Cancer-specific loss of *TERT* activation sensitizes glioblastoma to DNA damage

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Abstract

Most glioblastomas (GBMs) achieve cellular immortality by acquiring a mutation in the telomerase reverse transcriptase (*TERT*) promoter. *TERT* promoter mutations create a binding site for a GA binding protein (GABP) transcription factor complex, whose expression is associated with *TERT* reactivation and telomere maintenance. Here, using biochemical and cell biology approaches, we show direct evidence that a specific GABP complex containing the subunit protein GABPB1L forms predominantly at the mutant *TERT* promoter, leading to *TERT* re-expression. Furthermore, we find that *TERT* promoter mutant GBM cells, unlike wild-type cells, are immediately dependent on GABPB1L for proliferation in cell culture and post-tumor establishment *in vivo*. Notably, when combined with frontline temozolomide (TMZ) chemotherapy, GABPB1L knockdown and the associated *TERT* reduction lead to an impaired DNA damage response that results in profoundly reduced growth of intracranial GBM tumors. Together, these findings provide new insights into the mechanism of cancer-specific *TERT* regulation, uncover rapid effects of *TERT* suppression in GBM maintenance, and establish GABPB1L inhibition, alone or in combination with chemotherapy, as a therapeutic strategy for *TERT*p mutant GBM.

Introduction

Primary glioblastoma (GBM) is the most common and lethal form of malignant brain cancer in adults. Current treatment strategies are limited, with a median survival of 12-15 months, and GBM progression leading to death within two years of diagnosis in 90% of cases[1-3]. In GBM, as well as the vast majority of other cancers, transcriptional activation of the telomerase reverse transcriptase (*TERT*) gene, which is normally silenced in somatic cells, is a key step in tumorigenesis[4, 5]. *TERT* encodes the catalytic subunit of telomerase, and its reactivation in cancer is thought to contribute to cell survival and immortalization[5-8]. Reduction of *TERT* thus has the potential to directly affect both short- and long-term cell viability, through telomere length-dependent and independent pathways[9-15]. However, therapeutic telomerase inhibition is toxic to normal telomerase-dependent stem and germline cells[16-18]. Therefore, understanding genetic contributors to aberrant *TERT* expression, as well as consequences of cancer-specific ablation of *TERT*, could provide new therapeutic avenues for GBM.

A major mechanism by which *TERT* is reactivated in cancer involves the acquisition of somatic mutations in the promoter of the *TERT* gene, which are the most common non-coding mutation in cancer, and the third most common mutation overall[19-24]. In particular, ~80% of primary GBMs contain one of two common single-nucleotide mutations that are both associated with *TERT* re-expression, and are referred to as G228A or G250A[20, 22, 25]. Both G-to-A transitions generate an identical 11 base pair sequence (plus strand: CCCGGAAGGGG) that creates a binding site for GA binding protein A (GABPA), an ETS-family transcription factor[26,27]. Interestingly, this *de novo* ETS binding site occurs in close proximity to a native ETS binding site in the *TERT* promoter (*TERT*p), which is also required for *TERT* reactivation[26]. GABP transcription factors are obligate multimers that consist of the DNA-binding GABPA subunit and a transactivating GABPB subunit. Reduced function of the long isoform of GABPB1 (GABPB1L) via indel mutations has previously been linked to *TERT* downregulation and long-term telomere

attrition[9]. This may provide a cancer-specific way to target TERT, particularly given that GABPB1L is dispensable for normal murine development, while GABPA and total GABPB1 are not[28, 29]. However, the extended time period required to induce cell death via progressive telomere shortening could limit the therapeutic potential of this approach for high-grade GBM. Thus, our understanding of the therapeutic potential of targeting this axis is limited. Here, we examine the specificity and binding affinity of GABPB1L for the mutant *TERT*p, as well as functional effects of its loss in a rapid, clinically relevant timeframe. Excitingly, we observe a synergistic effect between GABPB1L reduction and standard-of-care chemotherapy in *TERT*p mutant cells only, which may be due to an inhibited DNA damage response in cells with reduced *TERT* expression.

Results

GABPB1L-containing transcription factor complexes bind the mutant TERT promoter

Previous work has demonstrated that *TERT* promoter (*TERT*p) mutations create a binding site for the GABPA-B transcription factor complex[26]. However, the assembly and binding properties of GABP complexes at the wild-type and mutant *TERT*p have not previously been examined. *GABPB1* encodes two main transcript isoforms, a short isoform (*GABPB1S*) and a long isoform (*GABPB1L*). GABPB1S functions as a heterodimer with GABPA ($GABPA_1B_1$), while GABPB1L forms a heterotetramer ($GABPA_2B_2$) due to its unique terminal exon that contains a leucine zipper-like domain[30, 31]. The necessity for two proximal GABP binding sites in *TERT*p mutant cells for *TERT* reactivation is suggestive of the recruitment of a GABPB1L-containing heterotetramer[9, 26, 32] (Fig. 1A). *TERT* re-expression is regulated in part by GABPB1L[9], but presence of GABPB1L at the mutant *TERT* promoter has not been tested. To explore this, we purified the human GABPA-B1L heterodimer to directly assay *TERT* promoter DNA binding *in vitro* using an electrophoretic mobility shift assay (EMSA) (Fig. 1B). To determine the equilibrium binding constants, we titrated the heterodimer separately against a fixed

concentration of radiolabeled wild-type *TERT*p DNA or mutant *TERT*p DNA (G228A). We observed that GABPB1L-containing complexes bind with increased affinity to the mutant *TERT*p (Kd=109.4nM) compared to the wild-type *TERT*p (Kd=194.4nM) (Fig. 1B). These data demonstrate that *TERTp* mutations are sufficient to confer enhanced binding of the GABPA-B1L heterodimer. Furthermore, the required presence of the second GABP binding site alongside the tetramer forming B1L subunit suggest a model where a $GABPA_2B1L_2$ heterotetramer regulates the mutant *TERT*p.

In order to confirm the specificity of *TERT* regulation by GABPB1L in *TERT*p mutant cells, we made use of a doxycycline-inducible, microRNA-embedded short hairpin RNA (shRNA) expression system with a green fluorescent protein (GFP) marker reporting shRNA induction[33-35]. We used a machine learning algorithm[33] to design four shRNAs each targeting either GABPB1L or GABPB1S specifically. Doxycycline-mediated induction of GABPB1L and GABPB1S shRNA expression led to reductions in the respective mRNA and protein levels in a *TERT*p mutant cell line (Fig. 1C and Fig. S1A). Interestingly, only GABPB1L inhibition led to reductions in *TERT* and telomerase activity (Fig. 1D and Fig. S1B,C). In fact, GABPB1S inhibition resulted in moderately increased *TERT* expression, perhaps through a resultant increase in GABPB1L (Fig. 1D). Together with the biochemical findings, these data demonstrate that *TERT*p mutations are sufficient to enable preferential binding of a probable $GABPA_2B1L_2$ tetramer complex even in the absence of other regulatory factors, which is specifically associated with *TERT* re-expression in *TERT*p mutant cells.

GABPB1L knockout rapidly impairs proliferation of TERT promoter mutant GBM cells

For GBM therapy, short-term effects of *TERT* reduction, rather than canonical long-term consequences, would significantly increase the clinical relevance of targeting the GABP-TERT axis. Given the direct and specific role of GABPB1L on *TERT* reactivation in the context of *TERT*p mutations, our data raise the question of immediate functional consequences of

GABPB1L inhibition on *TERT*p mutant cell growth and viability. To assess short-term effects of complete GABPB1L deletion, we used CRISPR-Cas9 and single-guide RNAs (sgRNAs) targeting the intron and 3'UTR flanking the unique exon 9 of *GABPB1L*, or targeting *GABPB1L* exon 9 internally proximal to both termini (Fig. 2A, Fig. S2A,B, and Table S1). In competitive proliferation assays (Fig. 2B), targeting of *GABPB1L* with sgRNA pairs led to a significant dropout of *TERT*p mutant U-251 GBM cells over 14 days, while lesser to no effect was observed for *TERT*p wild-type LN-18 GBM cells (Fig. 2C). These results suggest that loss of *GABPB1L* quickly and selectively limits growth of *TERT*p mutant GBM.

To more broadly and specifically investigate the effects of complete, validated loss of *GABPB1L* in *TERT*p mutant versus wild-type (WT) cells, we compared a set of three *TERT*p mutant GBM cell lines (U-251, LN-229, and T98G) and four *TERT*p WT cell lines (HEK293T, HAP1, NHA-S2, and LN-18) by generating a total of 169 monoclonal isogenic derivatives. The *TERT*p WT lines include an immortalized human astrocyte line (NHA-S2) as well as a GBM line (LN-18). Sequencing of the *TERT* promoter confirmed *TERT*p mutation status (Fig. S2C,D). CRISPR-Cas9 methods enable the precise dissection of genetic dependencies through the generation and analysis of specific heterozygous and homozygous genetic deletions in isogenic cell lines. Therefore, all possible combinations of three intronic and three 3'UTR targeting guides were compared in HEK293T and U-251 cells to assess editing efficiency (Fig. S2E,F). Next, the two most efficient pairs of sgRNAs were used to edit all seven cell lines, and 169 monoclonal cell line derivatives were established, genotyped, and subjected to further analysis (Fig. S3A-J, and Fig. S4A-C).

Strikingly, we observed that while full homozygous knockout of GABPB1L occurred at an appreciable rate across *TERT*p WT cell lines (13-36%), we were able to generate only one full GABPB1L knockout line, LN229-C19, among the 76 *TERT*p mutant clones that were screened (Fig. 2D and Fig. S3A-J). Importantly, the discrepancy between *TERT*p WT and mutant cell lines did not appear to result from differences in overall editing efficacy between cell lines, as

equal numbers of heterozygous knockouts were observed (Fig. 2D and Fig. S3H). These data support the hypothesis that *TERT*p mutant cells with full knockout of GABPB1L may be either very slow-growing or rapidly nonviable.

