126/science.1207313 (2011).
- 34 Arndt, G. M. & MacKenzie, K. L. New prospects for targeting telomerase beyond the telomere. *Nat Rev Cancer* **16**, 508-524, doi:10.1038/nrc.2016.55 (2016).
- 35 Bell, R. J. A. *et al.* Understanding TERT Promoter Mutations: A Common Path to Immortality. *Mol Cancer Res* **14**, 315-323, doi:10.1158/1541-7786.Mcr-16-0003 (2016).
- 36 Beroukhi, R. *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* **463**, 899-905, doi:10.1038/nature08822 (2010).
- 37 Blokzijl, F. *et al.* Tissue-specific mutation accumulation in human adult stem cells during life. *Nature* **538**, 260+, doi:10.1038/nature19768 (2016).
- 38 Carcano, F. M. *et al.* Hotspot TERT promoter mutations are rare events in testicular germ cell tumors. *Tumor Biol* **37**, 4901-4907, doi:10.1007/s13277-015-4317-y (2016).
- 39 Cowan, M. L. *et al.* Detection of TERT promoter mutations in primary adenocarcinoma of the urinary bladder. *Hum Pathol* **53**, 8-13, doi:10.1016/j.humpath.2016.02.009 (2016).
- 40 Critelli, R. *et al.* Detection of multiple mutations in urinary exfoliated cells from male bladder cancer patients at diagnosis and during follow-up. *Oncotarget* **7**, 67435-67448, doi:10.18632/oncotarget.11883 (2016).
- 41 Hoffmann, M. J. K., E.; Skowron, M.A.; Pinkerleil, M.; Niegisch, G.; Brandt, A.; Stepanow, S.; Rieder, H.; Schulz, W.A. . The New Immortalized Uroepithelial Cell Line HBLAK Contains Defined Genetic Aberrations Typical of Early Stage Urothelial Tumors. *Bladder Cancer* **27**, 449-463 (2016).
- 42 Hu, Y. *et al.* Switch telomerase to ALT mechanism by inducing telomeric DNA damages and dysfunction of ATRX and DAXX. *Sci Rep-Uk* **6**, doi:ARTN 3228010.1038/srep32280 (2016).
- 43 Kumar, M., Lechel, A. & Gunes, C. Telomerase: The Devil Inside. *Genes-Basel* **7**, doi:ARTN 4310.3390/genes7080043 (2016).
- 44 Kurtis, B. *et al.* Recurrent TERT promoter mutations in urothelial carcinoma and potential clinical applications. *Ann Diagn Pathol* **21**, 7-11, doi:10.1016/j.anndiagpath.2015.12.002 (2016).
- 45 Man, R. J., Chen, L. W. & Zhu, H. L. Telomerase inhibitors: a patent review (2010-2015). *Expert Opin Ther Pat* **26**, 679-688, doi:10.1080/13543776.2016.1181172 (2016).
- 46 Patel, P. L., Suram, A., Mirani, N., Bischof, O. & Herbig, U. Derepression of hTERT gene expression promotes escape from oncogene-induced cellular senescence. *P Natl Acad Sci USA* **113**, E5024-E5033, doi:10.1073/pnas.1602379113 (2016).
- 47 Plantinga, M. J. *et al.* Telomerase Suppresses Formation of ALT-Associated Single-Stranded Telomeric C-Circles. *Mol Cancer Res* **11**, 557-567, doi:10.1158/1541-7786.Mcr-13-0013 (2013).
- 48 Heaphy, C. M. *et al.* Prevalence of the Alternative Lengthening of Telomeres Telomere Maintenance Mechanism in Human Cancer Subtypes. *Am J Pathol* **179**, 1608-1615, doi:10.1016/j.ajpath.2011.06.018 (2011).

