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Non-canonical NF- κ B signalling and ETS1/2 cooperatively drive C250T mutant *TERT* promoter activation

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Abstract

Transcriptional reactivation of *TERT*, the catalytic subunit of telomerase, is necessary for cancer progression in about 90% of human cancers. The recent discovery of two prevalent somatic mutations—C250T and C228T—in the *TERT* promoter in various cancers has provided insight

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AUTHOR CONTRIBUTIONS

V.T. conceptualized the ideas for this manuscript. Y.L. and V.T. planned and devised the experiments. Y.L., Q.-L.Z. and H.S.C. performed CHIP and real-time PCR experiments along with all biochemical and molecular analysis. W.S. and S.P. performed TACO and all bioinformatics analysis. P.C. and K.-H.C. conducted the orthotopic glioma injections and imaging. M.Lakshmanan and A.R. did the tumour xenograft experiments. Z.Y., J.L. and M.Li genotyped the primary tumours and performed IHC on primary tumours. D.G.T. supported Q.-L.Z.'s studentship. S.-Y.C. provided GBM cell lines. Y.L. wrote the manuscript and V.T. edited it.

COMPETING FINANCIAL INTERESTS

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into a plausible mechanism of *TERT* reactivation. Although the two hotspot mutations create a similar binding motif for E-twenty-six (ETS) transcription factors, we show that they are functionally distinct, in that the C250T unlike the C228T *TERT* promoter is driven by non-canonical NF- κ B signalling. We demonstrate that binding of ETS to the mutant *TERT* promoter is insufficient in driving its transcription but this process requires non-canonical NF- κ B signalling for stimulus responsiveness, sustained telomerase activity and hence cancer progression. Our findings highlight a previously unrecognized role of non-canonical NF- κ B signalling in tumorigenesis and elucidate a fundamental mechanism for *TERT* reactivation in cancers, which if targeted could have immense therapeutic implications.

The telomerase reverse transcriptase (*TERT*) gene encodes the catalytic subunit of the telomerase complex, which is necessary for subverting the adverse effects of progressive telomere shortening^{1–4}. Unlike in stem cells, telomerase activity is typically low or absent in most somatic cells owing to negligible levels of *TERT* arising from transcriptional repression of its promoter⁵. Hence, reactivation of the *TERT* gene through transcriptional de-repression of its promoter is the rate-limiting step in reconstituting telomerase activity, which is an essential prerequisite for the progression of most human cancers^{6–8}. Distinct from its role in telomere elongation, various extra-telomeric functions of *TERT* critical for cancer development have also been described^{9–12}. Clearly, understanding how the human *TERT* (*hTERT*—hereafter referred as *TERT*) promoter is reactivated in cancers is key to unravelling a fundamental mechanism of cancer progression. Recently, two mutually exclusive and highly recurrent mutations in the core *TERT* promoter—C250T or C228T (refs 13, 14)—were described, providing a plausible handle for studying the mechanism of *TERT* reactivation.

Several transcription factors including Myc, β -catenin and NF- κ B have been proposed to be drivers of the *TERT* promoter, on the basis of evidence that binding sites for these factors exist on the *TERT* promoter^{15,16}. NF- κ B is a transcription factor well known for regulating many cellular pathways including tumorigenesis^{17,18}. Although NF- κ B signalling can regulate *TERT* expression *in vitro*⁹ and two potential NF- κ B-binding motifs have been identified within the *TERT* promoter¹⁹, regulation of *TERT* by NF- κ B in human cancers is not documented. As NF- κ B-dependent activation of tumour-promoting pathways has been implicated in the pathogenesis of glioblastoma multiforme²⁰ (GBM), the most aggressive form of brain malignancy with high telomerase activity²¹, we examined whether NF- κ B mediates transcription of *TERT* in GBM. Surprisingly, we uncover a NF- κ B-binding site in the *TERT* promoter that specifically maps to one of the two hotspot mutations (C250T or C228T; refs 13, 14) in the core *TERT* promoter and show that this binding is essential for activation of the mutant *TERT* promoter. Although enhanced transcriptional activity of the *TERT* promoter has been correlated with C250T or C228T mutations^{22–24}, the definite mechanism(s) underscoring the induction of *TERT* transcription by these mutations remain poorly understood. It is not clear whether both mutations lead to activation of the promoter by similar mechanisms. Our data suggest that the two *TERT* promoter mutations that were predicted to generate the same ETS-binding site are functionally distinct, in that the C250T unlike the C228T *TERT* promoter is driven by non-canonical NF- κ B signalling. Consistent with literature^{25,26} that suggests that ETS factors require dimerization with other

transcription factors for transactivation, we find that binding of ETS alone to the C250T mutant *TERT* promoter is insufficient to activate transcription. Efficient reactivation of *TERT* at this mutant promoter requires cooperation with p52, downstream of non-canonical NF- κ B signalling.

RESULTS

Non-canonical NF- κ B signalling selectively induces *TERT* expression in C250T-mutant cells

The NF- κ B signalling pathway is functionally segregated into canonical and non-canonical arms²⁷. Results from GBM cells stimulated with TNF- α and TWEAK (tumour necrosis factor-like weak inducer of apoptosis), which activate the canonical and non-canonical NF- κ B arms respectively, showed that although TNF- α caused a strong induction of NF- κ B target genes—*IL-8* and *I κ B α* in all GBM cell lines—it did not cause changes in *TERT* expression (Supplementary Fig. 1a). In contrast, a subset of GBM cell lines exhibited *TERT* induction following TWEAK exposure (Fig. 1a). Sequencing the *TERT* promoter region of these cell lines unexpectedly led to a distinct segregation of these lines that induce *TERT* after TWEAK stimulation, based on their *TERT* promoter mutation status (Fig. 1b). Upregulation of *TERT* in GBM cells containing the C250T mutation correlated with a strong induction of telomerase activity in C250T-mutant cells (Fig. 1c). It is noteworthy that small changes in *TERT* transcription are sufficient for a significant increase in telomerase activity^{28,29}.

The soluble form of tumour necrosis factor (TNF) superfamily member, tumour necrosis factor-like weak inducer of apoptosis (TWEAK), at low physiological concentration (10–30 ng ml⁻¹) predominantly activates non-canonical NF- κ B signalling through the Fn14 receptor^{30,31}. Activation of the non-canonical NF- κ B pathway induces NF- κ B-inducing kinase (NIK) to stimulate the processing of NF- κ B2 p100 to p52 (ref. 32), which forms a heterodimer with RelB, leading to transcriptional activation of selective NF- κ B target genes^{33,34}. GBM cells carrying the C228T mutation did not show induction of *TERT* or significant differences in telomerase activity following TWEAK stimulation (Fig. 1a,c), although these cell lines express similar levels of Fn14 and levels of TWEAK-induced nuclear p52 and RelB accumulation seen were comparable to C250T cells (Supplementary Fig. 1b,c). These results suggest that although similar levels of p52/RelB are activated in all GBM cells, only the C250T-mutant *TERT* promoter is responsive to these dimers. Consistent with the induction of *TERT* transcription and telomerase activity, C250T-mutant GBM cells exhibited a significant increase in p52 occupancy on the *TERT* promoter whereas no p52 enrichment was seen in C228T GBM cells (Fig. 1d). Specific enrichment of p52 at the C250T *TERT* promoter following TWEAK stimulation also led to increased RNA polymerase II (Pol II) recruitment (Fig. 1d). In contrast to the *TERT* promoter, TWEAK-induced p52 and Pol II enrichment at a known p52 target gene promoter, namely *BLC* (B lymphocyte chemoattractant or CXCL13), occurred with similar efficiency in both C250T and C228T GBM lines (Supplementary Fig. 2a). Lentiviral knockdown of p52, RelB and NIK abolished the induction of *TERT* in TWEAK-stimulated C250T GBM cells, verifying that TWEAK-mediated effects on the C250T *TERT* promoter are specifically mediated

through non-canonical NF- κ B signalling (Fig. 1e and Supplementary Fig. 2b). Association of p65 (RelA) at the *TERT* promoter was not observed in TWEAK-induced C250T GBM cells (Fig. 1d). Furthermore, TNF- α stimulation failed to recruit p52 to the *TERT* promoter (Supplementary Fig. 2c) although efficient p65 and Pol II recruitment to the canonical target gene promoter, *NF- κ B1A*, was observed (Supplementary Fig. 2d).