GABPB1L inhibition slows growth of existing TERTp mutant tumors

We next assessed the therapeutic potential of GABPB1L inhibition by examining its effect on existing GBM tumor growth *in vivo*. GABPB1L was inhibited in established orthotopic intracranial GBM xenografts using our doxycycline-inducible shRNAs. We first tested the system for *in vivo* efficacy using a *bona fide* essential gene, Replication Protein A1 (RPA1), along with controls. Addition of doxycycline to U-251 cells stably transduced with an inducible vector encoding RPA1-targeting shRNAs resulted in reduced *RPA1* mRNA (Fig. S5A) and loss of cell fitness (Fig. S5B) *in vitro*, and prolonged animal survival *in vivo* (Fig. S5C,D). *Post mortem* tumor analysis showed that *in vivo* expression of the RPA1-targeting shRNA construct was reduced compared to controls (Fig. S5E), consistent with a survival advantage for loss of RPA1 inhibition[34].

We then made use of our GABPB1L-targeting shRNAs in order to assess the effect of GABPB1L inhibition on tumor growth. *In vitro*, shRNA-mediated GABPB1L reduction did not cause loss of cell fitness over a short 7-day timeframe (Fig. S5B). In contrast, two separate GABPB1L-targeting shRNAs tested in orthotopic intracranial xenografts of U-251 cells slowed tumor growth when induced seven days post tumor implantation (Fig. 2E) and increased median survival by 70-90% (Fig. 2F). Additionally, GFP and linked shRNA expression was maintained, but relative fluorescence intensity significantly decreased, for GABPB1L-targeting shRNAs compared to controls when analyzed *ex vivo* in dissociated tumor cells *post mortem* (Fig. S5f,g). This suggests a survival advantage for cells that reduce GABPB1L suppression. Similarly, while the third tested GABPB1L-targeting shRNA did not impact survival, its expression was downregulated in tumor cells on *post mortem* analysis (Fig. S5g,h). The context-dependency of

growth effects following shRNA-mediated GABPB1L reduction (as opposed to full loss) is in line with numerous studies reporting differences between *in vitro* and *in vivo* phenotypes[36-38]. Together, these data show that GABPB1L shRNA-mediated suppression in established tumors significantly prolongs animal survival, highlighting its therapeutic value.

GABPB2 promotes resistance to GABPB1L loss in TERTp mutant GBM cells

Given the potential therapeutic benefit of GABPB1L inhibition in *TERT*p mutant GBM, we chose to analyze heterozygous knockout clones generated from *TERT*p mutant lines compared to knockouts from *TERT*p WT lines in order to better understand the molecular impact of GABPB1L loss (Fig. 2D and Fig. S3I,J). As expected, full or heterozygous GABPB1L knockout led to reduction or loss of *GABPB1L* mRNA and protein. We also observed unchanged or upregulated total *GABPB1* mRNA (all transcript variants), and substantially upregulated *GABPB1S* mRNA and protein in all cell lines (Fig. S6A-F), supporting a scenario where all *GABPB1* transcripts are now *GABPB1S*. Notably, GABPB1L heterozygous knockout was associated with a decrease in *TERT* mRNA expression and reduced telomerase activity in *TERT*p mutant cells only (Fig. S6G-J), confirming regulation of *TERT* by GABPB1L in a *TERT*p mutant-specific manner.

The single *TERT*p mutant GABPB1L full knockout cell line (LN229-C19) that we obtained did not exhibit reduction of either *TERT* or telomerase activity (Fig. S5G,I,J), suggesting a compensatory mechanism to regulate *TERT* independent of GABPB1L. *GABPB1* and *GABPB2* are distinct genes with high sequence conservation that perform similar but separate functions, though GABPB2 functions exclusively as a heterotetramer with GABPA[30, 31], likely due to the presence of a leucine zipper-like domain in both GABPB1L and GABPB2 proteins (Fig. 3A, Fig. S7A). Since GABPB2 is expressed at lower levels than GABPB1 in GBM based on data from the TCGA Research Network (https://www.cancer.gov/tcga), we probed for possible GABPB2 upregulation. While deletion of GABPB1L did not affect *GABPB2* mRNA in

any other line, the LN229-C19 clone showed a 4.5-fold elevation in *GABPB2* transcript levels (Fig. 3B and Fig. S7B). In support of a possible compensatory upregulation mechanism, stable GABPB2 overexpression in wild-type U-251 cells rescued cell dropout following CRISPR editing of the *GABPB1* locus (Fig. 3C). In addition, small interfering RNA (siRNA)-mediated inhibition of *GABPB2* showed that *TERT* mRNA levels are responsive to GABPB2 knockdown in LN229-C19, but not in wild-type LN-229 cells or heterozygous knockout clones (Fig. 3D). These data suggest that GABPB2 upregulation may compensate functionally for *GABPB1L* deletion in *TERT*p mutant cells, and indicate that *TERT*p mutant GBM can tolerate total loss of GABPB1L in the context of alternative mechanisms rescuing *TERT* expression. This finding therefore reveals a possible resistance mechanism for GABPB1L-targeted therapies.

Increased response of GABPB1L-reduced GBM tumors to chemotherapy

Currently, most patients diagnosed with primary GBM are treated with a regimen that includes temozolomide (TMZ), a DNA alkylating chemotherapeutic agent that is most effective at eliminating cells that lack O-6-methylguanine-DNA methyltransferase (MGMT) through generation of single- and double-strand DNA breaks[39, 40]. Prior studies have indicated that reduction of *TERT* in *TERT*-expressing cells may sensitize cells to DNA damaging agents, including ionizing radiation and chemotherapy[41, 42]. Reduced *TERT* has been shown to prevent the repair of DNA breaks *in vitro* by inhibiting central components of the DNA damage response (DDR), such as histone H2AX phosphorylation (yH2AX), through an incompletely understood mechanism. Loss of yH2AX-mediated DNA damage signaling increases DNA-damage related senescence or cell death, possibly in part through a reduction of normal cell cycle arrest[41-44]. Therefore, we wondered whether GABPB1L-mediated *TERT* reduction in *TERT*p mutant GBM might promote TMZ response, enabling a possible path to GBM cell-specific chemotherapy sensitization.

U-251 cells expressing doxycycline-inducible shRNAs targeting either TERT or GABPB1L were treated with a dose titration of TMZ, and DDR was assessed through analysis of γH2AX levels. Compared to control, shRNA-mediated TERT inhibition significantly reduced upregulation of γH2AX upon treatment with TMZ for 20 hours (Fig. 4A and Fig. S8A,B). Strikingly, GABPB1L inhibition led to an equally blunted γH2AX DDR following TMZ treatment (Fig. 4A and Fig. S8B). Similarly, γH2AX upregulation was reduced in both U-251 and LN-229 clones with heterozygous GABPB1L knockout, compared to wild-type cells, in response to TMZ (Fig. S8C,D). The full GABPB1L knockout clone LN229-C19 with GABPB2 upregulation and maintained *TERT* expression exhibited γH2AX induction following TMZ that was similar to wild-type, consistent with this effect being mediated by *TERT* downregulation (Fig. S8D). Abnormal DDR signaling can lead to a blunting of the G2 cell cycle arrest commonly observed following treatment with DNA damaging agents such as TMZ, causing an increased loss of viability as cells continue to divide[43, 45, 46]. Here, we also observe reduced G2 arrest in both U-251 and LN-229 GABPB1L heterozygous knockout clones compared to both wild-type cells and LN-229 full GABPB1L knockout clone C19 (Fig. 4B, Fig. S8E-G). Lack of MGMT expression was confirmed in all cell lines and corresponding clones (Fig. S8H).

Both partial and full loss of H2AX, or inhibition of its phosphorylation, have been shown to significantly impact cell survival following exposure to DNA damage[47-49]. Similarly, inhibition of G2 arrest following TMZ administration is associated with decreased cell viability and growth, resulting in TMZ sensitization[45]. Therefore, we hypothesized that *TERT*p mutant GBM cells with reduced GABPB1L would be more sensitive to TMZ chemotherapy. We assessed the therapeutic implications of this finding *in vivo* through administration of a TMZ regimen following orthotopic intracranial xenograft of either U-251 or LN-229 wild-type and heterozygous GABPB1L knockout cells (Fig. 4C). Initial analysis of GABPB1L heterozygous knockout cells in the absence of combination therapy *in vivo* demonstrated that GABPB1L reduction is sufficient to slow growth of both U-251 and LN-229 tumors and increase animal survival (Fig. S8I-L),

while full knockout LN229-C19 with maintained *TERT* formed tumors at a similar rate to WT cells.

Surprisingly, *in vivo* administration of chemotherapy in combination with GABPB1L heterozygous status ablated tumor growth of U-251 clones (Fig. 4D,E and Fig. S8M,N). Specifically, the combination of TMZ treatment and GABPB1L reduction increased median animal survival beyond that of a simple addition of effects (Fig. 4D,E). This potentiation of treatment efficacy was also observed, though to a lesser extent, in GABPB1L-reduced LN-229 tumors, again with the expected exception of the full knockout LN229-C19 clone that maintained *TERT* expression (Fig. 4F,G and Fig. S8O,P). The dramatic anti-tumor effects of TMZ combined with GABPB1L reduction and associated *TERT* downregulation are of significant clinical interest, as they indicate a path to increased TMZ response in *TERT*p mutant GBM cells specifically. Thus, together, our data suggest that *MGMT*-silenced *TERT*p mutant GBM could be effectively targeted through a combination of GABPB1L reduction and TMZ treatment.