- 49 Hakin-Smith, V. *et al.* Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. *Lancet* **361**, 836-838, doi:Doi 10.1016/S0140-6736(03)12681-5 (2003).
- 50 Henson, J. D. *et al.* A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. *Clin Cancer Res* **11**, 217-225 (2005).
- 51 Henson, J. D. & Reddel, R. R. Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers. *Febs Lett* **584**, 3800-3811, doi:10.1016/j.febslet.2010.06.009 (2010).
- 52 Choi, J. K. *et al.* TERT promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program. *Plos Genet* **4**, doi:ARTN e1010.1371/journal.pgen.0040010 (2008).
- 53 Gazzaniga, F. S. & Blackburn, E. H. An antiapoptotic role for telomerase RNA in human immune cells independent of telomere integrity or telomerase enzymatic activity. *Blood* **124**, 3675-3684, doi:10.1182/blood-2014-06-582254 (2014).
- 54 Haendeler, J. *et al.* Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage. *Arterioscl Throm Vas* **29**, 929-U400, doi:10.1161/Atvbaha.109.185546 (2009).
- 55 Jin, X. *et al.* Human telomerase catalytic subunit (hTERT) suppresses p53-mediated anti-apoptotic response via induction of basic fibroblast growth factor. *Exp Mol Med* **42**, 574-582, doi:10.3858/emm.2010.42.8.058 (2010).
- 56 Kedde, M. *et al.* Telomerase-independent regulation of ATR by human telomerase RNA. *J Biol Chem* **281**, 40503-40514, doi:10.1074/jbc.M607676200 (2006).
- 57 Listerman, I., Gazzaniga, F. S. & Blackburn, E. H. An Investigation of the Effects of the Core Protein Telomerase Reverse Transcriptase on Wnt Signaling in Breast Cancer Cells. *Mol Cell Biol* **34**, 280-289, doi:10.1128/Mcb.00844-13 (2014).
- 58 Liu, X. L. *et al.* Highly prevalent TERT promoter mutations in aggressive thyroid cancers. *Endocr-Relat Cancer* **20**, 603-610, doi:10.1530/Erc-13-0210 (2013).
- 59 Park, J. I. *et al.* Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature* **460**, 66-U77, doi:10.1038/nature08137 (2009).
- 60 Singhapol, C. *et al.* Mitochondrial Telomerase Protects Cancer Cells from Nuclear DNA Damage and Apoptosis. *Plos One* **8**, doi:ARTN e5298910.1371/journal.pone.0052989 (2013).
- 61 Zhu, H. Y., Fu, W. M. & Mattson, M. P. The catalytic subunit of telomerase protects neurons against amyloid beta-peptide-induced apoptosis. *J Neurochem* **75**, 117-124, doi:DOI 10.1046/j.1471-4159.2000.0750117.x (2000).
- 62 Sarin, K. Y. *et al.* Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature* **436**, 1048-1052, doi:10.1038/nature03836 (2005).
- 63 Strong, M. A. *et al.* Phenotypes in mTERT(+/-) and mTERT(-/-) Mice Are Due to Short Telomeres, Not Telomere-Independent Functions of Telomerase Reverse Transcriptase. *Mol Cell Biol* **31**, 2369-2379, doi:10.1128/Mcb.05312-11 (2011).
- 64 Ghosh, A. *et al.* Telomerase directly regulates NF-kappa B-dependent transcription. *Nat Cell Biol* **14**, 1270+, doi:10.1038/ncb2621 (2012).
- 65 Gonzalez, O. G. *et al.* Telomerase stimulates ribosomal DNA transcription under hyperproliferative conditions. *Nature communications* **5**, 4599, doi:10.1038/ncomms5599 (2014).
- 66 Hewitt, G. *et al.* Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nature communications* **3**, 708, doi:10.1038/ncomms1708 (2012).
- 67 Meena, J. K. *et al.* Telomerase abrogates aneuploidy-induced telomere replication stress, senescence and cell depletion. *Embo J* **34**, 1371-1384, doi:10.15252/emboj.201490070 (2015).
- 68 Suram, A. *et al.* Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. *Embo J* **31**, 2839-2851, doi:10.1038/emboj.2012.132 (2012).
- 69 Meena, J. K. R., K.L.; Günes, C. Telomere Dysfunction, Chromosomal Instability and Cancer. *Recent Results Cancer Res* **200**, 61-79, doi:10.1007/978-3-319-20291-4_3.
- 70 Suram, A. *et al.* Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. *Embo J* **31**, 2839-2851, doi:10.1038/emboj.2012.132 (2012).
- 71 Gellert, M., Lipsett, M. N. & Davies, D. R. Helix Formation by Guanylic Acid. *P Natl Acad Sci USA* **48**, 2013-&, doi:DOI 10.1073/pnas.48.12.2013 (1962).
- 72 Parkinson, G. N., Lee, M. P. H. & Neidle, S. Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature* **417**, 876-880, doi:DOI 10.1038/nature755 (2002).