These observations were also recapitulated when T98G and U251 cells, containing the C250T and C228T *TERT* mutations respectively, were stimulated with agonistic human LT β R antibody, another ligand that activates non-canonical NF- κ B signalling^{34,35}. Although anti-LT β R stimulated a similar pattern of p100 processing to p52 in both cell lines (Supplementary Fig. 3a), significant induction of *TERT* messenger RNA was seen in T98G but not in U251 cells (Supplementary Fig. 3b). These findings corresponded with a significant enrichment of p52 and Pol II at the *TERT* promoter in T98G cells but not in U251 cells (Supplementary Fig. 3c), although similar levels of p52 and Pol II recruitment at the *BLC* promoter were detected in all cells with anti-LT β R (Supplementary Fig. 3d). Increased occupancy of p52 on the C250T *TERT* promoter correlated with their enhanced proliferation (Supplementary Fig. 3e) as well as increased telomerase activity after anti-LT β R treatment (Supplementary Fig. 3f). In contrast, anti-LT β R-treated U251 cells exhibited no difference in proliferation and telomerase activity (Supplementary Fig. 3e,f). Taken together, these findings suggest that non-canonical NF- κ B signalling specifically activates the C250T *TERT* promoter in human GBM cells.

C250T *TERT* promoter mutation creates a p52-binding site

NF- κ B dimers bind the consensus sequence 5'-GGGRN(Y)YYCC-3'. However, the nucleotide sequence spanning the C250T mutation site does not form a complete NF- κ B consensus motif. Although NF- κ B dimers have been observed to bind only one half-site (5'-GGGGG-3' or 5'-GGAA-3') *in vitro*³⁶, *in vivo* half-site binding of NF- κ B dimers, especially p52/RelB, has not been investigated. We thus examined whether p52 can bind half-site at the C250T *TERT* promoter using the only available *in vivo* p52 ChIP-seq data from lymphoblastoid B cells³⁷. Through *de novo* motif analysis^{38–40}, we identified the palindromic p52 motif from 12,239 p52 ChIP-seq peaks (Fig. 2a) and analysed these binding regions for p52 half-site binding *in vivo*. The total binding energy (G-score) of the 11-base-pair p52 motif was decomposed into left and right half-site G_L and G_R (Fig. 2b) and p52 motif enrichment was computed in two-dimensional G_L - G space (Fig. 2b)^{38,41}. From this plot, 11 base pairs with high binding affinity (low G-score) were recognized as full-site binding regions and were uniformly distributed along anti-diagonal lines (Fig. 2b—area in triangle)⁴¹. In contrast, 11 base pairs with lower full-site binding affinity regions tend to enrich in the half-site binding regions (Fig. 2b—area in rectangles) where only one of the half-site G_L or G_R is low⁴¹. We therefore provide evidence that p52 binds to half-sites *in vivo*.

The electrophoretic mobility shift assay (EMSA) demonstrated enhanced binding on the DNA probe containing the C250T *TERT* promoter sequence relative to the wild-type (WT) *TERT* promoter (Fig. 2c, lanes 1–2) using nuclear extracts from the C250T line T98G. Whereas activation of NF- κ B DNA binding to its established consensus motif was

dependent on TNF- α stimulation (Fig. 2d, lanes 3–4), DNA binding to the C250T *TERT* promoter was unperturbed by TNF- α stimulation (Fig. 2d, lanes 7–8). This is consistent with earlier observations that regulation of the C250T *TERT* promoter is independent of canonical NF- κ B signalling. Supershift EMSA with antibodies targeting NF- κ B2 (p100/p52) and RelB diminished protein binding to the C250T *TERT* promoter (Fig. 2e, lanes 3–5). In contrast, antibodies targeting c-Rel and unspecific IgG did not affect protein binding on the C250T *TERT* promoter (Fig. 2e, lanes 6–7). To verify the functional implications of p52/RelB binding on the C250T *TERT* promoter, we performed short interfering RNA (siRNA) experiments on T98G and U251 cells. Significant downregulation of *TERT* was observed in T98G cells but not in U251 cells following downregulation of NF- κ B2 and RelB (Supplementary Fig. 3g). Enhanced binding to the C250T *TERT* promoter was observed with TWEAK stimulation (Fig. 2f, lanes 1–2) and this binding was disrupted by antibodies against NF- κ B2 (p100/p52) and RelB but not c-Rel and IgG (Fig. 2f, lanes 3–6). RelB does not possess DNA-binding activity as a homodimer but rather mediates its transactivation function by forming a heterodimer with p52 (refs 42,43). Recombinant p52 robustly bound to the C250T *TERT* promoter in a dose-dependent manner (Supplementary Fig. 4a, lanes 4–6) whereas GST protein did not (Supplementary Fig. 4a, lane 7). A DNA probe containing the known human immunodeficiency virus (HIV) κ B binding sites for p52 (ref. 43) was used as a control for p52 binding (Supplementary Fig. 4a, lanes 2–3). Binding of p52 to the C250T *TERT* promoter was completely abolished when two residues in the rel homology region (RHR) critical for DNA binding were mutated (Supplementary Fig. 4b, lane 6). Taken together, these results provide evidence for a p52 binding site on the C250T *TERT* promoter and demonstrate a previously unrecognized function of non-canonical NF- κ B signalling in regulation of *TERT* transcription.

Non-canonical NF- κ B activation enhances tumorigenicity of C250T-mutant GBMs by increasing telomerase activity

NIK is a MAP3K kinase that signals downstream of TWEAK/Fn14 and Lt β R to induce phosphorylation of p100 by I κ B kinase α (IKK α), leading to p100 ubiquitylation and processing to p52 (refs 33,34). Thus, NIK overexpression activates the non-canonical NF- κ B pathway, resulting in the generation of mature p52 (ref. 44). Ectopically expressed NIK upregulated *TERT* expression in T98G cells whereas expression of kinase-inactive mutant NIK (NIK KK) did not (Fig. 3a and Supplementary Fig. 5a), indicating that NIK-induced processing of p100 was necessary for activation of the C250T *TERT* promoter. This was consistent with the significant enrichment of p52 and Pol II at the *TERT* promoter (Supplementary Fig. 5b) and the induction of telomerase activity in NIK-expressing T98G cells (Supplementary Fig. 5c). In contrast, NIK expression in U251 cells did not affect *TERT* expression (Fig. 3a), telomerase activity (Supplementary Fig. 5c) or recruitment of p52 or Pol II at the *TERT* promoter (Supplementary Fig. 5b), although both complexes were significantly enriched at the *BLC* promoter (Supplementary Fig. 5d). These findings were consistent with reporter assays using these *TERT* promoters. Ectopic expression of NIK augmented the transcriptional activity at the C250T *TERT* promoter-driven but not WT *TERT* promoter-driven luciferase and this effect was not seen when NIK KK was expressed (Supplementary Fig. 5e). Furthermore, TWEAK treatment resulted in increased

transcriptional activity at the C250T *TERT* promoter but not the WT *TERT* promoter (Supplementary Fig. 5f).

Ectopic expression of NIK in T98G cells caused increased cell proliferation (Fig. 3b,c), which was abolished following treatment with siRNA against *TERT* (Fig. 3d,e), suggesting that the NIK-mediated growth advantage depends on upregulation of *TERT* and hence telomerase activity. Induction of *TERT* mRNA and cell proliferation in NIK-expressing C250T cells were abrogated following treatment with siRNA against NF- κ B2 and RelB but not RelA, further suggesting that NIK-regulated activation of the C250T *TERT* promoter is mediated through the non-canonical NF- κ B pathway (Fig. 3f,g and Supplementary Fig. 5g,h). Ectopic expression of NIK (Supplementary Fig. 6a) markedly enhanced *in vivo* tumour growth of T98G cells (Fig. 4a,b and Supplementary Fig. 6b,c), which were previously reported to be non-tumorigenic in immunodeficient mice⁴⁵. In contrast, T98G cells expressing vector control or NIK in combination with short hairpin RNA (shRNA) targeting p52 (Supplementary Fig. 6a) were not tumorigenic *in vivo* (Fig. 4a,b and Supplementary Fig. 6b). Enhanced tumorigenic potential of NIK-expressing T98G cells in NOD-SCID mice was consistent with their increased telomerase activity relative to vector control that was abolished in NIK p52 shRNA cells (Supplementary Fig. 6d). Gliomagenesis and invasiveness of these human cells in orthotopic glioma xenograft models showed accelerated tumour growth in mice that were xenotransplanted with NIK-expressing T98G cells (Fig. 4c). One of three mice implanted with vector T98G cells developed an intracranial tumour, but all three mice bearing NIK-expressing T98G cells developed gliomas and this increased tumorigenesis was completely abolished by p52 knockdown (Fig. 4c). These observations therefore support the critical role of the non-canonical NF- κ B pathway in activating *TERT* expression and telomerase function through the C250T *TERT* promoter, hence potentiating *in vivo* tumorigenicity. We also analysed NIK and *TERT* expression levels in primary human tumours derived from GBM patients using immunohistochemical (IHC) staining, and simultaneously sequenced genomic DNA extracted from the same tumour material for *TERT* promoter mutations. From the 29 GBM tumours analysed, 28% and 38% of the samples harboured C250T ($n=8$) and C228T ($n=11$) *TERT* mutations respectively, 34% of tumours were WT for the *TERT* promoter ($n=10$) (Fig. 4d). Tumours containing *TERT* promoter mutations exhibited increased *TERT* expression compared with WT tumours (Fig. 4d). In particular, C250T-positive tumours had significantly higher *TERT* expression compared with WT tumours ($P=0.0017$; *t*-test, two-tailed; Fig. 4d). Tumours with the C228T mutation showed a trend of higher *TERT* levels as compared with WT tumours, although the difference was not statistically significant ($P=0.0859$; *t*-test, two-tailed; Fig. 4d). Further IHC analysis of these tumours for NIK expression revealed a positive correlation ($P=0.028$; Linear regression test) between increased NIK expression and high *TERT* levels particularly in C250T tumours (Fig. 4e,f). This association between elevated NIK levels and *TERT* overexpression in GBM tumours was not seen in WT tumours or in C228T tumours (Fig. 4e,f). These findings highlight the significant concordance between enhanced NIK expression and increased telomerase expression in GBM tumours carrying the C250T mutation.