Discussion

A major challenge in glioblastoma therapy is finding methods to reduce growth of established tumors while leaving normal cells unaffected. Here, our data demonstrate direct and specific binding of a GABPB1L-containing tetramer to the mutant but not wild-type *TERT* promoter (*TERT*p) for *TERT* reactivation. We show that this regulatory mechanism results in rapid anti-growth effects upon GABPB1L inhibition *in vitro* and *in vivo* on *TERT*p mutant cells, in the absence of alternative compensation for *TERT* expression. Thus, these results provide the first evidence that even after tumor initiation, *GABPB1L* inhibition rapidly impairs growth of GBM tumors *in vivo*. Surprisingly, we also find that reduced *GABPB1L* combined with TMZ treatment dramatically potentiates anti-tumor effects, emphasizing the clinical potential of GABPB1L inhibition *in vivo*. Targeting of *TERT* through the long isoform of GABPB1 (GABPB1L) specifically is particularly promising given that both GABPA and total GABPB1 (the short and

long isoform together) are required for normal murine development, while the GABPB1L isoform is dispensable[9, 28, 29].

Mechanistically, the early effects seen in response to full knockout of GABPB1L are inconsistent with the expected timeframe associated with canonical telomere shortening[50, 51]. Prior studies have demonstrated an association between reduced GABPB1L function and telomere attrition over a protracted timeframe in GABPB1L-edited clones[9], but this may in part have resulted from clones that were less sensitive to *TERT* downregulation preferentially surviving through the selection process, as well as differences between indel-based editing and full knockout. Our work here demonstrates that that GABPB1L loss has rapid, near-term effects on cell viability and growth. This could be due to an immediate vulnerability of cells with short telomeres, or involvement of non-canonical *TERT* and telomerase functions[10-12], which future studies should explore. Additionally, whether these effects are due to reduction of *TERT* alone, or result from a combination of *TERT* reduction and other effects of GABPB1L loss, remains to be tested.

Precision medicine approaches for targeted cancer therapy have the potential to be highly impactful, but cancer cells commonly develop resistance to these anti-tumor drugs[52]. Uncovering mechanisms underlying such effects often allows for the generation of combination therapies to circumvent resistance. In this context, our work points to sporadic GABPB2 upregulation as a possible resistance mechanism to loss of GABPB1L. Thus, our results provide preliminary evidence that GABPB2, which is typically expressed only at very low levels in GBM, could be targeted in conjunction with GABPB1L for increased treatment robustness. Multiple other transcription factors have also been linked to TERT regulation, both in normal and oncogenic contexts[53-56]. Such factors could play a role in basal expression of *TERT* off the mutant promoter, compensation for loss of GABPB1L, or both, and merit future study.

Previous studies have observed that *TERT* reduction sensitizes cells to chemotherapy and radiation by inhibiting the normal DDR, measured in part by a loss of yH2AX upregulation[42],

which is involved in DDR signaling. Here, our data show that GABPB1L inhibition and associated *TERT* downregulation reduces the yH2AX response and normal G2 cell cycle arrest following exposure of *TERT*p mutant cells to standard-of-care TMZ chemotherapy. The mechanism of action of TMZ is largely through mismatch repair pathway-associated single strand breaks, but also results in DNA double strand breaks, and yH2AX signaling may occur in response to single as well as canonically to double strand breaks[39, 40, 57, 58]. Loss of yH2AX signaling leads to reduced cancer cell viability following exposure to DNA damaging agents such as radiation and chemotherapy[43, 44, 49]. Accordingly, we find that *TERT*p mutant GBM tumors with reduced GABPB1L and *TERT* show a dramatically increased response to standard of care TMZ chemotherapy *in vivo.* Further confirming these findings, we observe that a *TERT*p mutant GABPB1L knockout clone with maintained *TERT* expression (through endogenous GABPB2 upregulation) does not exhibit a loss of yH2AX upregulation, change in cell cycle dynamics, or increased sensitivity in response to TMZ. We hypothesize that inhibiting GABPB1L in a *TERT*p wild-type line would also not promote sensitivity to TMZ, due to lack of *TERT* downregulation. However, we were unable to test this given the scarcity of *TERT*p wild-type GBM lines, the vast majority of which maintain *MGMT* expression, making them TMZ insensitive.

Together with our prior work[9], these data suggest that GABPB1L inhibition is a viable therapeutic strategy for *TERT*p mutant GBM, and provide new mechanistic and functional insights into the consequences of GABPB1L ablation. Importantly, the effects of combining GABPB1L reduction and TMZ treatment *in vivo* highlight the potential to improve disease outcomes in a tumor-specific manner. These findings have exciting implications for the treatment of primary GBM, and possibly the many other cancers harboring *TERT*p mutations.

Methods

Protein purification

GABPB1L and GABPA subunits were cloned into pET-Duet-1 vector with gibson assembly. The GABPB1L subunit was inserted after the first ribosome binding site and a plasmid encoded HisTag. HisTag and GABPB1L were separated by insertion of TEV cleavage sequence. GABPA was inserted after the second ribosome binding site without any affinity purification tags. GABPB1L and GABPA were expressed in Rosetta DE3 cells. A single colony was used for an overnight starter culture in Luria-Bertani media (LB) with 100 µg/ml Ampicillin. Next, bacteria from starter culture was grown in Terrific Broth (TB) media with 100 µg/ml of Ampicillin at 37°C until OD_{600} 0.8-1. Cells were chilled on ice and induced with 0.5 mM IPTG and grown overnight at 16°C. Cells were harvested via centrifugation, resuspended in 50 mM HEPES pH 7 500 mM NaCl, 1 mM TCEP, 5% (v/v) glycerol and flash frozen. Cell pellets were thawed and 0.5 mM PMSF and cOmplete protease inhibitor (Roche) were added. Cells were lysed by sonication and lysates were cleared by centrifugation. GABPA-B1L heterodimers were purified first through Ni-NTA Superflow resin (Qiagen). Column was equilibrated in 50 mM HEPES pH 7, 500 mM NaCl, 1mM TCEP, 5% glycerol, 10mM imidazole. Cleared lysate was incubated with resin for 30 min on a rocker at 4°C. Resin was then washed with the above equilibration buffer and protein was eluted with 50 mM HEPES pH 7, 500 mM NaCl, 1 mM TCEP, 5% (v/v) glycerol, 500 mM imidazole. Fractions containing both subunits were combined and dialysed overnight in Slide-A-Lyzer (ThermoFisher) with addition of TEV protease in 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP, 5% (v/v) glycerol. Heterodimers were then purified through Ni HiTrap HP (GE) and Heparin HiTrap column (GE) or Q HiTrap HP using dialysis buffer as buffer A and 50 mM HEPES pH 7.5 1 M KCl, 1 mM TCEP, 5% (v/v) glycerol as eluting buffer B. Fractions containing heterodimers were combined, concentrated and loaded on Superdex 200 Increase 10/300 equilibrated with 20 mM HEPES pH 7.5, 200 mM KCl, 1 mM TCEP, 5% (v/v) glycerol. Purified protein was concentrated and flash frozen for storage at -80°C.

Electrophoretic Mobility Shift Assay

Single stranded 60bp oligonucleotides with wild type *TERT*p including native ETS sites (5'-AGGGCGGGGCCG**CGGAAAGGAA**GGGGAGGGGCTGGGAGGGCCCGGAGGGGGCTG GGCCGG - 3') and mutant *TERT*p with native and *de novo* ETS sites created by G228A mutation (5'- AGGGCGGGGCCG**CGGAAAGGAA**GGGGAGGGGCTGGGAGGGCC**CGGAa**G GGGCTGGGCCGG - 3') were obtained from Integrated DNA Technologies (IDT) and were Urea - PAGE purified, ethanol precipitated and resuspended in DEPC. One of the strands of each sequence was then labeled with P^{32} as follows: 10 pmols of single stranded DNA was incubated with 5 Units of T4 PNK (NEB) and 4.76µCi of P^{32}-γ-ATP (Perkin-Elmer) in PNK buffer in total volume of 25µl for 30 min at 37°C and heat inactivated at 65°C for 20min. Reaction was diluted by addition of 25µl of DEPC. Excess of ATP was removed on desalt G25 (GE) column. Purified oligos were then further diluted by addition of 50µl of DEPC. Labeled oligos were annealed with corresponding unlabeled oligo in 20 mM Tris pH 7.5, 100 mM LiCl, 1 mM $MgCl_2$. These double stranded substrates were used in binding experiments with GABPA-B1L heterodimers.

GABPA-B1L heterodimers were thawed and diluted to stock concentrations of 2 µM, 1 µm and 750 nM in Superdex 200 buffer. DNA oligonucleotides were diluted to 1 nM stock concentration in annealing buffer. Reactions were performed by mixing 0.2 nM 60bp DNA with increasing concentrations of GABPA-B1L heterodimers (0-900nM) with reaction buffer and KCl to obtain 20 mM Tris pH7.5, 100 mM KCl, 5.2 mM $MgCl_2$, 1 mM TCEP, 5% (v/v) glycerol in 15 µl final reaction volume. Reactions were incubated at room temperature for 1 hr and then 5µl were loaded on 5% (v/v) native 0.5X TBE PAGE gel with 5 mM $MgCl_2$, which was prerun at 8W for ~1hr in 4°C. Gels were running in 0.5X TBE buffer with 5 mM $MgCl_2$ for ~2.5hr at 8W in 4°C. Gels were dried and phosphorous screen was exposed with the gel overnight. Gels were imaged on Typhoon and quantified using ImageQuant software. Fraction of promoter sequence

bound was calculated by dividing the signal of bound DNA fraction by the sum of bound and unbound signals in each lane. Data points were plotted and fitted using GraphPad Prism8 software with the following equation:

$$Y = \frac{Bmax \cdot X^h}{Kd^h + X^h}$$

to obtain dissociation constant (Kd) and Hill coefficient (h). Statistics for the analysis were obtained from three replicates per condition tested. Replicates were obtained by preparing three 2 μM heterodimer stocks which were further serially diluted to create 1 μM and 750 nM concentration stocks. These three different sets of stocks were used to perform one replicate of heterodimer titration from 0-900 nM protein concentration per condition tested.