- 73 Sen, D. & Gilbert, W. Formation of Parallel 4-Stranded Complexes by Guanine-Rich Motifs in DNA and Its Implications for Meiosis. *Nature* **334**, 364-366, doi:DOI 10.1038/334364a0 (1988).
- 74 Biffi, G., Tannahill, D., McCafferty, J. & Balasubramanian, S. Quantitative visualization of DNA G-quadruplex structures in human cells. *Nat Chem* **5**, 182-186, doi:10.1038/Nchem.1548 (2013).
- 75 Mohaghegh, P., Karow, J. K., Brosh, R. M., Bohr, V. A. & Hickson, I. D. The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. *Nucleic Acids Res* **29**, 2843-2849, doi:DOI 10.1093/nar/29.13.2843 (2001).
- 76 Fletcher, T. M., Sun, D. K., Salazar, M. & Hurley, L. H. Effect of DNA secondary structure on human telomerase activity. *Biochemistry-Us* **37**, 5536-5541, doi:DOI 10.1021/bi972681p (1998).
- 77 Gunes, C. & Rudolph, K. L. The Role of Telomeres in Stem Cells and Cancer. *Cell* **152**, 390-393, doi:10.1016/j.cell.2013.01.010 (2013).
- 78 Kan, Z. Y. *et al.* G-quadruplex formation in human telomeric (TTAGGG)₄ sequence with complementary strand in close vicinity under molecularly crowded condition. *Nucleic Acids Res* **35**, 3646-3653, doi:10.1093/nar/gkm203 (2007).
- 79 Sfeir, A. *et al.* Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. *Cell* **138**, 90-103, doi:10.1016/j.cell.2009.06.021 (2009).
- 80 Tang, J. *et al.* G-quadruplex preferentially forms at the very 3' end of vertebrate telomeric DNA. *Nucleic Acids Res* **36**, 1200-1208, doi:10.1093/nar/gkm1137 (2008).
- 81 Zhou, W. J. *et al.* G-quadruplex ligand SYUIQ-5 induces autophagy by telomere damage and TRF2 delocalization in cancer cells. *Mol Cancer Ther* **8**, 3203-3213, doi:10.1158/1535-7163.Mct-09-0244 (2009).
- 82 Zimmermann, M., Kibe, T., Kabir, S. & de Lange, T. TRF1 negotiates TTAGGG repeat-associated replication problems by recruiting the BLM helicase and the TPP1/POT1 repressor of ATR signaling. *Gene Dev* **28**, 2477-2491, doi:10.1101/gad.251611.114 (2014).
- 83 Chang, M. *et al.* Telomerase is essential to alleviate pif1-induced replication stress at telomeres. *Genetics* **183**, 779-791, doi:10.1534/genetics.109.107631 (2009).
- 84 Hewitt, G. *et al.* Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nature communications* **3**, doi:ARTN 70810.1038/ncomms1708 (2012).
- 85 Ferguson, L. R. *et al.* Genomic instability in human cancer: Molecular insights and opportunities for therapeutic attack and prevention through diet and nutrition. *Seminars in cancer biology* **35 Suppl**, S5-24, doi:10.1016/j.semcancer.2015.03.005 (2015).