Targeted reversal of C250T mutation abolishes activation of *TERT* by p52

To confirm whether p52 binds the C250T loci and no other location on this promoter, we targeted this mutation site using CRISPR/Cas9 technology to reverse this point mutation to the WT in the genome of T98G cells. Two guide RNAs targeting the *TERT* promoter were each selected to mediate a single Cas9 endonuclease-induced double-stranded break at either loci adjacent to C250T mutation site (Fig. 5a) and single-stranded oligonucleotides containing the WT *TERT* promoter sequence were co-transfected into T98G cells to mediate genome editing. We obtained three T98G clones that carried the WT *TERT* promoter sequence—two het clones with one copy of the thymine residue repaired to cytosine and one clone with both alleles edited (Fig. 5b). Reversal of the C250T mutation to WT resulted in an appreciable reduction of telomerase activity (Fig. 5c) and proliferation (Fig. 5d,e) of all three WT clones, relative to a C250T-positive clone that was similarly isolated following the CRISPR/Cas9 screen. Furthermore, C250T reversal abolished TWEAK-mediated induction of *TERT* expression (Fig. 5f) and telomerase function (Fig. 5g) in all three WT clones but not the C250T-positive clone, which retained similar telomerase activity as control T98G cells (Fig. 5c). Consistently, TWEAK-induced recruitment of p52 and Pol II to the *TERT* promoter was abrogated following C250T reversal (Fig. 5h) although similar enrichment of the two factors was found at the *BLC* promoter, on TWEAK stimulation, in both the C250T-positive and WT clones (Fig. 5i). These findings therefore reaffirm that this single hotspot mutation generates a binding site for p52 that drives *TERT* reactivation in GBMs.

p52 interacts with ETS factors at the C250T *TERT* promoter to mediate *TERT* reactivation

The C>T transition is predicted to create a binding motif for the E-twenty-six (ETS) family of transcription factors (GGAA, opposite strand)^{13,14}. As NF- κ B factors have been previously demonstrated to interact with ETS proteins^{46,47}, we performed immunoprecipitation with anti-Flag beads in T98G cells expressing Flag-tagged p52. p52 co-immunoprecipitated ETS1 and ETS2 in T98G cells (Fig. 6a), confirming the physical interaction between p52 and ETS factors. Consistent with this, ChIP re-ChIP showed ETS1/2 association with p52 at the *TERT* promoter in T98G cells (Fig. 6b). Furthermore, knockdown of ETS1/ETS2 or NIK similarly reduced proliferation in C250T-mutant GBM cells (Fig. 6c–f). ETS1/ETS2 are known to be auto-inhibited and require cooperative binding with another transcription factor by forming a homodimer^{25,26} or a heterodimer⁴⁸ for DNA-binding activities. Our experimental results show that ETS1/2 and p52 co-bind the C250T region but not the C228T region. As both C250T and C228T *TERT* mutations generate the same ETS consensus motif *de novo*, we examined the *TERT* promoter region spanning both mutation sites for cooperative binding between ETS and p52/RelB to explain the differential response observed. Using TACO to predict co-association of ETS–p52/RelB heterodimers at the chromatin⁴⁹, we found all three dimers to be highly enriched in two cell lines (adjusted *P* value of less than 0.05; Fig. 7a). The consensus of the three dimers 5'-nTCCnn(T/A)TCC-3' indicates that p52 and ETS1 may form a heterodimer with a 2-base-pair (bp) space. As the p52 half-site motif is very similar to the ETS1 consensus site, it is not feasible to pinpoint which half-site is bound by p52. However, it is worth noting that both 5'-T1C2C3-3' half-sites require a T at position 1 instead of a C. No enrichment of another p52/RelB half-site motif 5'-NCCCC-3' in the dimers also highlights the importance of T in

both half-sites. We therefore aligned the consensus of the enriched dimer to the C250T and C228T regions and found that the C250T region perfectly matches the two ‘TCC’ half-sites with the correct spacing. In contrast, the C228T region lacks a T on the right half-site, which may explain the less favourable binding of the p52/ETS1 heterodimer to the C228T region (Fig. 7a).

To verify cooperative binding between p52 and ETS factors *in vitro*, we performed EMSA using recombinant p52 and ETS1 proteins. Whereas p52 bound both the WT and C250T *TERT* promoters (Fig. 7b, lanes 1 and 4), we found that ETS1 bound specifically to the mutant *TERT* promoter (Fig. 7b, lanes 2 and 5), which supports current evidence that *TERT* promoter mutations generate ETS binding sites *de novo*^{13,14,50}. Our ChIP analysis further confirmed that ETS1 can bind the C250T *TERT* promoter without stimulation (Fig. 7c). Notably, addition of p52 and ETS1 proteins to the mutant *TERT* promoter resulted in enhanced binding of p52/ETS and a complex of higher mobility (Fig. 7b, lane 6), thus validating our bioinformatics prediction. siRNA against ETS1/ETS2 reduced the induction of *TERT* expression in TWEAK-treated T98G cells (Fig. 7d), and decreased p52 and Pol II enrichment at the *TERT* promoter (Fig. 7e), suggesting that ETS1/2 occupancy at the C250T *TERT* promoter is critical for p52-mediated activation of *TERT* transcription. Notably, p52 enrichment at the *TERT* promoter was completely abolished in T98G cells treated with ETS1 siRNA (Fig. 7e), which is probably attributed to the predominant expression of ETS1 and its association with p52 in T98G cells (Fig. 6a; red asterisk). Correspondingly, the increase in telomerase activity was abrogated in all three TWEAK-induced C250T GBM cell lines that were pre-treated with siRNAs targeting ETS1 or ETS2 (Fig. 7f).

DISCUSSION

We uncover a NF- κ B-binding site in the C250T mutant *TERT* promoter that is essential for its activation. We show that the C250T *TERT* promoter, unlike C228T, is driven by non-canonical NF- κ B signalling. Binding of ETS factors to the C250T-mutant *TERT* promoter in the absence of non-canonical NF- κ B signalling is insufficient to activate *TERT* transcription and efficient reactivation requires cooperation with the p52 subunit of NF- κ B, downstream of non-canonical NF- κ B signalling. We also show that ETS–p52 cooperation occurs only in context of the mutant C250T sequence (Fig. 7a,b) and these data explain why *TERT* is not activated by NF- κ B signalling in somatic cells with the WT *TERT* promoter sequence. On the basis of the results presented, we propose a model for telomerase reactivation in human cancers harbouring C250T *TERT* promoter mutations (Fig. 7g). *TERT* expression is low when ETS1/2 alone is bound at the C250T promoter. On activation of non-canonical NF- κ B signalling through exogenous ligands (for example, TWEAK) or constitutive NIK expression, p52 is recruited to the C250T promoter and cooperates with ETS factors to drive efficient *TERT* transcription (Fig. 7g). Enhanced *TERT* expression promotes telomerase activity, necessary for cancer progression (Fig. 4a–c). Strong support for this model is provided by the evidence that the C250T mutation site when reversed to WT status by CRISPR/Cas9 loses ETS1/2 binding and p52 is no longer recruited or stabilized at the *TERT* promoter (Fig. 5h,i), resulting in the loss of *TERT* expression (Fig. 5f), telomerase activity (Fig. 5g) and cancer cell growth (Fig. 5d,e). Although recombinant p52 binds *in vitro* to the WT *TERT* promoter (Fig. 7b), this half-site binding at non-physiological concentrations is

not reflective of *in vivo* interactions as demonstrated by ChIP (Fig. 5h). The non-canonical NF- κ B pathway is predominantly known to regulate lymphoid organ development⁵¹, B cell activity and differentiation⁵², dendritic cell function^{53,54}, and bone metabolism^{34,52}. There has been accumulating evidence documenting the involvement of NIK in tumorigenesis of melanoma⁵⁵, breast cancer⁵⁶ and multiple myeloma^{35,57}. The canonical NF- κ B pathway has been shown to be associated with progression of many cancers and thought to be required for acquisition of resistance to chemotherapy^{58–61}. Unlike the canonical NF- κ B activity that is transient⁶² but strong, the non-canonical NF- κ B signalling is known to be slow but persistent. We reason that given that the *TERT* promoter needs to be open persistently following reactivation, tumour cells co-opt to use the non-canonical NF- κ B activity (much like it is used in organogenesis) for continued expression of *TERT* in the proliferating tumour mass. Indeed recent studies have documented increased levels of RelB and NIK in human GBMs (refs 63,64), implicating the role of non-canonical NF- κ B signalling in GBM survival and tumour progression. We conclude that the non-canonical NF- κ B pathway is essential for telomerase reactivation in human cancers harbouring the highly prevalent C250T *TERT* promoter mutations.