Mammalian cell culture

All mammalian cell cultures were maintained in a 37°C incubator at 5% CO_2. HEK293T human kidney cells (293FT; Thermo Fisher Scientific, #R70007; RRID:CVCL_6911), NHA-PC5 normal human astrocyte cells (a kind gift from Russell Pieper, UCSF), and derivatives thereof were grown in Dulbecco's Modified Eagle Medium (DMEM; Corning Cellgro, #10-013-CV) supplemented with 10% fetal bovine serum (FBS; Seradigm #1500-500), and 100 Units/ml penicillin and 100 μg/ml streptomycin (100-Pen-Strep; Gibco, #15140-122). U-251 human glioblastoma cells (Sigma-Aldrich, #09063001; RRID:CVCL_0021), LN-229 human glioblastoma cells (American Type Culture Collection (ATCC), #CRL-2611; RRID:CVCL_0393), T98G human glioblastoma cells (ATCC, #CRL-1690; RRID:CVCL_0556), LN-18 human glioblastoma cells (ATCC, #CRL-2610; RRID:CVCL_0392), and derivatives thereof were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco, #11320-033 or Corning Cellgro, #10-090-CV) supplemented with 10% FBS and 100-Pen-Strep. HAP1 cells (a kind gift from Jan Carette, Stanford University) and derivatives thereof were grown in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, #12440-053 or HyClone, #SH30228.01) supplemented with

10% FBS and 100-Pen-Strep. HAP1 cells had been derived from the near-haploid chronic myeloid leukemia cell line KBM7[59].

U-251, LN-229, T98G, LN-18, HEK293T, and HAP1 cells were authenticated using short tandem repeat DNA profiling (STR profiling; UC Berkeley Cell Culture/DNA Sequencing facility). STR profiling was carried out by PCR amplification of nine STR loci plus amelogenin (GenePrint 10 System; Promega, #B9510), fragment analysis (3730XL DNA Analyzer; Applied Biosystems), comprehensive data analysis (GeneMapper software; Applied Biosystems), and final verification using supplier databases including ATCC and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

U-251, LN-229, T98G, LN-18, NHA-PC5, HEK293T, and HAP1 cells were initially tested for absence of mycoplasma contamination (UC Berkeley Cell Culture facility) by fluorescence microscopy of methanol fixed and Hoechst 33258 (Polysciences, #09460) stained samples. Lack of mycoplasma contamination was confirmed every 6 months using MycoAlert mycoplasma detection kit (Lonza, #LT07-418).

Establishment of a puromycin-sensitive normal human astrocyte cell line

The normal human astrocyte cell line NHA-PC5 was previously described[51], and is puromycin resistant. For our experiments, we established a puromycin-sensitive monoclonal derivative, termed NHA-S2, through CRISPR editing of the previously inserted puromycin resistance cassette. In brief, NHA-PC5 cells were transiently transfected in 6-well plates with a pair of plasmid vectors (pCF120), each encoding Cas9 and a guide RNA targeting the puromycin resistance marker (sgPuro5 and sgPuro6, Table S1), or a negative control. Transfections were carried out using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's procedures (500 ng vector 1, 500 ng vector 2). For construction of pCF120 see corresponding section (Plasmid and lentiviral vectors). Starting at 24 h post-transfection, NHA-PC5 cells were selected on hygromycin B (500 µg/ml; Thermo Fisher Scientific, #10687010) for four days.

Subsequently, the selected cells were seeded using limiting dilution, and 16 clones picked 17 days later. Clones were further expanded and tested for puromycin (1.0 µg/ml; InvivoGen, #ant-pr-1) sensitivity. All 11 out of 11 edited NHA-PC5 clones that grew well were puromycin sensitive. Five of these clones, NHA-S1/S2/S3/S7/S9 (S for puromycin sensitive), were further expanded and tested for hygromycin B (500 µg/ml) sensitivity to ensure that the plasmid vectors (pCF120) used for editing did not integrate into the genome. NHA-S3 were hygromycin B resistant, NHA-S1 were partially resistant, and NHA-S2/S7/S9 showed sensitivity. From the latter three, NHA-S2 were chosen as the cell line for further experiments based on morphology and cell growth. NHA-S2 were authenticated using short tandem repeat DNA profiling (STR profiling; UC Berkeley Cell Culture/DNA Sequencing facility) to be derived from NHA-PC5. NHA-PC5 and NHA-S2 were stored in freezing medium consisting of 5% DMSO, 40% FBS, and 55% growth medium.

Sequencing of *TERT* promoter mutation status

To sequence *TERT* promoter mutation status, genomic DNA was extracted from cell lines using QuickExtract DNA Extraction Solution (Epicentre, #QE09050). Specifically, samples were incubated at 65°C for 20 minutes followed by 20 minutes at 98°C. The *TERT* promoter locus was amplified with the primers oCF1039_TERTp-fw2 (CAGGGCCTCCACATCATGG) and oCF1038_TERTp-rev1 (CCTCGCGGTAGTGGCTG), yielding a 532 bp product. PCRs were carried out using Q5 High-Fidelity DNA polymerase (New England Biolabs, #M0491) with an annealing temperature of 70°C, 20 seconds elongation time, 32 PCR cycles, and standard Q5 buffer with 10% High-GC Enhancer due to elevated G/C content of this locus. Sanger sequencing of the *TERT* promoter locus was carried out, after gel purification of the PCR bands, using the primers oCF1039_TERTp-fw2 and oCF1038_TERTp-rev1. Sequences were analyzed using SnapGene software (GSL Biotech).

Plasmid and lentiviral vectors

To generate monoclonal knockout cell lines, sgRNAs and Cas9 were expressed from either the pCF120 or pCF123 vectors. In brief, the plasmid vector pCF120, expressing a U6 promoter driven sgRNA and an EF1-alpha short (EFS) promoter driven Cas9-P2A-Hygro cassette, was derived from pX459[60] by replacing the chicken beta-actin promoter with an EFS promoter from pCF204[61], swapping the T2A-Puro cassette with a P2A-Hygro cassette, and exchanging the guide RNA scaffold for a more efficient variant[62]. The plasmid vector pCF123, expressing a U6 driven sgRNA and an EFS driven Cas9-T2A-Puro cassette, was generated based on pX459[60] by replacing the chicken beta-actin promoter with an EFS promoter and exchanging the guide RNA scaffold for a more efficient variant[62].

For CRISPR-Cas9-mediated competitive proliferation assays, single-guide RNAs (sgRNAs) were expressed from the previously established pCF221 lentiviral vector and Cas9 from the pCF226 lentiviral vector[61]. For low-level stable overexpression of *GABPB2* (NCBI gene ID: 126626) and mTagBFP2 (negative control), we first modified the pCF525-mTagBFP2 lentiviral vector[63], encoding an EF1a-Hygro-P2A-mTagBFP2 cassette, by replacing the strong EF1-alpha promoter with a weak EF1-alpha short (EFS) promoter. This new vector, EFS-Hygro-P2A-mTagBFP2, was named pCF554. Next, we cloned the coding sequence (CDS) of homo sapiens GA binding protein transcription factor subunit beta 2 (GABPB2), transcript variant 1, mRNA (NCBI reference sequence: NM_144618.2) into pCF554 by replacing mTagBFP2, yielding lentiviral vector pCF553. The *GABPB2* CDS was synthesized as gBlocks (Integrated DNA Technologies). For doxycycline-inducible miR-E shRNA expression, both in cell culture and *in vivo*, we used the lentiviral vector LT3GEPIR[35]. LT3GEPIR provides a GFP marker reporting shRNA expression. Full sequences of all vectors are provided (Table S1). All vectors were cloned and/or sequence verified using standard molecular biology techniques, enzymes from New England Biolabs (NEB), and oligonucleotides from Integrated DNA Technologies (IDT).

Lentiviral transduction

Lentiviral particles were produced in HEK293T cells using polyethylenimine (PEI; Polysciences #23966) based transfection of plasmids, as previously described[61]. In brief, lentiviral vectors were co-transfected with the lentiviral packaging plasmid psPAX2 (Addgene #12260) and the VSV-G envelope plasmid pMD2.G (Addgene, #12259). Transfection reactions were assembled in reduced serum media (Opti-MEM; Gibco, #31985-070). For lentiviral particle production on 6-well plates, 1 µg lentiviral vector, 0.5 µg psPAX2 and 0.25 µg pMD2.G were mixed in 0.4 ml Opti-MEM, followed by addition of 5.25 µg PEI. After 20-30 min incubation at room temperature, the transfection reactions were dispersed over the HEK293T cells. Media was changed 12-14 h post-transfection, and virus harvested at 42-48 h post-transfection. Viral supernatants were filtered using 0.45 µm polyethersulfone (PES) membrane filters, diluted in cell culture media as appropriate in order to obtain similar levels of viral expression between cell lines and conditions, and added to target cells. Polybrene (5 µg/ml; Sigma-Aldrich) was supplemented to enhance transduction efficiency, if necessary.