- 86 Lavelle, J. *et al.* Bladder permeability barrier: recovery from selective injury of surface epithelial cells. *Am J Physiol-Renal* **283**, F242-F253, doi:10.1152/ajprenal.00307.2001 (2002).
- 87 Varley, C. *et al.* Autocrine regulation of human urothelial cell proliferation and migration during regenerative responses in vitro. *Exp Cell Res* **306**, 216-229, doi:10.1016/j.yexer.2005.02.004 (2005).
- 88 Wezel, F., Pearson, J. & Southgate, J. Plasticity of In Vitro-Generated Urothelial Cells for Functional Tissue Formation. *Tissue Eng Pt A* **20**, 1358-1368, doi:10.1089/ten.tea.2013.0394 (2014).
- 89 Chapman, E. J. *et al.* Expression of hTERT immortalises normal human urothelial cells without inactivation of the p16/Rb pathway. *Oncogene* **25**, 5037-5045, doi:10.1038/sj.onc.1209513 (2006).
- 90 Chapman, E. J., Kelly, G. & Knowles, M. A. Genes involved in differentiation, stem cell renewal, and tumorigenesis are modulated in telomerase-immortalized human urothelial cells. *Mol Cancer Res* **6**, 1154-1168, doi:10.1158/1541-7786.Mcr-07-2168 (2008).
- 91 Chapman, E. J. *et al.* Integrated Genomic and Transcriptional Analysis of the In Vitro Evolution of Telomerase-Immortalized Urothelial Cells (TERT-NHUC). *Gene Chromosome Canc* **48**, 694-710, doi:10.1002/gcc.20672 (2009).
- 92 Kyo, S., Kunimi, K., Uchibayashi, T., Namiki, M. & Inoue, M. Telomerase activity in human urothelial tumors. *Am J Clin Pathol* **107**, 555-560 (1997).
- 93 Mayfield, M. P., Shah, T., Flannigan, G. M., Stewart, P. A. H. & Bibby, M. C. Telomerase activity in malignant and benign bladder conditions. *Int J Mol Med* **1**, 835-840 (1998).
- 94 Yoshida, K. *et al.* Telomerase activity in bladder carcinoma and its implication for noninvasive diagnosis by detection of exfoliated cancer cells in urine. *Cancer* **79**, 362-369, doi:Doi 10.1002/(Sici)1097-0142(19970115)79:2<362::Aid-Cncr20>3.0.Co;2-Y (1997).
- 95 Zou, L., Zhang, P., Luo, C. L. & Tu, Z. G. Mad1 suppresses bladder cancer cell proliferation by inhibiting human telomerase reverse transcriptase transcription and telomerase activity. *Urology* **67**, 1335-1340, doi:10.1016/j.urology.2005.12.029 (2006).
- 96 Horn, S. *et al.* TERT Promoter Mutations in Familial and Sporadic Melanoma. *Science* **339**, 959-961, doi:10.1126/science.1230062 (2013).