METHODS

Methods and any associated references are available in the online version of the paper.

METHODS

Cell lines and reagents

All GBM cell lines were obtained from S.-Y. Cheng's laboratory, Northwestern University and authenticated by short tandem repeat fingerprinting at IDEXX RADIL and Services at the SYSU Forensic Medicine Lab. No cell lines used in this study are found in the database of commonly misidentified cell lines (ICLAC and NCBI Biosample). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone) using standard tissue culture techniques. Cell lines were tested using MycoAlert Plus Mycoplasma Detection Kit (Lonza, no. LT07-710) and confirmed to be free of mycoplasma contamination. For TWEAK stimulation, recombinant human TWEAK (PeproTech; 310-06) was reconstituted and administered according to the manufacturer's recommendations. All GBM cell lines except D247mg and LN229 were treated with 10 ng ml⁻¹ of TWEAK. D247mg and LN229 cells were stimulated with 30 ng ml⁻¹ of TWEAK. For agonistic human Lt β R antibody treatment, anti-Lt β R (R&D Systems; AF629) was reconstituted with PBS and added to cells at a concentration of 0.4 μ g ml⁻¹. For siRNA treatment, GBM cells were seeded overnight to 60% confluency on the following day in 12-well plates and transfected with RNA–lipid complexes containing non-targeting siRNA, siRNAs targeting NF- κ B2 (L-003918), RelB (L-004767), RelA (L-003533) ETS1 (L-003887) or ETS2 (L-003888) (Dharmacon smartpool siRNA) and Lipofectamine RNAiMAX reagent (Life Technologies). siRNA resuspension and transfection procedures were performed according to the manufacturers' recommendations.

Plasmids

TERT promoter DNA fragments were PCR amplified using genomic DNA of cancer cell lines containing either the WT or C250T *TERT* promoter sequence and subcloned into pGL3 luciferase reporter vectors. Human WT p52 was cloned from cDNA into the pBobi mammalian expression vector with an amino-terminal Flag tag. Lentiviral mammalian expression constructs for human WT NIK and kinase-dead NIK mutant (NIK KK) were cloned from cDNA into the pLenti-CMV GFP-zeocin vector (Addgene). Lentiviral shRNA expression plasmids of NF- κ B2 (p52), RelB and NIK were cloned into the pLKO.1-puro vector (Addgene). Targeting sequences are available on request.

Genomic DNA isolation and Sanger sequencing

Genomic DNA of GBM cell lines was extracted by digesting cell pellets with lysis buffer (supplemented with proteinase K) and performing DNA precipitation thereafter using isopropanol. The proximal *TERT* promoter fragment spanning C250T and C228T was PCR amplified from genomic DNA using the following primers: forward 5'-CCCTGGGTCTCCG GATCA-3'; reverse 5'-CACGCACACCAGGCACT-3'. The purified PCR product was subsequently sent for Sanger sequencing using the primer 5'-CCCACAGCTTAGGCCGATTC-3' to check for *TERT* promoter mutation status.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from control or treated cells were isolated using standard methods. EMSA was performed as described previously⁹. The following radiolabelled probes were used for EMSA: WT *TERT* promoter (double stranded, 5'-GTCCCGACCCCTCCCGGGTCCCGGC-3'), C250T *TERT* promoter (double stranded, 5'-GTCCCGACCCCTTCCGGGTCCCGGC-3'), NF- κ B consensus (double stranded, 5'-TCAACAGAGGGGACTTTCCGAGAGGCC-3'), Oct-1 (double stranded, 5'-TGTCGAATGCAAATCACTAGAA-3') and HIV- κ B (double stranded, 5'-GCTACAAGGGACTTTCCGCTGGGGACTTTCCAGAGAGG-3'). For supershift assays, 1 μ g of polyclonal antibodies targeting NF- κ B2 (Cell Signaling; 4882 and Santa Cruz; sc-298), RelB (Cell Signaling; 4954), c-Rel (Santa Cruz; sc-70) and nonspecific IgG (Santa Cruz) were added to nuclear lysates and incubated on ice for 20 min, before addition of radiolabelled probes. For the cooperative binding assay, 10 μ g each of recombinant p52 and ETS1 proteins were incubated alone in EMSA binding buffer or mixed together and incubated for 30 min at 4 °C before DNA-labelled probes were added.

De novo motif discovery and half-site binding analysis

For the motif analysis of p52 half-site binding *in vivo*, p52 ChIP-seq data generated from lymphoblastoid B cells³⁷ were used. Duplicate reads were filtered out using SAMtools⁴⁰ and only reads with a mapping quality score ≥ 10 were kept. Peaks were called using DFilter³⁹. A total of 12,239 peaks were called with the *P* value threshold of 10^{-10} . Then *de novo* motif discovery was performed using TherMos³⁸ by forcing a palindrome on the p52 ChIP-seq library (accession number: GSE55105). On the basis of the palindromic p52 motif discovered by TherMos, the 12,239 p52-binding regions were scanned for evidence of half-site binding of p52 *in vivo* as described previously⁴¹. Briefly, from the position-specific

energy matrix predicted by TherMos, the total binding energy (G-score) of any 11 base pairs was calculated by summing over the free energy contributions of each nucleotide in the 11 base pairs. The G-score of the 11-base-pair p52 motif was decomposed into left and right half-sites G_L and G_R and p52 motif enrichment was calculated in the two-dimensional G_L – G_R space. In the two-dimensional space, the foreground is represented by 11 base pairs in the 100-base-pair regions centred on the 12,239 ChIP-seq peaks (Fig. 2b) while the background consists of 11 base pairs in random 4 million 100-base-pair regions without overlapping with the p52 ChIP-seq binding regions. Any 11-base-pairs on the same anti-diagonal line has the same binding affinity. The bottom left corner of the plot (area in the triangle) is recognized as the full-site binding region where the 11 base pairs have high binding affinity (low G-score) and uniformly distribute along the anti-diagonal lines. However, at lower full-site binding affinity regions, 11 base pairs tend to enrich in the half-site binding region (area in the rectangles) where only one of the half-site G_L or G_R is low.

TACO (transcription factor association from complex overrepresentation)

For the bioinformatics prediction of cell-type-specific cooperative binding between ETS factors and p52/RelB dimers, TACO (ref. 49) was used to scan regulatory regions in 44 cell types. Regulatory regions in these cell types were annotated on the basis of chromatin openness using ENCODE DNase-seq data sets from the University of Washington⁶⁶. The two p52/RelB half-site motifs (5'-TCCCN-3' and 5'-NCCCC-3') and the ETS1 consensus motif (5'-[T/A]TCC-3') were provided as input to TACO (Fig. 6c, top panel). All possible dimers constructed from these three half-sites (all possible orientations within 50-base-pair spacing) were tested by TACO for enrichment in regulatory regions. TACO tests for dimer enrichment in regulatory regions of one specific cell type relative to a background set constructed by taking the union of regulatory elements from all 44 cell types. Dimers with an adjusted *P* value of less than 0.05 are annotated as significantly enriched.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated using the RNeasy Kit (Qiagen) according to the manufacturer's recommendations. Complementary DNA was synthesized using Superscript Vilo reverse transcriptase (Life Technologies) and real-time PCR (qPCR) was performed in triplicates using SYBR GreenER (Life Technologies). The sequences of the qPCR primers used are listed in Supplementary Table 1.

Telomere repeat amplification protocol (TRAP) assay

Five thousand viable cells per sample were collected and lysed in 1× CHAPS buffer containing RNase inhibitor (Promega). The telomerase reaction was performed in 1× TRAP buffer (20 mM Tris.HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 0.1 mg ml⁻¹ BSA) supplemented with dNTPs and TS primers⁶⁷ at 30 °C for 30 min. PCR was subsequently conducted using ACX primers²⁸ for 45 cycles of 95 °C for 5 s, 50 °C for 6 s and 72 °C for 10 s. All samples were run in triplicates.