Design of sgRNAs for CRISPR-Cas9 genome editing

Standard sgRNA sequences were either designed manually or using GuideScan[64]. To generate specific genomic excisions, rather than indels, pairs of sgRNAs were designed and used in tandem. This strategy was applied to remove sequences specific to the long isoform of *GABPB1L*, while not affecting the short isoform. All sgRNAs were designed with a G preceding the 20 nucleotide spacer for better expression from U6 promoters. All sgRNA sequences are shown (Table S1), and were cloned into the pCF120 and pCF123 vector using BbsI restriction sites and enzymes (New England Biolabs) or into the pCF221 vector using Esp3I restriction sites and enzymes (New England Biolabs). Control sequencing of sgRNAs was carried out with

the primer oCF112_U6seq2-fw (ACGATACAAGGCTGTTAGAGAG). Oligonucleotides for sgRNA cloning were purchased from IDT.

CRISPR-Cas9 competitive proliferation assay

CRISPR-Cas9 competitive proliferation assays were used to assess whether Cas9-mediated editing of genes of interest leads to a change in proliferation speed compared to unedited cells. First, cell lines (U-251, LN-18) were stably transduced with a lentiviral vector expressing Cas9 (pCF226) and selected on puromycin (1.0-2.0 µg/ml), as previously described[61]. Subsequently, Cas9 expressing cell lines were further stably transduced with pairs of lentiviral vectors (pCF221) expressing various mCherry-tagged sgRNAs. Volume of virus was adjusted as appropriate between cell lines to establish similar levels of infectivity, with ~2x more virus used in LN-18 cells than U-251 cells. At day two post-transduction, sgRNA expressing populations were mixed approximately 80:20 with parental cells and the fraction of mCherry-positive cells was quantified over time by flow cytometry (Attune NxT flow cytometer, Thermo Fisher Scientific). Different non-targeting sgRNAs (sgNT) and sgRNAs targeting a neutral gene (sgOR2B6), an essential gene (sgRPA1), and genes-of-interest were compared.

Design of shRNAs for reversible target inhibition

MicroRNA-embedded miR-E shRNA sequences were predicted using SplashRNA[33] and cloned into LT3GEPIR[35], an all-in-one doxycycline-inducible miR-E shRNA expression vector. All shRNA sequences are shown (Table S1), and were cloned as previously described[65]. Oligonucleotides for shRNA cloning were purchased from IDT.

RNAi competitive proliferation assay

To assess effects on cell proliferation mediated by reversible target inhibition, RNAi competitive proliferation assays were used. Cells stably transduced with LT3GEPIR inducibly expressing

various shRNAs were mixed with non-transduced parental cells and cultured in the absence or presence of doxycycline (1.0 µg/ml, Sigma-Aldrich). GFP fluorescence, functionally linked to shRNA expression, was quantified over time by flow cytometry (Attune NxT, Thermo Fisher Scientific).

Generation of isogenic GABPB1L knockout cell lines

To generate isogenic monoclonal knockout cell lines of the long isoform of GABPB1 (NCBI gene ID: 2553), pairs of sgRNAs flanking the long isoform-specific exon 9 of *GABPB1* were used (Fig. 1a). In brief, the sgRNAs sgGB.2, sgGB.3, sgGB.4, sgGB.12, sgGB.13, and sgGB.14 as well as sgPTEN.3 and sgPTEN.12 (negative controls) were cloned into the pCF123 plasmid vector (Table S1). To test genome editing and locus excision efficiency, every possible combination of a 5' flanking (intron) and a 3' flanking (3'UTR) sgRNA were tested alonside controls by transient transfection into both HEK293T and U-251 cells. Transfections were carried out in 12-well plates using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's procedures (250 ng vector 1, 250 ng vector 2). Starting at 24 h post-transfection, HEK293T and U-251 cells were selected on puromycin (HEK293T: 1.0 µg/ml, U-251: 0.5 µg/ml) for two days. Subsequently, cells were expanded and genomic DNA extracted (QuickExtract DNA Extraction Solution; Epicentre, #QE09050), as described above, for genotyping analysis. Genotyping, as described below, determined sgGB.2-12 and sgGB.3-14 to be the two most efficient guide pairs (Fig. S1f).

GABPB1L locus editing was assessed by genotyping with the primers oCF862_GAL-fw1 (AAAAGTACAGGTGCCCAGTTTG) and oCF863_GAL-rev2 (GCCTAACCAACAACGATCAC), yielding an 808 bp band in wild-type cells. Genotyping PCRs were carried out using Q5 High-Fidelity DNA polymerase (New England Biolabs, #M0491) with an annealing temperature of 65°C, 20 seconds elongation time, and 32 PCR cycles. Where mentioned, Sanger sequencing of the GABPB1L locus and TIDE analysis[66] were also carried out using the primers

oCF862_GAL-fw1 and oCF863_GAL-rev2. Perfect GABPB1L editing with the sgRNA pair sgGB.2-12 yields a 472 bp genotyping band, and with the sgGB.3-14 pair a 387 bp band.
To establish isogenic monoclonal GABPB1L knockout cell lines, TERT promoter wild-type (HEK293T, HAP1, NHA-S2, LN-18) and TERT promoter mutant (U-251, LN-229, T98G) cell lines were edited with pCF123-sgGB.2-12 and pCF123-sgGB.3-14 pairs in 12-well (250 ng vector 1, 250 ng vector 2) or 6-well (500 ng vector 1, 500 ng vector 2) plates, as described above. After selection on puromycin (0.5-3.0 µg/ml), the selected cells were seeded using limiting dilution, and clones picked approximately 10-15 days later. Clones were then expanded, genotyped (Fig. S2), and further cultured for secondary analysis and storage. A set of clones from each cell line were selected for further analysis based on clean sequencing of the edited locus, and normal morphology and growth. A mix of clones generated using each of the 2 different pairs of sgRNAs were selected for each cell line. Selected clones were genotyped a second time (Fig. 1d,e) and authenticated using short tandem repeat DNA profiling (STR profiling; UC Berkeley Cell Culture/DNA Sequencing facility) to ensure that monoclonal lines are derived from the intended parental cell line. Specifically, the following clones were authenticated by STR profiling: LN-229 C6, C10, C11, C19, C24, C26, C28, C30, LN-18 C4, C8, C11, C14, C21, C23, C24, C32, T98G C3, C4, C5, C9, C13, C20, and NHA-S2 C1, C4, C14, C23, C24, C29, C32.

Flow cytometry and fluorescence microscopy

Percentages of fluorophore-positive cells were quantified by flow cytometry (Attune NxT flow cytometer, Thermo Fisher Scientific), regularly acquiring 10,000-30,000 events per sample. Phase contrast imaging and fluorescence microscopy was carried out following standard procedures (EVOS FL Cell Imaging System, Thermo Fisher Scientific), routinely at least 48 h post-transfection or post-transduction of target cells with fluorophore expressing constructs.

RT-qPCR

Cells were seeded in 96 well plates in a volume of 100ul, at a concentration of 50 cells/ul. When cells reached ~80% confluency (3-4 days post-seeding), RNA isolation and cDNA generation was performed using the Cells-to-CT kit (Invitrogen, #4402954), per the manufacturer's instructions. Real-time quantitative PCR was performed using the POWER SYBR Green PCR Master Mix (Applied Biosystems, #4367659) to measure expression levels of *GUSB, TERT*, *GABPB1L*, total *GABPB1*, *GABPB1S*, *GABPB2*, *RPA1,* and *MGMT*. Each sample was measured in technical and biological triplicate on a QuantStudio 5 Real-Time PCR machine (Thermo Fisher Scientific). Manual inspection of melting curves was performed to confirm PCR specificity. Relative expression levels of biological triplicates were quantified using the deltaCT method against *GUSB*. Experiments were performed three times with separate RNA isolations for final quantification. Primer sequences are available in Table S1.

Immunoblotting

Cells were washed two times in cold PBS and harvested on ice with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, #78501), plus freshly added Turbonuclease (Sigma Aldrich, #T4330), and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, #1861282). Protein concentration was determined by Bicinchoninic acid (BCA) assay. Equal protein amounts were resolved on SDS-PAGE gels and electrotransferred to a PVDF membrane. Membranes were blocked with 5% BSA in Tris-buffered saline tween 20 (TBST 0.1%) or 5% milk in Tris-buffered saline tween 20 (TBST 0.1%) for 1 hour, and then probed with primary antibodies towards GABPB1 (Proteintech, #12597-1-AP; https://www.ptglab.com/products/GABPB1-Antibody-12597-1-AP.htm#validation), yH2AX (Cell Signaling, #80312S https://www.cellsignal.com/products/primary-antibodies/phospho-histone-h2a-x-ser139-d7t2v-mouse-mab/80312), or GAPDH (Millipore, #CB1001; http://www.emdmillipore.com/US/en/product/Anti-GAPDH-Mouse-mAb-6C5,EMD_BIO-

CB1001). Each immunoblot was performed a minimum of three times with separate protein samples.

Telomerase Repeated Amplification Protocol (TRAP)

Cy5-based TRAP assays were performed as previously described[67]. Briefly, 150,000 cells per condition were collected via trypsinization and resuspended in NP-40 lysis buffer (10mM Tris-HCl pH 8.0, 1mM $MgCl_2$, 1mM EDTA, 1% NP-40, 0.25mM sodium deoxycholate, 10% glycerol, 150mM NaCl, 5mM 2-mercaptoethanol, 0.1mM AEBSF) at a concentration of 2,500 cells/ul to maintain telomerase activity. 1 ul of lysate (or 1 ul of NP-40 lysis buffer for the negative control) was added to a PCR master mix containing a Cy5-labeled telomeric substrate primer, an internal standard control, and an extension primer mix, at the described ratios[67]. The following PCR protocol was used to amplify Cy5 telomeric primer extension products: 1) 25°C for 40 min; 2) 95°C for 5 min; 3) 27 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec; 4) 72°C for 10 min. Post-PCR samples were resolved on a polyacrylamide gel, and visualized using a BioRad Chemidoc Imager on the Cy5 setting. Data was visualized and analyzed using BioRad Image Lab software 6.0 and ImageJ with n=3-4 biological replicates.