- 97 Huang, D. S. *et al.* Recurrent TERT promoter mutations identified in a large-scale study of multiple tumour types are associated with increased TERT expression and telomerase activation. *Eur J Cancer* **51**, 969-976, doi:10.1016/j.ejca.2015.03.010 (2015).
- 98 Killela, P. J. *et al.* TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *P Natl Acad Sci USA* **110**, 6021-6026, doi:10.1073/pnas.1303607110 (2013).
- 99 Nault, J. C. *et al.* High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions (vol 4, 2218, 2013). *Nature communications* **4**, doi:ARTN 257710.1038/ncomms3577 (2013).
- 100 Vinagre, J. *et al.* Frequency of TERT promoter mutations in human cancers. *Nature communications* **4**, doi:ARTN 218510.1038/ncomms3185 (2013).
- 101 Vinagre, J. *et al.* Telomerase promoter mutations in cancer: an emerging molecular biomarker? *Virchows Arch* **465**, 119-133, doi:10.1007/s00428-014-1608-4 (2014).
- 102 Allory, Y. *et al.* Telomerase Reverse Transcriptase Promoter Mutations in Bladder Cancer: High Frequency Across Stages, Detection in Urine, and Lack of Association with Outcome. *Eur Urol* **65**, 360-366, doi:10.1016/j.eururo.2013.08.052 (2014).
- 103 Bell, R. J. A. *et al.* GABP selectively binds and activates the mutant TERT promoter across multiple cancer types. *Cancer Res* **75**, doi:10.1158/1538-7445.Brain15-B12 (2015).
- 104 Borah, S. *et al.* TERT promoter mutations and telomerase reactivation in urothelial cancer. *Science* **347**, 1006-1010, doi:10.1126/science.1260200 (2015).
- 105 Wu, S. *et al.* Telomerase Reverse Transcriptase Gene Promoter Mutations Help Discern the Origin of Urogenital Tumors: A Genomic and Molecular Study. *Eur Urol* **65**, 274-277, doi:10.1016/j.eururo.2013.10.038 (2014).
- 106 Wu, W. J., Liu, L. T., Huang, C. N., Huang, C. H. & Chang, L. L. The clinical implications of telomerase activity in upper tract urothelial cancer and washings. *Bju Int* **86**, 213-219, doi:DOI 10.1046/j.1464-410x.2000.00830.x (2000).
- 107 Wang, K. *et al.* TERT promoter mutations are associated with distant metastases in upper tract urothelial carcinomas and serve as urinary biomarkers detected by a sensitive castPCR. *Oncotarget* **5**, 12428-12439 (2014).
- 108 Nguyen, D. *et al.* High prevalence of TERT promoter mutations in micropapillary urothelial carcinoma. *Virchows Arch* **469**, 427-434, doi:10.1007/s00428-016-2001-2 (2016).
- 109 Melton, C., Reuter, J. A., Spacek, D. V. & Snyder, M. Recurrent somatic mutations in regulatory regions of human cancer genomes. *Nat Genet* **47**, 710+, doi:10.1038/ng.3332 (2015).
- 110 Totoki, Y. *et al.* Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. *Nat Genet* **46**, 1267-1273, doi:10.1038/ng.3126 (2014).
- 111 Weinhold, N., Jacobsen, A., Schultz, N., Sander, C. & Lee, W. Genome-wide analysis of noncoding regulatory mutations in cancer. *Nat Genet* **46**, 1160-1165, doi:10.1038/ng.3101 (2014).
- 112 Weinstein, J. N. *et al.* The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet* **45**, 1113-1120, doi:10.1038/ng.2764 (2013).
- 113 Stern, J. L., Theodorescu, D., Vogelstein, B., Papadopoulos, N. & Cech, T. R. Mutation of the TERT promoter, switch to active chromatin, and monoallelic TERT expression in multiple cancers. *Gene Dev* **29**, 2219-2224, doi:10.1101/gad.269498.115 (2015).
- 114 Hurst, C. D., Platt, F. M. & Knowles, M. A. TERT promoter mutations are highly prevalent in bladder cancer and represent a potential new urinary biomarker. *Cancer Res* **74**, doi:10.1158/1538-7445.Am2014-2240 (2014).
- 115 Rachakonda, P. S. *et al.* TERT promoter mutations in bladder cancer affect patient survival and disease recurrence through modification by a common polymorphism. *P Natl Acad Sci USA* **110**, 17426-17431, doi:10.1073/pnas.1310522110 (2013).
- 116 Kinde, I. *et al.* TERT Promoter Mutations Occur Early in Urothelial Neoplasia and Are Biomarkers of Early Disease and Disease Recurrence in Urine. *Cancer Res* **73**, 7162-7167, doi:10.1158/0008-5472.Can-13-2498 (2013).
- 117 Dahmcke, C. M. *et al.* A Prospective Blinded Evaluation of Urine-DNA Testing for Detection of Urothelial Bladder Carcinoma in Patients with Gross Hematuria. *Eur Urol* **70**, 916-919, doi:10.1016/j.eururo.2016.06.035 (2016).
- 118 Rossi, D. J., Jamieson, C. H. M. & Weissman, I. L. Stems cells and the pathways to aging and cancer. *Cell* **132**, 681-696, doi:10.1016/j.cell.2008.01.036 (2008).