Cell proliferation and colony formation assay

For colony formation assay, 300 cells per condition were plated in triplicates and cultured for 10 days before staining viable colonies with crystal violet. For the MTT assay, 3,000 cells per well were seeded in 96-well plates for 48 h before performing the assay with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide reagent (Sigma; M2128) according to standard procedures.

Western blot analysis

Western blotting was performed using standard methods and the following antibodies were used for analysis: anti-NF- κ B2 (Cell Signaling; 4882), anti-RelB (Cell Signaling; 4954), anti-p65 (Santa Cruz; sc-372), anti-NIK (Cell Signaling; 4994), anti-Flag (Sigma; F7425), anti-ETS1/2 (Santa Cruz; sc-112), anti-actin (Santa Cruz; sc-1616), anti-GAPDH (Santa Cruz; sc-32233) and anti-TRF2 (Upstate; 05-521). Except for anti-GAPDH (clone 1315) and anti-TRF2 (clone 4A794) monoclonal antibodies, all other antibodies are polyclonal. Antibody dilutions used were 1:2,000 for anti-Flag and 1:1,000 for all other antibodies.

Co-immunoprecipitation and chromatin immunoprecipitation (ChIP) assays

For co-immunoprecipitation, cells were lysed in immunoprecipitation lysis buffer (10 mM Tris at pH 8, 170 mM NaCl and 0.5% NP40, supplemented with protease inhibitors) on ice for 15 min before sonication for 5 cycles. Lysates were centrifuged at 14,000 r.p.m. (Eppendorf, centrifuge 5417R) for 15 min and 500 mg of each supernatant was used for immunoprecipitation. For immunoprecipitation of Flag-tagged proteins, 20 μ l packed gel volume of anti-Flag M2 magnetic beads (Sigma; M8823) was used per reaction, according to the manufacturer's instructions.

ChIP was performed on GBM cell lines as described previously⁹, using the following antibodies (2 μ g per ChIP): anti-NF- κ B2 (p100/p52) (Abcam; ab7972), anti-Pol II (Santa Cruz; sc-899), anti-p65 (Santa Cruz; sc-372X), anti-ETS1/2 (Santa Cruz; sc-112X), anti-ETS1 (Santa Cruz; sc-350) and nonspecific IgG (Santa Cruz). For re-ChIP, anti-NF- κ B2 (p100/p52) was used for the first immunoprecipitation. After elution with recombinant p52 protein and washing, the ChIP eluate was used for the second immunoprecipitation using anti-ETS1/2 or nonspecific IgG antibodies. The sequences of the ChIP primers for the *TERT* and *BLC* promoter regions and the negative control promoter fragment (*TERT* +2000) are listed in Supplementary Table 1.

Mouse tumour xenograft and orthotopic glioma models

All animal experiments were carried out in accordance with ethical guidelines and approved by the Institutional Animal Care and Use Committee (IACUC), Biological Resource Center (BMRC) A*STAR. For the mouse tumour xenograft assay, 5 million cells per sample were mixed with Matrigel (BD) in a 1:1 ratio and injected subcutaneously into 5–6-week-old, female NOD-SCID mice ($n=5$ mice per group). Mice implanted with human GBM cells were euthanized after 10 weeks and their tumours isolated for subsequent analysis.

The orthotopic tumour model was created by stereotaxic injection of T98G cells into the brains of 8-week-old, female NOD-SCID mice ($n=3$ mice per group). In brief, 1×10^5 cells

(at a concentration of 2×10^4 cells μl^{-1}) were prepared in sterile $1\times$ PBS. After anaesthetization by 2–3% isoflurane, the mouse head was fixed to a digital stereotaxic system (Stoelting). A burr hole was made on the skull using a 23G sterile needle at 2 mm behind the bregma, 1.5 mm on the right of the midline. A 10 μl NANOFIL syringe (World Precision Instruments) pre-filled with cells was prepared and the needle was inserted 3.5 mm into the brain parenchyma through the burr whole. A 5 ± 0.3 μl volume of cells was injected at a rate of 1 $\mu\text{l min}^{-1}$ using a infusion pump (KD Scientific). After injection, the needle was removed and the burr hole was covered with bone wax, and then the incision was sutured with poly-lysine thread. The mice were given analgesics (buprenorphine) and antibiotics (enrofloxacin) over 5 days post surgery.

The tumour growth was monitored periodically using a 7 T MRI (ClinScan, Bruker BioSpin GmbH). A 72 mm volume transmit coil along with a mouse brain array coil was used for imaging. T2-weighted images were acquired using a turbo-spin echo sequence with $\text{TR}=4,000$ ms $\text{TE}=34$ ms, 100 100 300 μm resolution and two averages. The tumour volume was manually segmented and calculated using ImageJ.

CRISPR/Cas9 reversal of the *TERT* promoter mutation

The CRISPR design tool (<http://crispr.mit.edu>) was used to identify CRISPR/Cas9 sites targeting the *TERT* promoter region and highly specific targets were chosen according to recommendations from a previous study⁶⁸. Guide RNAs (gRNAs) were subsequently designed and cloned into the BbsI sites of the PX459 (pSpCas9 (BB)-2A-Puro) vector for coexpression with Cas9, according to the previously described protocol⁶⁸. The targeting efficiency of each gRNA in inducing cleavage of the *TERT* promoter was tested by co-transfecting each pSpCas9-gRNA vector with the pCAG-EGxxFP plasmid⁶⁵ containing a genomic fragment spanning the *TERT* promoter (TF2-TR2) (Fig. 5a) in T98G cells. The sequences of the oligonucleotides used to generate two of the double-stranded gRNAs (containing BbsI overhangs) with the best efficiency are listed in Supplementary Table 1.

gRNA2 and gRNA8 were then cloned into PX458 (pSpCas9 (BB)-2A-GFP) for more efficient selection of clonal cell lines by FACS. For CRISPR/Cas9 reversal of the C250T mutation by homology-directed repair (HDR), T98G cells were co-transfected with pSpCas9 (PX458)-gRNA2 or -gRNA8 and single-stranded HDR oligonucleotides containing the WT *TERT* promoter sequence. Isolation of clonal cell populations from transfected cells was then performed 48 h after transfection by FACS sorting through GFP expression. Single-cell clones that grow after 2–3 weeks were picked, expanded and genotyped for *TERT* promoter mutation status as described earlier.

Clinical samples

Twenty-nine archived glioma specimens (Grade IV astrocytoma) were clinically and histopathologically diagnosed at the First Affiliated Hospital of the Sun Yat-sen University. The study was approved by the Sun Yat-sen University Zhongshan School of Medicine Research Ethics Committee. Prior informed consent was obtained from every donor.

Protein expression and purification

Human WT p52 and ETS1 were cloned from cDNA into the pGEX-KG vector with an N-terminal GST tag. Mutant p52 was generated by mutating amino acids 52 and 53 in the Rel homology domain (RHR) using site-directed mutagenesis. GST-tagged ETS1, WT and mutant p52 proteins were expressed by *Escherichia coli* BL21 and purified using glutathione Sepharose beads (GE Healthcare) followed by size-exclusion chromatography.

Luciferase reporter assay

293T HEK (human embryonic kidney) cells were seeded at a density of 3×10^4 cells per well in 24-well plates and transfected the following day using Lipofectamine LTX (Life Technologies) with 0.75 μ g of pGL3-basic, pGL3-*TERT* WT promoter or pGL3-*TERT* C250T promoter and 30 ng of *Renilla* luciferase constructs. Cells were lysed 48 h later and assayed for luciferase activity using the Dual Luciferase Reporter Kit (Promega) according to the manufacturer's instructions. Triplicate wells per sample were assayed and relative luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity.

Immunohistochemistry

Tumours extracted from mouse xenograft models were fixed in 10% neutral-buffered formalin, paraffin embedded and H&E and immunohistochemical staining with NIK (Abcam; ab7204), TERT (Abcam; ab32020) and Ki-67 (Abcam; ab15580)-specific antibodies were performed by the Advanced Molecular Pathology Laboratory (AMPL) of IMCB (A*STAR). The expression of NIK and TERT in tumour tissues from GBM patients was evaluated by performing immunohistochemical staining with anti-NIK and anti-TERT antibodies and subsequent quantification of the staining intensity was calculated using Image Pro Plus 6.0. For each set of specimens examined, the relative expression of TERT was plotted as a function of NIK relative expression and linear regression analysis was performed.