Orthotopic xenografts and bioluminescence imaging

Animal procedures conformed to care guidelines approved by the UCSF Institutional Animal Care and Use Committee (IACUC protocol number: AN179550 and AN17599). U-251 and LN-229 cell lines were stably transduced with Firefly Luciferase Lentifect Purified Lentiviral Particles (Genecopoiea, #LPP-FLUC-Lv105) at an MOI of 3. All cell lines were verified to be stably expressing luciferase at similar levels *in vitro* 48 hours prior to xenograft. Either $4x10^5$ U-251 cells or $1x10^5$ LN-229 cells were injected in a 4ul total volume into the right frontal cortex (ML:1.5mm, AP:1mm, DV:3.5mm) of 6-7 week old female athymic *nu/nu* mice (The Jackson

laboratory; RRID:IMSR_JAX:002019). Sample sizes were estimated based on both the standard in the field, as well as power calculations using previously observed effect sizes published in experiments using similar techniques. Later experiments were re-estimated based on observed effect sizes in our own work. Animals were excluded from analysis if surgical complications arose immediately (within 48 hours) post-surgery. Approximate tumor size was monitored via bioluminescence imaging (Xenogen IVIS Spectrum Imaging System) 1-2x per week following intraperitoneal injection of luciferin reagent (GOLDBIO, #LUCK-1G) at 150mg/kg. Animals were weighed 3x per week, and once daily after initial weight loss was observed. General behavior and symptomology for all mice were recorded daily. Humane endpoints for sacrifice were hunched posture, >15% weight loss from maximum recorded weight, or neurologic symptoms, and were verified by approved UCSF veterinary technicians.

***In vivo* doxycycline administration and analysis**

Mice bearing xenografts of U-251 cells expressing doxycycline-inducible shRNAs were placed on either control or doxycycline chow at a dosage of 625 mg/kg (Dox Diet; Bio-Serv, #F5829) seven days post xenograft, when bioluminescence readings were in the $1x10^6$ (radiance) range. Mice were placed into an experimental condition post-xenograft (control versus doxycycline chow) via randomization based on tumor volume, as determined by bioluminescence imaging. Mice were maintained on appropriate chow until humane endpoint. For analysis of induced GFP expression in U-251 orthotopic xenografts, tumors were dissected from the brain *post mortem*, and dissociated into a single cell suspension through manual trituration followed by passage through a 100um cell strainer. Cells were washed 2x in FACS buffer (PBS + 2% fetal bovine serum + 0.05% Sodium Azide), then resuspended in FACS buffer containing 2 ug/ml APC/Cy7 anti-human HLA-A,B,C (BioLegend, #311425). Cells were incubated with the antibody on ice for 20 minutes in the dark. Cells were then washed 2x with FACS buffer, and resuspended in FACS buffer for flow cytometry analysis. GFP and Far Red (APC-Cy7) fluorescence were measured

by flow cytometry (Attune NxT, Thermo Fisher Scientific), routinely acquiring 10,000 events per sample. Percent of GFP positive tumor cells was calculated as the percentage of Far Red positive cells (human cells) that were also GFP positive.

Temozolomide administration *in vitro*

U-251 and LN-229 cells were seeded in 24 (600ul/well) well plates at a density of 500 cells/ul. For cells transduced with doxycycline-inducible shRNA constructs, cells were maintained on doxycycline at 1 ug/ml for 5 days prior to plating and through TMZ treatment paradigm. 24 hours post-seeding, cells were treated with the described concentrations of TMZ (Tocris, #85622-93-1), or a DMSO control, in a total volume of 2ul/ml, for 3 hours. TMZ drug was then removed. For yH2AX immunoblots, cells were harvested 20 hours post TMZ treatment. At the end of the 20 hour incubation, cells were harvested for analysis by western blot as described above and analyzed by quantification using Image J. For cell cycle analysis, cells in 24 well plates were harvested 72 hours post-TMZ treatment and fixed in 70% ethanol at 4°C overnight. Fixed cell pellets were washed 2x with cold PBS, resuspended in 300 ul FxCycle PI/RNAse solution (ThermoFisher Scientific, #F10797), and incubated at room temperature in the dark for 15 minutes. Samples were then analyzed by flow cytometry, using the Attune NxT (Thermo Fisher Scientific) flow cytometer, routinely acquiring 10,000 events per sample. Experiments were done in biological triplicates.

Temozolomide administration *in vivo*

Mice were placed into an experimental condition post-xenograft (temozolomide versus vehicle) via randomization based on tumor volume, as determined by bioluminescence imaging. Mice assigned to the temozolomide condition were given per os (p.o.) doses of Temodar temozolomide capsules (NDC, #43975-257-05) at either 50mg/kg for 10 days total (5 days on, 2 days off, for a period of 12 days; U-251 xenografts) or 10mg/kg for 5 days (5 consecutive days;

LN-229 xenografts). Temodar was resuspended in Ora-Plus oral suspension vehicle at either 5mg/ml (for 50mg/kg) or 1mg/ml (for 10mg/kg), to be administered at 10ml/kg (~0.2ml per 20g mouse). Mice assigned to the vehicle condition were given an equivalent volume of Ora-Plus without drug. Both Temodar and vehicle solutions were administered to mice by an experimenter blind to the xenograft condition. Simple additive effects of TMZ combined with GABPB1L knockout were calculated as $1+((M_y/M_x-1)+(M_z/M_x-1))$, where M_x=median survival of wild-type vehicle condition, M_y=median survival of wild-type TMZ condition, and M_z=median survival of GABPB1L knockout vehicle condition.

siRNA knockdown

Non-targeting (#D-001206-13-20), GABPA (#M-011662-01), and GABPB2 (#M-016074-00-0005) directed siGENOME SMARTsiRNA pools were purchased from Dharmacon. LN-229 cells were seeded in a 100ul volume in 96 well plates at a concentration of 500 cells/ul. 24 hours post-seeding, cells were transfected using 50 nM siRNA and 0.1 ul Dharmafect I reagent (Dharmacon, #T-2001-01). At 72 hours post-transfection, cells were lysed and harvested for qPCR analysis, as described above.

Statistical analysis

Two-tailed, unpaired student's *t* tests were used with alpha = 0.01 or alpha = 0.05, as specified. Statistical significance between Kaplan Meyer survival curves was performed using a Kaplan Meyer log-rank test with alpha = 0.01 or alpha = 0.05, as specified. Statistical analysis for qPCR data was performed among specific samples, rather than relative to wild-type control due to normalization on wild-type control. Quantified data represent mean +/- standard deviation or mean +/- SEM, as referenced in figure legends. Values of 'n' are listed in relevant methods and/or figure legends.

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Author Contributions

A.M.A., C.F., J.A.D, and J.F.C. conceived the project. A.M.A. and C.F. designed, planned, and carried out experiments, and analyzed and interpreted data. K.M.S, G.J.K, and J.E.P. conceived, designed, and carried out protein purification and DNA binding assays. S.M.R. and R.J.L. helped plan experiments, and carried out experiments. A.M.M. and A.M. helped plan experiments and interpret data. J.A.D. and J.F.C supervised all experiments. A.M.A., C.F., J.A.D, and J.F.C. wrote the manuscript with input from all authors.

Competing Interests

C.F. is a co-founder of Mirimus, Inc. J.A.D. is a co-founder of Caribou Biosciences, Editas Medicine, Intellia Therapeutics, Scribe Therapeutics, and Mammoth Biosciences. J.A.D. is a scientific advisory board member of Caribou Biosciences, Intellia Therapeutics, eFFECTOR Therapeutics, Scribe Therapeutics, Synthego, Metagenomi, Mammoth Biosciences, and Inari. J.A.D. is a Director at Johnson & Johnson and has sponsored research projects by Pfizer, Roche Biopharma, and Biogen. J.F.C is a co-founder of Telo Therapeutics Inc., and has ownership interests. The other authors declare no competing interests.

References

1. Reardon, D.A., Rich, J.N., Friedman, H.S. & Bigner, D.D. Recent advances in the treatment of malignant astrocytoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **24**, 1253-1265 (2006).
2. Stupp, R. *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine* **352**, 987-996 (2005).
3. Louis, D.N. *et al.* The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta neuropathologica* **131**, 803-820 (2016).
4. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
5. Kim, N.W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-2015 (1994).
6. Li, Y. & Tergaonkar, V. Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. *Cancer research* **74**, 1639-1644 (2014).
7. Greider, C.W. & Blackburn, E.H. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* **43**, 405-413 (1985).
8. Shay, J.W. & Bacchetti, S. A survey of telomerase activity in human cancer. *European journal of cancer* **33**, 787-791 (1997).
9. Mancini, A. *et al.* Disruption of the beta1L Isoform of GABP Reverses Glioblastoma Replicative Immortality in a TERT Promoter Mutation-Dependent Manner. *Cancer cell* **34**, 513-528 e518 (2018).
10. Celeghin, A. *et al.* Short-term inhibition of TERT induces telomere length-independent cell cycle arrest and apoptotic response in EBV-immortalized and transformed B cells. *Cell death & disease* **7**, e2562 (2016).
11. Sahin, E. *et al.* Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* **470**, 359-365 (2011).
12. Stewart, S.A. *et al.* Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12606-12611 (2002).
13. Cao, Y., Li, H., Deb, S. & Liu, J.P. TERT regulates cell survival independent of telomerase enzymatic activity. *Oncogene* **21**, 3130-3138 (2002).
14. Anderson, R. *et al.* Length-independent telomere damage drives post-mitotic cardiomyocyte senescence. *The EMBO journal* **38** (2019).
15. Li, S., Crothers, J., Haqq, C.M. & Blackburn, E.H. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. *The Journal of biological chemistry* **280**, 23709-23717 (2005).
16. Tefferi, A. *et al.* A Pilot Study of the Telomerase Inhibitor Imetelstat for Myelofibrosis. *The New England journal of medicine* **373**, 908-919 (2015).
17. Shay, J.W. & Wright, W.E. Telomerase therapeutics for cancer: challenges and new directions. *Nature reviews. Drug discovery* **5**, 577-584 (2006).
18. Jager, K. & Walter, M. Therapeutic Targeting of Telomerase. *Genes* **7** (2016).
19. Huang, F.W. *et al.* Highly recurrent TERT promoter mutations in human melanoma. *Science* **339**, 957-959 (2013).
20. 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. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 6021-6026 (2013).
21. Horn, S. *et al.* TERT promoter mutations in familial and sporadic melanoma. *Science* **339**, 959-961 (2013).