- 119 Tomasetti, C. & Vogelstein, B. Variation in cancer risk among tissues can be explained by the number
of stem cell divisions. *Science* **347**, 78-81, doi:10.1126/science.1260825 (2015).
- 120 Barker, N. *et al.* Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* **457**, 608-U119,
doi:10.1038/nature07602 (2009).
- 121 Lynch, M. Rate, molecular spectrum, and consequences of human mutation. *P Natl Acad Sci USA* **107**,
961-968, doi:10.1073/pnas.0912629107 (2010).
- 122 Chaffer, C. L. & Weinberg, R. A. How Does Multistep Tumorigenesis Really Proceed? *Cancer Discov* **5**,
22-24, doi:10.1158/2159-8290.Cd-14-0788 (2015).
- 123 Schwitalla, S. *et al.* Intestinal Tumorigenesis Initiated by Dedifferentiation and Acquisition of Stem-
Cell-like Properties. *Cell* **152**, 25-38, doi:10.1016/j.cell.2012.12.012 (2013).
- 124 Gandhi, D. *et al.* Retinoid Signaling in Progenitors Controls Specification and Regeneration of the
Urothelium. *Dev Cell* **26**, 469-482, doi:10.1016/j.devcel.2013.07.017 (2013).
- 125 Pignon, J. C. *et al.* p63-expressing cells are the stem cells of developing prostate, bladder, and
colorectal epithelia. *P Natl Acad Sci USA* **110**, 8105-8110, doi:10.1073/pnas.1221216110 (2013).
- 126 Shin, K. *et al.* Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in
bladder. *Nature* **472**, 110-U145, doi:10.1038/nature09851 (2011).
- 127 Van Batavia, J. *et al.* Bladder cancers arise from distinct urothelial sub-populations. *Nat Cell Biol* **16**,
982-991, doi:10.1038/ncb3038 (2014).
- 128 Papafotiou, G. *et al.* KRT14 marks a subpopulation of bladder basal cells with pivotal role in
regeneration and tumorigenesis. *Nature communications* **7**, doi:ARTN 1191410.1038/ncomms11914
(2016).
- 129 Colopy, S. A., Bjorling, D. E., Mulligan, W. A. & Bushman, W. A Population of Progenitor Cells in the
Basal and Intermediate Layers of the Murine Bladder Urothelium Contributes to Urothelial
Development and Regeneration. *Dev Dynam* **243**, 988-998, doi:10.1002/Dvdy.24143 (2014).
- 130 Li, C. *et al.* The C228T mutation of TERT promoter frequently occurs in bladder cancer stem cells and
contributes to tumorigenesis of bladder cancer. *Oncotarget* **6**, 19542-19551 (2015).
- 131 Chiba, K. *et al.* Cancer-associated TERT promoter mutations abrogate telomerase silencing. *Elife* **4**,
doi:ARTN e0791810.7554/eLife.07918 (2015).
- 132 Campanella, N. C. *et al.* Low frequency of TERT promoter mutations in gastrointestinal stromal tumors
(GISTs). *Eur J Hum Genet* **23**, 877-879, doi:10.1038/ejhg.2014.195 (2015).
- 133 Epel, E. S. *et al.* Dynamics of telomerase activity in response to acute psychological stress. *Brain Behav
Immun* **24**, 531-539, doi:10.1016/j.bbi.2009.11.018 (2010).
- 134 Fu, W. M., Lee, J., Guo, Z. H. & Mattson, M. P. Seizures and tissue injury induce telomerase in
hippocampal microglial cells. *Exp Neurol* **178**, 294-300, doi:10.1006/exnr.2002.8030 (2002).
- 135 Igarashi, H. & Sakaguchi, N. Telomerase activity is induced in human peripheral B lymphocytes by the
stimulation to antigen receptor. *Blood* **89**, 1299-1307 (1997).
- 136 Ueda, M. *et al.* Evidence for UV-associated activation of telomerase in human skin. *Cancer Res* **57**, 370-
374 (1997).
- 137 Fantini, D. *et al.* A Carcinogen-induced mouse model recapitulates the molecular alterations of human
muscle invasive bladder cancer. *Oncogene*, doi:10.1038/s41388-017-0099-6 (2018).
- 138 Vail, E. *et al.* Telomerase reverse transcriptase promoter mutations in glandular lesions of the urinary
bladder. *Ann Diagn Pathol* **19**, 301-305, doi:10.1016/j.anndiagpath.2015.06.007 (2015).
- 139 Di Meo, A., Bartlett, J., Cheng, Y. F., Pasic, M. D. & Yousef, G. M. Liquid biopsy: a step forward towards
precision medicine in urologic malignancies. *Mol Cancer* **16**, doi:ARTN 8010.1186/s12943-017-0644-5
(2017).
- 140 Cavallo, D. *et al.* Assessment of DNA Damage and Telomerase Activity in Exfoliated Urinary Cells as
Sensitive and Noninvasive Biomarkers for Early Diagnosis of Bladder Cancer in Ex-Workers of a Rubber
Tyres Industry. *Biomed Res Int*, doi:Artn 37090710.1155/2014/370907 (2014).
- 141 Hosen, I. *et al.* TERT promoter mutations in clear cell renal cell carcinoma. *Int J Cancer* **136**, 2448-2452,
doi:10.1002/ijc.29279 (2015).
- 142 Theodorescu, D. & Cech, T. R. Telomerase in Bladder Cancer: Back to a Better Future? *Eur Urol* **65**, 370-
371, doi:10.1016/j.eururo.2013.10.019 (2014).
- 143 Damm, K. *et al.* A highly selective telomerase inhibitor limiting human cancer cell proliferation. *Embo J*
20, 6958-6968, doi:DOI 10.1093/emboj/20.24.6958 (2001).