Statistical analyses and reproducibility of experiments

Student's *t*-test (two-tailed) was performed to determine the significance of difference shown in every data figure, using GraphPad Prism software, unless otherwise mentioned. Normal distribution was assumed for all statistical analyses. Every cell culture, CHIP or *in vitro* DNA-binding experiment, including western blots and luciferase assays, was reproduced at least twice independently with similar results. The number of independent sets for each experiment is indicated in all figure legends and raw data are shown in Supplementary Table 2. No particular method of randomization was used in the experiments. Samples/animals were randomly assigned to experimental groups and processed. Investigators were not blinded to allocation during experiments and outcome assessment. For cell culture experiments, no statistical method was used to predetermine sample size. For all *in vivo* experiments, animals of the same age, gender and strain were used and thus equal variance was assumed. The experiments were not randomized. Healthy NOD-SCID mice of similar age and gender were randomly assigned to experimental groups in the tumour xenograft and orthotopic glioma models. Power analysis was used to estimate the sample size for *in vivo* experiments. Assuming that 90% of mice xenografted with NIK-

overexpressing T98G cells would exhibit oncogenic properties, compared with 20% of mice xenografted with vector control T98G cells, we calculated that a sample size of at least 5 per group would be needed to detect a significant difference in proportions, at an alpha of 0.05 and power of 0.80. On the basis of the results of the tumour xenograft experiment, we calculated that a sample size of a minimum of 2 per group would be needed to detect a significant difference in proportions, at an alpha of 0.05 and power of 0.80, assuming that 100% and 20% of the mice engrafted with NIK-overexpressing and vector control T98G cells, respectively, would exhibit oncogenic properties. Hence, we performed the orthotopic glioma animal studies using a sample size of 3 per experimental group.

Accession numbers

The accession number of the p52 ChIP-seq library³⁷ analysed in this study is GSE55105 (repository name: NCBI Gene Expression Omnibus (GEO); Title: The NF- κ B genomic landscape in lymphoblastoid B cells). ENCODE DNase-seq data sets from the University of Washington⁶⁶ were analysed in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature*. 1990; 345:458–460. [PubMed: 2342578]
2. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. *Microbiol Mol Biol Rev*. 2002; 66:407–425. [PubMed: 12208997]
3. Stewart SA, Weinberg RA. Telomeres: cancer to human aging. *Annu Rev Cell Dev Biol*. 2006; 22:531–557. [PubMed: 16824017]
4. Verdun RE, Karlseder J. Replication and protection of telomeres. *Nature*. 2007; 447:924–931. [PubMed: 17581575]
5. Günes C, Rudolph KL. The role of telomeres in stem cells and cancer. *Cell*. 2013; 152:390–393. [PubMed: 23374336]
6. Counter CM. The roles of telomeres and telomerase in cell life span. *Mutat Res*. 1996; 366:45–63. [PubMed: 8921986]
7. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]
8. Low KC, Tergaonkar V. Telomerase: central regulator of all of the hallmarks of cancer. *Trends Biochem Sci*. 2013; 38:426–434. [PubMed: 23932019]
9. Ghosh A, et al. Telomerase directly regulates NF- κ B-dependent transcription. *Nat Cell Biol*. 2012; 14:1270–1281. [PubMed: 23159929]
10. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer*. 2011; 11:161–176. [PubMed: 21346783]

11. Koh CM, et al. Telomerase regulates MYC-driven oncogenesis independent of its reverse transcriptase activity. *J Clin Invest.* 2015; 125:2109–2122. [PubMed: 25893605]
12. Li Y, Tergaonkar V. Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. *Cancer Res.* 2014; 74:1639–1644. [PubMed: 24599132]
13. Horn S, et al. TERT promoter mutations in familial and sporadic melanoma. *Science.* 2013; 339:959–961. [PubMed: 23348503]
14. Huang FW, et al. Highly recurrent TERT promoter mutations in human melanoma. *Science.* 2013; 339:957–959. [PubMed: 23348506]
15. Greider CW. Wnt regulates TERT—putting the horse before the cart. *Science.* 2012; 336:1519–1520. [PubMed: 22723405]
16. Hoffmeyer K, et al. Wnt/ β -catenin signaling regulates telomerase in stem cells and cancer cells. *Science.* 2012; 336:1549–1554. [PubMed: 22723415]
17. Shin EM, et al. DEAD-box helicase DP103 defines metastatic potential of human breast cancers. *J Clin Invest.* 2014; 124:3807–3824. [PubMed: 25083991]
18. Tong L, Tergaonkar V. Rho protein GTPases and their interactions with NF κ B: crossroads of inflammation and matrix biology. *Biosci Rep.* 2014; 34:283–295.
19. Yin L, Hubbard AK, Giardina C. NF- κ B regulates transcription of the mouse telomerase catalytic subunit. *J Biol Chem.* 2000; 275:36671–36675. [PubMed: 10970902]
20. Yang W, et al. EGFR-induced and PKC ϵ monoubiquitylation-dependent NF- κ B activation upregulates PKM2 expression and promotes tumorigenesis. *Mol Cell.* 2012; 48:771–784. [PubMed: 23123196]
21. Sampl S, et al. Expression of telomeres in astrocytoma WHO grade 2 to 4: TERRA level correlates with telomere length, telomerase activity, and advanced clinical grade. *Transl Oncol.* 2012; 5:56–65. [PubMed: 22348177]
22. Brennan CW, et al. The somatic genomic landscape of glioblastoma. *Cell.* 2013; 155:462–477. [PubMed: 24120142]
23. Heidenreich B, et al. Telomerase reverse transcriptase promoter mutations in primary cutaneous melanoma. *Nat Commun.* 2014; 5:3401. [PubMed: 24569790]
24. Killela PJ, et al. TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc Natl Acad Sci USA.* 2013; 110:6021–6026. [PubMed: 23530248]
25. Babayeva ND, et al. Structural basis of Ets1 cooperative binding to palindromic sequences on stromelysin-1 promoter DNA. *Cell Cycle.* 2010; 9:3126–3134.
26. Newman JA, Cooper CDO, Aitkenhead H, Gileadi O. Structural insights into the auto-regulation and cooperativity of the human transcription factor Ets-2. *J Biol Chem.* 2015; 290:8539–8549. [PubMed: 25670864]
27. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF- κ B signaling pathways. *Nat Immunol.* 2011; 12:695–708. [PubMed: 21772278]
28. Akincilar SC, et al. Quantitative assessment of telomerase components in cancer cell lines. *FEBS Lett.* 2015; 589:974–984. [PubMed: 25749370]
29. Yi X, Shay JW, Wright WE. Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. *Nucleic Acids Res.* 2001; 29:4818–4825. [PubMed: 11726691]
30. Roos C, et al. Soluble and transmembrane TNF-like weak inducer of apoptosis differentially activate the classical and noncanonical NF- κ B pathway. *J Immunol.* 2010; 185:1593–1605. [PubMed: 20610643]
31. Salzmann S, et al. Fibroblast growth factor inducible (Fn14)-specific antibodies concomitantly display signaling pathway-specific agonistic and antagonistic activity. *J Biol Chem.* 2013; 288:13455–13466. [PubMed: 23532848]
32. Heusch M, Lin L, Geleziunas R, Greene WC. The generation of nfkb2 p52: mechanism and efficiency. *Oncogene.* 1999; 18:6201–6208. [PubMed: 10597218]
33. Ghosh S, Karin M. Missing pieces in the NF- κ B puzzle. *Cell.* 2002; 109:S81–S96. [PubMed: 11983155]