22. Zehir, A. *et al.* Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nature medicine* **23**, 703-713 (2017).
23. Arita, H. *et al.* TERT promoter mutations rather than methylation are the main mechanism for TERT upregulation in adult gliomas. *Acta neuropathologica* **126**, 939-941 (2013).
24. Arita, H. *et al.* Upregulating mutations in the TERT promoter commonly occur in adult malignant gliomas and are strongly associated with total 1p19q loss. *Acta neuropathologica* **126**, 267-276 (2013).
25. Chiba, K. *et al.* Cancer-associated TERT promoter mutations abrogate telomerase silencing. *eLife* **4** (2015).
26. Bell, R.J. *et al.* Cancer. The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. *Science* **348**, 1036-1039 (2015).
27. 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. *Genes & development* **29**, 2219-2224 (2015).
28. Xue, H.H. *et al.* GA binding protein regulates interleukin 7 receptor alpha-chain gene expression in T cells. *Nature immunology* **5**, 1036-1044 (2004).
29. Xue, H.H. *et al.* Targeting the GA binding protein beta1L isoform does not perturb lymphocyte development and function. *Molecular and cellular biology* **28**, 4300-4309 (2008).
30. Rosmarin, A.G., Resendes, K.K., Yang, Z., McMillan, J.N. & Fleming, S.L. GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein-protein interactions. *Blood cells, molecules & diseases* **32**, 143-154 (2004).
31. de la Brousse, F.C., Birkenmeier, E.H., King, D.S., Rowe, L.B. & McKnight, S.L. Molecular and genetic characterization of GABP beta. *Genes & development* **8**, 1853-1865 (1994).
32. Chiba, K. *et al.* Mutations in the promoter of the telomerase gene TERT contribute to tumorigenesis by a two-step mechanism. *Science* **357**, 1416-1420 (2017).
33. Pelossof, R. *et al.* Prediction of potent shRNAs with a sequential classification algorithm. *Nature biotechnology* **35**, 350-353 (2017).
34. Zuber, J. *et al.* Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. *Nature biotechnology* **29**, 79-83 (2011).
35. Fellmann, C. *et al.* An optimized microRNA backbone for effective single-copy RNAi. *Cell reports* **5**, 1704-1713 (2013).
36. Meacham, C.E. *et al.* A genome-scale in vivo loss-of-function screen identifies Phf6 as a lineage-specific regulator of leukemia cell growth. *Genes & development* **29**, 483-488 (2015).
37. Camphausen, K. *et al.* Influence of in vivo growth on human glioma cell line gene expression: convergent profiles under orthotopic conditions. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8287-8292 (2005).
38. Miller, T.E. *et al.* Transcription elongation factors represent in vivo cancer dependencies in glioblastoma. *Nature* **547**, 355-359 (2017).
39. Hegi, M.E. *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *The New England journal of medicine* **352**, 997-1003 (2005).
40. Ochs, K. & Kaina, B. Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. *Cancer research* **60**, 5815-5824 (2000).
41. Nakamura, M. *et al.* Efficient inhibition of human telomerase reverse transcriptase expression by RNA interference sensitizes cancer cells to ionizing radiation and chemotherapy. *Human gene therapy* **16**, 859-868 (2005).

42. Masutomi, K. *et al.* The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8222-8227 (2005).
43. Fragkos, M., Jurvansuu, J. & Beard, P. H2AX is required for cell cycle arrest via the p53/p21 pathway. *Molecular and cellular biology* **29**, 2828-2840 (2009).
44. Meador, J.A. *et al.* Histone H2AX is a critical factor for cellular protection against DNA alkylating agents. *Oncogene* **27**, 5662-5671 (2008).
45. Hirose, Y., Berger, M.S. & Pieper, R.O. Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer research* **61**, 5843-5849 (2001).
46. Hirose, Y., Berger, M.S. & Pieper, R.O. p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer research* **61**, 1957-1963 (2001).
47. Celeste, A. *et al.* H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* **114**, 371-383 (2003).
48. Celeste, A. *et al.* Genomic instability in mice lacking histone H2AX. *Science* **296**, 922-927 (2002).
49. Taneja, N. *et al.* Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. *The Journal of biological chemistry* **279**, 2273-2280 (2004).
50. Allsopp, R.C. *et al.* Telomere shortening is associated with cell division in vitro and in vivo. *Experimental cell research* **220**, 194-200 (1995).
51. Ohba, S. *et al.* Mutant IDH1 Expression Drives TERT Promoter Reactivation as Part of the Cellular Transformation Process. *Cancer research* **76**, 6680-6689 (2016).
52. Sabnis, A.J. & Bivona, T.G. Principles of Resistance to Targeted Cancer Therapy: Lessons from Basic and Translational Cancer Biology. *Trends in molecular medicine* **25**, 185-197 (2019).
53. Takakura, M., Kyo, S., Inoue, M., Wright, W.E. & Shay, J.W. Function of AP-1 in transcription of the telomerase reverse transcriptase gene (TERT) in human and mouse cells. *Molecular and cellular biology* **25**, 8037-8043 (2005).
54. Hoffmeyer, K. *et al.* Wnt/beta-catenin signaling regulates telomerase in stem cells and cancer cells. *Science* **336**, 1549-1554 (2012).
55. Wu, K.J. *et al.* Direct activation of TERT transcription by c-MYC. *Nature genetics* **21**, 220-224 (1999).
56. Song, Y.S. *et al.* Interaction of BRAF-induced ETS factors with mutant TERT promoter in papillary thyroid cancer. *Endocrine-related cancer* **26**, 629-641 (2019).
57. Kinner, A., Wu, W., Staudt, C. & Iliakis, G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic acids research* **36**, 5678-5694 (2008).
58. Ward, I.M. & Chen, J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *The Journal of biological chemistry* **276**, 47759-47762 (2001).
59. Carette, J.E. *et al.* Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* **477**, 340-343 (2011).
60. Ran, F.A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nature protocols* **8**, 2281-2308 (2013).
61. Oakes, B.L. *et al.* CRISPR-Cas9 Circular Permutants as Programmable Scaffolds for Genome Modification. *Cell* **176**, 254-267 e216 (2019).
62. Chen, B. *et al.* Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**, 1479-1491 (2013).
63. Watters, K.E., Fellmann, C., Bai, H.B., Ren, S.M. & Doudna, J.A. Systematic discovery of natural CRISPR-Cas12a inhibitors. *Science* **362**, 236-239 (2018).

64. Perez, A.R. *et al.* GuideScan software for improved single and paired CRISPR guide RNA design. *Nature biotechnology* **35**, 347-349 (2017).
65. Fellmann, C. *et al.* Functional identification of optimized RNAi triggers using a massively parallel sensor assay. *Molecular cell* **41**, 733-746 (2011).
66. Brinkman, E.K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic acids research* **42**, e168 (2014).
67. Mender, I. & Shay, J.W. Telomerase Repeated Amplification Protocol (TRAP). *Bio-protocol* **5** (2015).

Figure Legends

Fig. 1 | A GABP transcription factor tetramer binds directly to the mutant but not WT TERT promoter

A, Schematic of the wild-type versus mutant *TERT* promoter. The mutant promoter contains two adjacent ETS family transcription factor binding sites that are hypothesized to recruit a GABP heterotetramer complex.

B, GABP – *TERT*p binding curve analysis. The plot shows change in a fraction of bound wild type and mutant *TERT*p dependent on the concentration of GABPBA-B1L heterodimers with their respective Kd values.

C, Representative immunoblot of GABPB1 in U-251 cells with doxycycline-induced shRNAs targeting GABPB1S (shGB1S.82, shGB1S.77, shGB1S.32, shGB1S.110) and GABPB1L (shGB1L.377, shGB1L.699, shGB1L.968, shGB1L.1202) compared to negative control (olfactory receptor OR2B6, shOR2B6.910) and non-targeting (renilla luciferase, shRen.713) shRNAs. Cells were incubated with doxycycline for 6 days prior to harvest. The lower band represents GABPB1S, the upper band represents GABPB1L.

D, *TERT* mRNA expression measured via qRT-PCR in lines from (**C**).

Fig. 2 | Inhibition of GABPB1L rapidly prevents growth of *TERT* promoter mutant cells

A, *GABPB1L* exon 9 excision strategy. Diagram of the major human *GABPB1* transcript variants, encoding either a short (*GABPB1S*) or long (*GABPB1L*) isoform. Red rectangles represent the coding sequence (CDS), green rectangles the 3' untranslated region (3'UTR). For clarity, the first exon containing the 5' untranslated region (5'UTR) is not shown. *GABPB1L* sgRNA targeting sites are highlighted in purple.