- 144 Dikmen, Z. G. *et al.* In vivo inhibition of lung cancer by GRN163L: A novel human telomerase inhibitor. *Cancer Res* **65**, 7866-7873, doi:10.1158/0008-5472.Can-05-1215 (2005).
- 145 Djojsoebroto, M. W. *et al.* Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. *Hepatology* **42**, 1127-1136, doi:10.1002/hep.20822 (2005).
- 146 Kumar, M. *et al.* CEBP factors regulate telomerase reverse transcriptase promoter activity in whey acidic protein-T mice during mammary carcinogenesis. *Int J Cancer* **132**, 2032-2043, doi:10.1002/ijc.27880 (2013).
- 147 Norton, J. C., Piatyszek, M. A., Wright, W. E., Shay, J. W. & Corey, D. R. Inhibition of human telomerase activity by peptide nucleic acids. *Nat Biotechnol* **14**, 615-619, doi:DOI 10.1038/nbt0596-615 (1996).
- 148 Zahler, A. M., Williamson, J. R., Cech, T. R. & Prescott, D. M. Inhibition of Telomerase by G-Quartet DNA Structures. *Nature* **350**, 718-720, doi:DOI 10.1038/350718a0 (1991).
- 149 Baerlocher, G. M. *et al.* Telomerase Inhibitor Imetelstat in Patients with Essential Thrombocythemia. *New Engl J Med* **373**, 920-928, doi:10.1056/NEJMoa1503479 (2015).
- 150 Chiappori, A. *et al.* A randomized phase II study of the telomerase inhibitor imetelstat as maintenance therapy for advanced non-small cell lung cancer. *Cancer Res* **73**, doi:10.1158/1538-7445.Am2013-4660 (2013).
- 151 Kozloff, M. *et al.* Phase I study of imetelstat (GRN163L) in combination with paclitaxel (P) and bevacizumab (B) in patients (pts) with locally recurrent or metastatic breast cancer (MBC). *Journal of Clinical Oncology* **28**, 2598-2598, doi:10.1200/jco.2010.28.15_suppl.2598 (2010).
- 152 Mender, I., Gryaznov, S., Dikmen, Z. G., Wright, W. E. & Shay, J. W. Induction of Telomere Dysfunction Mediated by the Telomerase Substrate Precursor 6-Thio-2'-Deoxyguanosine. *Cancer Discov* **5**, 82-95, doi:10.1158/2159-8290.Cd-14-0609 (2015).
- 153 Mender I, G. S., Shay JW. A novel telomerase substrate precursor rapidly induces telomere dysfunction in telomerase positive cancer cells but not telomerase silent normal cells. *Oncoscience* **22**, 693-695 (2015).
- 154 de Lange, T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Gene Dev* **19**, 2100-2110, doi:10.1101/gad.1346005 (2005).
- 155 Arnoult, N. & Karlseder, J. Complex interactions between the DNA-damage response and mammalian telomeres. *Nat Struct Mol Biol* **22**, 859-866, doi:10.1038/nsmb.3092 (2015).
- 156 Muraki, K., Murnane, J. P. The DNA damage response at dysfunctional telomeres, and at interstitial and subtelomeric DNA double-strand breaks. *Genes Genet. Syst.*, 135-152 (2017).
- 157 Sfeir, A. & de Lange, T. Removal of Shelterin Reveals the Telomere End-Protection Problem. *Science* **336**, 593-597, doi:10.1126/science.1218498 (2012).
- 158 Karlseder, J., Broccoli, D., Dai, Y. M., Hardy, S. & de Lange, T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* **283**, 1321-1325, doi:DOI 10.1126/science.283.5406.1321 (1999).
- 159 Chang, J., Dinney, C. P., Huang, M. S., Wu, X. F. & Gu, J. Genetic Variants in Telomere-Maintenance Genes and Bladder Cancer Risk. *Plos One* **7**, doi:ARTN e3066510.1371/journal.pone.0030665 (2012).
- 160 Liu, J. Y. *et al.* PinX1 suppresses bladder urothelial carcinoma cell proliferation via the inhibition of telomerase activity and p16/cyclin D1 pathway. *Mol Cancer* **12**, doi:Artn 14810.1186/1476-4598-12-148 (2013).
- 161 Zhang, B. *et al.* Silencing PinX1 Compromises Telomere Length Maintenance As Well As Tumorigenicity in Telomerase-Positive Human Cancer Cells. *Cancer Res* **69**, 75-83, doi:10.1158/0008-5472.Can-08-1393 (2009).
- 162 Yamaguchi, S. *et al.* Eribulin Mesylate Targets Human Telomerase Reverse Transcriptase in Ovarian Cancer Cells. *Plos One* **9**, doi:ARTN e11243810.1371/journal.pone.0112438 (2014).
- 163 Hu, J. *et al.* Antitelomerase Therapy Provokes ALT and Mitochondrial Adaptive Mechanisms in Cancer. *Cell* **148**, 651-663, doi:10.1016/j.cell.2011.12.028 (2012).