34. Sun SC. Non-canonical NF- κ B signaling pathway. *Cell Res.* 2011; 21:71–85. [PubMed: 21173796]
35. Razani B, Reichardt AD, Cheng G. Non-canonical NF- κ B signaling activation and regulation: principles and perspectives. *Immunol Rev.* 2011; 244:44–54. [PubMed: 22017430]
36. Siggers T, et al. Principles of dimer-specific gene regulation revealed by a comprehensive characterization of NF- κ B family DNA binding. *Nat Immunol.* 2012; 13:95–102. [PubMed: 22101729]
37. Zhao B, et al. The NF- κ B genomic landscape in lymphoblastoid B cells. *Cell Rep.* 2014; 8:1595–1606. [PubMed: 25159142]
38. Sun W, et al. TherMos: estimating protein–DNA binding energies from *in vivo* binding profiles. *Nucleic Acids Res.* 2013; 41:5555–5568. [PubMed: 23595148]
39. Kumar V, et al. Uniform, optimal signal processing of mapped deep-sequencing data. *Nat Biotechnol.* 2013; 31:615–622. [PubMed: 23770639]
40. Li H, et al. The sequence alignment/map format and SAMtools. *Bioinformatics.* 2009; 25:2078–2079. [PubMed: 19505943]
41. Joseph R, et al. Integrative model of genomic factors for determining binding site selection by estrogen receptor- α . *Mol Syst Biol.* 2010; 6:456. [PubMed: 21179027]
42. Bonizzi G, et al. Activation of IKK α target genes depends on recognition of specific κ B binding sites by RelB:p52 dimers. *EMBO J.* 2004; 23:4202–4210. [PubMed: 15470505]
43. Fusco AJ, et al. Stabilization of RelB requires multidomain interactions with p100/p52. *J Biol Chem.* 2008; 283:12324–12332. [PubMed: 18321863]
44. Ramakrishnan P, Wang W, Wallach D. Receptor-specific signaling for both the alternative and the canonical NF- κ B activation pathways by NF- κ B-inducing kinase. *Immunity.* 2004; 21:477–489. [PubMed: 15485626]
45. Ishii N, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol.* 1999; 9:469–479. [PubMed: 10416987]
46. Bassuk AG, Anandappa RT, Leiden JM. Physical interactions between Ets and NF- κ B/NFAT proteins play an important role in their cooperative activation of the human immunodeficiency virus enhancer in T cells. *J Virol.* 1997; 71:3563–3573. [PubMed: 9094628]
47. Longoni N, et al. ETS transcription factor ESE1/ELF3 orchestrates a positive feedback loop that constitutively activates NF- κ B and drives prostate cancer progression. *Cancer Res.* 2013; 73:4533–4547. [PubMed: 23687337]
48. Kar A, Gutierrez-Hartmann A. Molecular mechanisms of ETS transcription factor-mediated tumorigenesis. *Crit Rev Biochem Mol Biol.* 2013; 48:522–543. [PubMed: 24066765]
49. Jankowski A, Prabhakar S, Tiurny J. TACO: a general-purpose tool for predicting cell-type-specific transcription factor dimers. *BMC Genomics.* 2014; 15:208. [PubMed: 24640962]
50. Bell RJ, et al. Cancer The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. *Science.* 2015; 348:1036–1039. [PubMed: 25977370]
51. Yilmaz ZB, Weih DS, Sivakumar V, Weih F. RelB is required for Peyer's patch development: differential regulation of p52–RelB by lymphotoxin and TNF. *EMBO J.* 2003; 22:121–130. [PubMed: 12505990]
52. Franzoso G, et al. Requirement for NF- κ B in osteoclast and B-cell development. *Genes Dev.* 1997; 11:3482–3496. [PubMed: 9407039]
53. Hofmann J, Mair F, Greter M, Schmidt-Supprian M, Becher B. NIK signaling in dendritic cells but not in T cells is required for the development of effector T cells and cell-mediated immune responses. *J Exp Med.* 2011; 208:1917–1929. [PubMed: 21807870]
54. Lind EF, et al. Dendritic cells require the NF- κ B2 pathway for cross-presentation of soluble antigens. *J Immunol.* 2008; 181:354–363. [PubMed: 18566401]
55. Thu YM, et al. NF- κ B inducing kinase (NIK) modulates melanoma tumorigenesis by regulating expression of pro-survival factors through the β -catenin pathway. *Oncogene.* 2012; 31:2580–2592. [PubMed: 21963849]
56. Zhang W, et al. A NIK-IKK α module expands ErbB2-induced tumor-initiating cells by stimulating nuclear export of p27/Kip1. *Cancer Cell.* 2013; 23:647–659. [PubMed: 23602409]

57. Keats JJ, et al. Promiscuous mutations activate the noncanonical NF- κ B pathway in multiple myeloma. *Cancer Cell*. 2007; 12:131–144. [PubMed: 17692805]
58. Baldwin AS. Regulation of cell death and autophagy by IKK and NF- κ B: critical mechanisms in immune function and cancer. *Immunol Rev*. 2012; 246:327–345. [PubMed: 22435564]
59. Perkins ND. The diverse and complex roles of NF- κ B subunits in cancer. *Nat Rev Cancer*. 2012; 12:121–132. [PubMed: 22257950]
60. Dey A, et al. Hexamethylene bisacetamide (HMBA) simultaneously targets AKT and MAPK pathway and represses NF κ B activity: implications for cancer therapy. *Cell Cycle*. 2008; 7:3759–3767. [PubMed: 19029824]
61. Tergaonkar V, Pando M, Vafa O, Wahl G, Verma I. p53 stabilization is decreased upon NF κ B activation: a role for NF κ B in acquisition of resistance to chemotherapy. *Cancer Cell*. 2002; 1:493–503. [PubMed: 12124178]
62. Saccani S, Marazzi I, Beg AA, Natoli G. Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor κ B response. *J Exp Med*. 2004; 200:107–113. [PubMed: 15226358]
63. Lee DW, et al. The NF- κ B RelB protein is an oncogenic driver of mesenchymal glioma. *PLoS ONE*. 2013; 8:e57489. [PubMed: 23451236]
64. Cherry E, Lee D, Jung JU, Sitcheran R. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) promotes glioma cell invasion through induction of NF- κ B-inducing kinase (NIK) and noncanonical NF- κ B signaling. *Mol Cancer*. 2015; 14:9. [PubMed: 25622756]
65. Mashiko D, et al. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci Rep*. 2013; 3
66. ENCODE. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012; 489:57–74. [PubMed: 22955616]
67. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res*. 1997; 25:2595–2597. [PubMed: 9185569]
68. Ran FA, et al. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013; 8:2281–2308. [PubMed: 24157548]

Figure 1.

TWEAK-induced non-canonical NF- κ B signalling regulates *TERT* expression and telomerase function in C250T GBM cells. **(a)** GBM cell lines were treated with TWEAK for one or two days and analysed for *TERT* expression by qPCR. Plots depict relative fold change in mRNA expression. Data from one experiment are shown, which is representative of three independent experiments for T98G and U251 and two independent experiments for remaining cell lines. **(b)** Table showing *TERT* promoter mutation status of cell lines. Representative sequences of *TERT* promoter from cell lines harbouring C250T and C228T *TERT* mutations respectively are shown. **(c)** Relative telomerase activity of TWEAK-treated cells was quantified using the TRAP (telomere repeat amplification protocol) assay. Data from one experiment are shown, which is representative of three independent experiments for T98G, LN444, D247mg and LN229 and two independent experiments for U251 and

LN382. **(d)** ChIP was performed in TWEAK-treated cells using p52-, p65- or Pol II-specific antibodies and IgG as a negative control. Enrichment of *TERT* promoter DNA fragments in ChIP DNA was normalized to DNA input. Data shown represent $n=3$ independent ChIP assays for all cell lines. Error bars represent s.d. **(e)** C250T-mutant GBM cells transduced with shRNAs targeting p52, RelB, NIK or vector control were treated with TWEAK and analysed for relative *TERT* expression. Data shown represent the mean of two independent experiments. Student's *t*-test (two-tailed) was used for all statistical analyses: * $P < 0.05$; ** $P < 0.01$. All raw data are shown in Supplementary Table 2.

Figure 2. C250T *TERT* promoter mutation creates a p52-binding site. **(a)** Sequence logo of the p52 motif *de novo* discovered by TherMos (ref. 38) from the p52 ChIP-seq library³⁷. The 11-base-pair motif is palindromic with the left and right half-sites separated by a 1-bp spacer. **(b)** *In vivo* enrichment plot of the 11-base-pair p52 motif in two-dimensional G_R - G_L space. G_R : G-scores from the right half-site. G_L : G-scores from the left half-site. Plot shows the log₁₀-scale enrichment of G_R - G_L pairs in 100-bp regions centred on p52 ChIP-seq peaks relative to randomly chosen non-p52 binding regions. The area in the triangle illustrates the

full-site binding region and the areas in the two rectangles illustrate the half-site binding regions. (c) Double-stranded 26 base pairs DNA oligonucleotides spanning the *TERT* promoter region containing either the WT or the C250T mutation were designed as probes for EMSA analysis. T98G nuclear lysates were analysed by EMSA using WT or C250T probes. (d) EMSA analysis using Oct-1 control, NF- κ B consensus, WT or C250T probes and nuclear extracts from untreated or TNF- α -treated T98G cells. (e) EMSA supershift analysis using the indicated antibodies was performed on T98G nuclear lysates with WT or C250T probes. For lanes 3 and 4, anti-NF- κ B2 (Cell Signaling, 4882) and anti-NF- κ B2 (Santa Cruz, sc-298) were used respectively. (f) Nuclear extracts from untreated or TWEAK-treated T98G cells were analysed for binding to the C250T *TERT* promoter DNA-labelled probe using EMSA and a supershift assay with the indicated antibodies was performed using TWEAK-induced nuclear extracts. All EMSA images shown are representative of three independent experiments.

Figure 3.