Figure Legends

[Au:As you can see in the proofs, I have made some edits to the Figures for clarity and consistency with the legends, OK?]

Figure 1 | Classical concept of telomere and telomerase functions in tumour suppression and initiation. During the life of differentiated cells, telomere length shortens owing to suppression of telomerase expression [Au:OK?]. Short, dysfunctional telomeres resemble chromosome breaks, and activate various DNA-damage response mechanism¹⁵⁵⁻¹⁵⁷. After a maximum number of cell replications (Hayflick limit), [Au:OK?] critically short telomeres induce replicative senescence in cells with a functional DNA damage response (DDR), serving as a mechanism to protect from tumorigenesis. [Au:Please add why cells with overly short telomeres are prone to tumorigenesis – why the DDR would be activated.] In DDR-deficient cells, overly short telomeres can result in genetic instabilities by repeated breakage-fusion-bridges (BFB) cycles, that lead to cellular transformation. Transformed cells require telomerase activity to stabilize telomere functionality and avoid apoptosis, and for unlimited proliferation. Transformed cells in culture and human cancers usually reactivate telomerase or employ alternative lengthening of telomeres (ALT) as telomere maintenance mechanisms.

Figure 2 | Alternative concept of telomere and Telomerase functions in tumour suppression and initiation. a | New data indicate that tumour-initiating mutations (for example, an oncogenic mutation) occurring in a telomerase-negative somatic cell (T-) can cause telomere replication stress, accumulation of fragile telomeres, and eventual senescence independent of telomere length (oncogene-induced senescence (OIS) or aneuploidy-induced senescence (AIS). This senescence response prevents tumorigenesis. b | When the same mutation occurs in a telomerase-positive somatic cell (T+) with a telomerase-reactivating *TERT* promoter mutation, the mutated cell can continue to proliferate. [Au:Please add why telomerase activation prevents senescence or apoptosis in a mutated cell.] During continued proliferation, the cell accumulates additional mutations, eventually leading to transformation and cancer.

Figure 3 | The cell of origin of cancer in UCB. In tissues with a defined stem cell compartment, stem cells have constitutive telomerase activity, supporting the proliferation of stem and transit-amplifying cells. Telomerase activity is subsequently down-regulated in differentiated cells arising from the progenitor cells. [Au:OK?] In the mitotically quiescent bladder urothelium, including in basal cells, telomerase activity is undetectable. Whether telomerase is activated under physiological regenerative conditions in human urothelium *in*

vivo remains to be clarified. In urothelial carcinoma of the bladder (UCB), telomerase is activated through *TERT* promoter mutations (red flash icon), which probably occur in proliferating urothelial cells. The activation of telomerase endows continued proliferation of cells by preventing telomere shortening and counteracting cellular differentiation. Specifically, it was demonstrated that forced expression of *TERT* in normal urothelial cells results in *loss of differentiation capacity*^{13,90}, potentially *by the down-regulation of components of polycomb response complex targets and genes associated with differentiation*⁹⁰. Cells with *TERT* mutations show sustained proliferation and can accumulate additional mutations (black flash icon) that promote malignant transformation. **[Au:For this Figure, please clarify the connection between the panel with the cells on the left and “Cellular differentiation” (how is differentiation blocked?), and with “Mutation load” (why does the mutation load decrease again?).]**

Author notes

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Fig 1

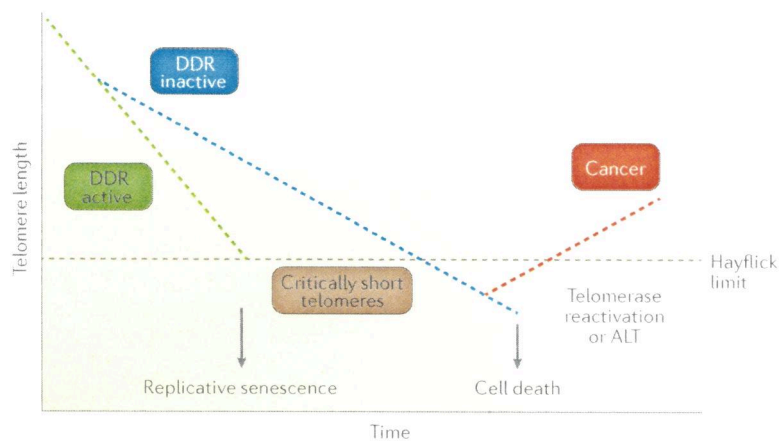
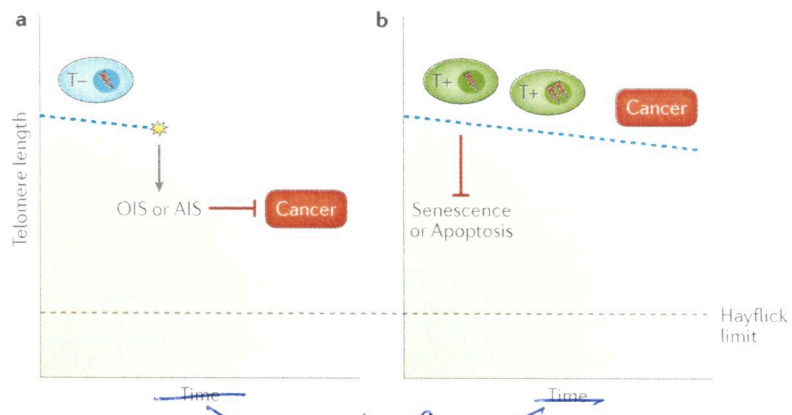


Fig 2



proliferation
(alternative: proliferation capacity)

Fig 3