Ectopic expression of NIK promotes telomerase function and proliferation of C250T GBM cells. (a) Relative *TERT* expression was analysed in T98G and U251 cells transfected with vector, human NIK WT or kinase-inactive mutant NIK (KK) vector constructs. Data are presented as mean $\pm$ s.e.m., $n=3$ extracts, each representing one independent experiment. (b) Colony-formation assay was performed in T98G and U251 cells transfected with vector, NIK WT or NIK KK plasmids. Representative images from two independent experiments are shown. (c) Plot depicting the average number of colonies counted (mean $\pm$ s.e.m.), $n=6$ fields assessed from two independent experiments in the above assay. (d) Colony-formation assay was performed in vector- or NIK WT-expressing T98G cells that were transfected with Ctrl siRNA or TERT siRNA. Representative images from two independent experiments are shown. (e) Plot depicting the average number of colonies counted (mean $\pm$ s.e.m.), $n=6$ fields from two independent experiments in the earlier assay. (f) Colony-formation assay was performed in vector- or NIK WT-expressing T98G cells that were transfected with Ctrl siRNA, RelA siRNA, RelB siRNA or NF- κ B2 siRNA. Representative images from two independent experiments are shown. (g) Plot depicting the average number of colonies counted per group (mean $\pm$ s.e.m.), $n=6$ fields from two independent

experiments in the previous assay. Student's *t*-test (two-tailed) was used for all statistical analyses where $**P < 0.01$, $***P < 0.001$. All raw data are shown in Supplementary Table 2.

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Figure 4.

Constitutive non-canonical NF- κ B activation promotes *in vivo* tumorigenicity of C250T GBM cells. **(a)** T98G cells expressing vector, NIK WT or NIK p52 shRNA were injected subcutaneously into 5 NOD-SCID mice per group. Picture shows tumours isolated from 4 mice implanted with the respective cells after 10 weeks. **(b)** Plot depicting average volume of subcutaneous xenograft tumours (mean $\pm$ s.e.m.) from $n=5$ mice per treatment group. **(c)** NOD-SCID mice were implanted with T98G cells expressing vector, NIK WT or NIK p52 shRNA ($n=3$ per group). Magnetic resonance imaging scans of representative orthotopic

glioma tumours (marked by white dashed lines) formed after 8 weeks are shown. Scale bar, 2 mm. **(d)** Plot of relative TERT IHC expression in GBM tumours containing the indicated *TERT* promoter mutation status—WT ($n=10$), C228T ($n=11$) and C250T ($n=8$), where n is the number of patient tumours; whiskers, maxima and minima number; box, upper/lower 25th percentile and centre line, median. $**P < 0.01$; Student's t -test (two-tailed). **(e)** Correlation between TERT and NIK expression in GBM tumours of the various *TERT* promoter mutation statuses. Linear regression test was performed for each analysis and the respective R and P values are indicated below. Data shown represent $n=10$ WT, $n=11$ C228T and $n=8$ C250T tumours. Refer to Supplementary Table 2 for raw data. **(f)** Representative IHC images of GBM tumours with the indicated *TERT* promoter mutation status are shown for NIK and TERT expression. IHC staining of GBM tumours with nonspecific IgG antibodies is presented in the last row. Scale bars, 200 μm .

Figure 5.

CRISPR/Cas9-mediated reversal of the C250T mutation abolishes p52 activation of the *TERT* promoter. (a) gRNAs targeting the *TERT* promoter at various loci adjacent to the C250T mutation site were cloned into pSpCas9 (BB) vector and co-transfected with a pCAG-EGFP plasmid^{28,65} containing a genomic fragment spanning the *TERT* promoter (TF2-TR2) to examine their efficiency. gRNA2 and gRNA8 target sequences that have high efficiency in targeting Cas9 endonuclease-mediated cleavage of the *TERT* promoter (shown here) were selected for CRISPR/Cas9-induced reversal of C250T mutation in T98G cells. Scale bars, 100 μ m. (b) The C250T mutation site was edited from a thymine to a cytosine

residue in the *TERT* promoter of T98G cells using the CRISPR/Cas9 system and DNA chromatograms spanning the C250T *TERT* promoter region of 4 different single-cell clones are shown. (c) Relative telomerase activity of C250T-positive and WT clones presented as fold change relative to T98G control cells. Data shown represent the mean of two independent experiments, each with three technical replicate samples. (d) Cell proliferation of C250T-positive, *TERT* het and WT clones was analysed by MTT assay. Plot depicts the mean absorbance from two independent assays, each with three technical replicate samples. (e) Colony-formation assay was performed in four CRISPR/Cas9 clones with various *TERT* promoter statuses. Representative images from two experiments are shown. (f) Relative *TERT* expression of untreated or TWEAK-stimulated CRISPR/Cas9 clones carrying either the C250T *TERT* mutation (C250T-positive clone) or the WT *TERT* promoter (het clones 1–2 and WT clone). Plots depict relative fold change in mRNA expression. Data shown represent the mean of two independent experiments. (g) Relative telomerase activity of untreated or TWEAK-treated C250T-positive and WT clones. Data shown represent the mean of two independent experiments. (h, i) ChIP was performed in control or TWEAK-treated C250T-positive and WT clones using p52-, p65- or Pol II-specific antibodies and IgG as a negative control. Enrichment of *TERT* promoter (h) and *BLC* promoter (i) DNA fragments in ChIP DNA was normalized to DNA input. $n=3$ independent ChIP assays per clone were performed. Error bars represent s.d. Student's *t*-test (two-tailed) was used for all statistical analyses; $*P < 0.05$; $**P < 0.01$. All raw data are shown in Supplementary Table 2.

Figure 6.

ETS factors regulate proliferation of GBM cells and interact with p52 at the C250T *TERT* promoter. **(a)** p52 interacts with both ETS1 and ETS2. Lysates of Flag-p52-overexpressing T98G cells were immunoprecipitated with anti-Flag beads and analysed by immunoblotting with the indicated antibodies. The relative molecular masses of ETS1 (red asterisks) and ETS2 are 55,412 and 53,001 respectively. Data shown are representative of three independent experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 7. **(b)** p52 ChIP followed by re-ChIP using ETS1/2-specific antibody and IgG was performed in control or TWEAK-treated cells. Enrichment of *TERT* promoter DNA is

represented as relative fold change to IgG. $n=3$ ChIP re-ChIPs were performed in three independent experiments. Error bars depict s.e.m. **(c, d)** Colony-formation assay was performed in two C250T-mutant GBM cell lines—T98G **(c)** and LN444 **(d)**—that were treated with Ctrl siRNA, ETS1 siRNA, ETS2 siRNA or NIK siRNA. Representative images from two experiments are shown. **(e, f)** Plot depicts average number of colonies counted (mean $\pm$ s.e.m.), $n=6$ fields per cell line assessed from two independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; Student's t -test, two-tailed. All raw data are shown in Supplementary Table 2.

Figure 7.

p52 cooperates with ETS factors at the C250T *TERT* promoter. **(a)** Cooperative binding between p52/RelB and ETS1 predicted by TACO (ref. 49). Top panel: motifs used in TACO. Middle panel: dimer motifs enriched in various cell lines with significant *P* values. Bottom panel: alignment of the C250T and C228T regions to the enriched dimer motifs. The mutated C250T and C228T residues are coloured red; the critical T residue is in blue. **(b)** Recombinant p52 and ETS1 proteins were analysed for cooperative binding on WT or C250T *TERT* promoter DNA-labelled probes with EMSA. Data shown are representative of

two independent experiments. (c) ChIP assay of unstimulated T98G cells using ETS1-specific antibody and IgG. Enrichment of TERT promoter DNA was normalized to input. Data shown represent the mean of two independent experiments that were performed with three technical replicates each. (d) T98G cells transfected with control siRNA, ETS1 siRNA or ETS2 siRNA were either untreated or TWEAK-stimulated and their percentage of *TERT* induction is shown. Data shown represent the mean of two independent experiments. (e) ChIP analysis of siRNA-treated T98G cells, following TWEAK induction, using p52, p65 or Pol II-specific antibodies and IgG as a negative control. Enrichment of *TERT* promoter DNA was normalized to input. Data shown are from one experiment that is representative of three independent experiments for p52 and Pol II ChIP and two independent p65 ChIP assays. (f) Relative telomerase activity of untreated or TWEAK-stimulated C250T-mutant GBM cells that were transfected with Ctrl siRNA, ETS1 siRNA or ETS2 siRNA. Data shown are from $n=3$ independent experiments for T98G, and the mean of two independent experiments for LN444 and D247mg. Error bars represent s.e.m. $**P < 0.01$; Student's *t*-test, two-tailed. All raw data are shown in Supplementary Table 2. (g) Proposed model for telomerase reactivation in human cancers harbouring the C250T *TERT* promoter mutation. The critical T residue for ETS1/2–p52 cooperative binding is indicated in blue in the dashed rectangle. The mutational hotspots are in red.