B, Schematic of competitive proliferation assays. Cas9-positive cells are transduced with pairs of lentiviral vectors expressing mCherry-tagged sgRNAs and mixed with parental cells. Percentage of mCherry positive cells over time is measured by flow cytometry.

C, Competitive proliferation assay in *TERT* promoter mutant (*TERT*p mut) U-251 and *TERT* promoter wild-type (*TERT*p WT) LN-18 GBM cells using pairs of sgRNAs targeting a negative control (sgOR2B6.2-3), a positive control (sgRPA1.2-3), total *GABPB1* (exon 3, sgGBV.33-34), internal excision of *GABPB1L* exon 9 (sgGB.21-24), and full *GABPB1L* exon 9 excision (sgGB.2-12 and sgGB.3-14). A pair of non-targeting control sgRNAs (sgNT.1-2) was used for normalization. Error bars show the standard deviation of triplicates with propagation of uncertainty. p, p-value (unpaired, two-tailed student's t-test). n.s., not significant (alpha level = 0.01).

D, Comparison of *GABPB1L* editing outcomes between *TERT*p wild-type (blue) and *TERT*p mutant (yellow) lines. While no significant difference between percentages of unedited (Retained) clones or heterozygous knockout clones (Het KOs) was observed, there were significantly fewer *TERT*p mutant full knockout clones than *TERT*p wild-type full knockout clones (Full KOs). Retained refers to clones where the *GABPB1L* exon 9 was overall retained, even though they may contain indels at either or both of the sgRNA target sites flanking exon 9.

E,F, Representative IVIS bioluminescence images (**E**) and Kaplan-Meier survival curve (**F**) of mice injected with U-251 cells expressing a control shRNA (shRen.713) or GABPB1L-targeting shRNAs (shGB1L.968, shGB1L.1202). Mice were put on doxycycline chow to induce shRNA expression starting at day 7 post-injection. n=10-12 mice per condition. *=p-value<0.01 compared to shRen.713 (Kaplan-Meyer log-rank test). M: median survival.

Fig. 3 | GABPB2 upregulation can compensate for loss of GABPB1L

A, Similarity between GABP proteins. Partial protein alignment (MUSCLE) of GABPB1L , GABPB1S, and GABPB2. GABPB1L is the long isoform , and GABPB1S the short isoform, of the *GABPB1* gene. *GABPB2* is a distinct gene. Amino acids matching across all three proteins are highlighted in blue, those matching across two proteins are highlighted in green. GABPA (alpha) subunits of the GABP complex bind to both the native ETS site (ETS) and the mutation-

derived ETS sites (G228A or G250A) at the TERT promoter locus. The GABPB1 short (beta-1S) or long (beta-1L) isoform subunits bind to the alpha subunits to form either heterodimers (beta-1S) or heterotetramers (beta-1L). Heterotetramer formation is presumably mediated through the leucine zipper-like domain of GABPB1L, which is also present in GABPB2.

B, *GABPB2* mRNA expression measured via qRT-PCR in GABPB1L knockout clones, plotted relative to control (wild-type) cells, in *TERT*p mutant cell lines. Data represent mean +/- SEM.

C, Competitive proliferation assay in U-251 cells using pairs of sgRNAs targeting a positive control locus (sgRPA1.2-3) and total GABPB1 (targeting exon 3, sgGABPV.33-34), in the presence of a lentiviral vector expressing either GABPB2 or mTagBFP2 (control). Data represent mean +/- standard deviation of triplicates.

D, *TERT* mRNA expression measured via qRT-PCR following siRNA-mediated knockdown of GABPA or GABPB2 in LN-229 wild-type, FKO, or heterozygous KO cells. p, p-value (unpaired, two-tailed student's t-test). n.s., not significant (alpha level = 0.01). Data represent mean +/- SEM.

Fig. 4 | Reduction of GABPB1L promotes a dramatic anti-tumor response of *TERT*p mutant GBM to chemotherapy

A, Representative immunoblots of yH2AX in U-251 cells expressing doxycycline-induced shRNAs targeting TERT (shTERT.3795, shTERT.3592) or GABPB1L (shGB1L.968, shGB1L.1202) compared to a non-targeting (renilla luciferase, shRen.713) shRNA. Cells were incubated with doxycycline for 6 days prior to harvest, and treated with the specified doses of TMZ during the final 20 hours.

B, Representative images from FACS-based cell cycle analysis of U-251 wild-type and GABPB1L heterozygous knockout cells treated with specified concentrations of TMZ 60 hours post-treatment.

C, Schematic of *in vivo* temozolomide (TMZ) treatment. Mice were treated with TMZ by oral gavage starting 7 (U-251) or 10 (LN-229) days post-orthotopic xenograft of *TERT*p mutant GBM cells. TMZ was dosed for a 10-day (U-251) or 5-day (LN-229) treatment course, with 5 days on 2 days off (U-251 only).

D, Kaplan-Meier survival curves for mice injected with either wild-type U-251 cells or one of two GABPB1L heterozygous knockout U-251 clones, and p.o. dosed with either TMZ or a vehicle control as described in (**B**). †: Survival of mice with no tumor burden was set as the experimental endpoint (135 days). n=6-7 mice per condition. *=p-value<0.05 relative to wild-type vehicle condition, **=p-value<0.001, relative to all vehicle conditions (Kaplan-Meyer log-rank test). M: median survival.

E, Comparison of the relative fold median survival between TMZ alone, GABPB1L heterozygous knockout, and combined TMZ with GABPB1L knockout in U-251 cells. Dashed line represents a simple addition of effects. Survival of mice xenografted with wild-type cells and treated with vehicle control is set as 1.0.

F, Kaplan-Meier survival curves for mice injected with either wild-type LN-229 cells, one of two GABPB1L heterozygous knockout LN-229 clones (C10, C26), or a full GABPB1L knockout clone (C19). Mice were p.o. dosed with a 5-day regimen of either TMZ or a vehicle control as described in Fig 4b. Survival of mice with no tumor burden was set as the experimental endpoint (135 days). n=6-8 mice per condition. **=p<0.001 between TMZ-treated compared to vehicle conditions (Kaplan-Meyer log-rank test). +: This animal was euthanized due to health problems unrelated to tumor burden. M: median survival.

G, Comparison of the relative fold median survival between TMZ alone, GABPB1L knockout, and combined TMZ with GABPB1L knockout in LN-229 cells. Dashed line represents a simple addition of effects. Survival of mice xenografted with wild-type cells and treated with vehicle control is set as 1.0.

Supplementary Figure Legends

Supplementary Fig. 1 | Assessment of GABPB isoforms at the mutant *TERT* promoter

A, GABPB1S and GABPB1L mRNA expression levels measured via qRT-PCR in U-251 cells with doxycycline-induced shRNAs targeting GABPB1S (shGB1S.82, shGB1S.77, shGB1S.32, shGB1S.110; left panel) and GABPB1L (shGB1L.377, shGB1L.699, shGB1L.968, shGB1L.1202; right panel) compared to negative control (olfactory receptor OR2B6, shOR2B6.910) and non-targeting (renilla luciferase, shRen.713) shRNAs. Cells were incubated with doxycycline for 6 days prior to harvest.

B,C, Representative gel (**B**) and quantification (**C**) of relative telomerase activity, measured via TRAP assay, in doxycycline-induced shGABPB1L-expressing U-251 cells compared to OR2B6 (negative control), or Renilla luciferase (non-targeting control). (**C**) Telomerase banding intensity is normalized to internal control band and plotted relative to shRen.713. p, p-value (unpaired, two-tailed student's t-test). Data represent mean +/- standard deviation.

Supplementary Fig. 2 | Editing of *GABPB1L* in *TERT* promoter wild-type and mutant cell lines.

A, Genotyping gels showing editing efficiency of sgRNA pairs used for competitive proliferation assays (Fig. 1c). U-251 cells stably expressing Cas9 from the lentiviral vector pCF226 were transduced with pairs of lentiviral vectors (pCF221) expressing the indicated sgRNAs. Genomic DNA was harvested at day 4 post-transduction. Genotyping PCRs were run at the indicated loci (Table S1) and locus excision efficiency quantified (ImageJ). D-cut, double-cut. C, PCR no template control.

B, Quantification of sgRNA editing efficiency in U-251 GBM cells. Indel generation efficiency of guide RNAs was assessed by T7 endonuclease I (T7E1) assay and quantified by deconvolution of sequencing reads (TIDE).

C, Sequencing of *TERT* promoter mutation status. Representative sequencing chromatograms of a select region of the *TERT* promoter for the indicated cell lines are shown.

D, Summary of *TERT* promoter mutation status and nearby single nucleotide polymorphisms (SNPs) in the indicated cell lines. wt, wild-type.

E, Editing strategy for scarless knockout of the long protein isoform of GABPB1**,** referred to as GABPB1L. In each case, a pair of sgRNAs were used targeting *GABPB1L* intron number 8 and the 3' UTR of *GABPB1L*, respectively.

F, Analysis of different sgRNA pairs targeting *GABPB1L* intron 8 and 3' UTR to excise exon 9 of *GABPB1L*. PCR-based genotyping analysis of pooled editing of a *TERT* promoter wild-type control cell line (HEK293T) and a *TERT* promoter mutant GBM cell line (U-251) through transient transfection of vectors (pCF123) expressing Cas9 and the indicated pairs of sgRNAs, followed by puromycin selection. C, PCR water control. wt, parental cell line wild-type control. D-cut, relative double-cut band intensity compared to full-length, quantified by densitometry (ImageJ). ADC, D-cut normalized to PCR fragment length.

Supplementary Fig. 3 | Generation of monoclonal GABPB1L full and heterozygous knockouts in *TERT* promoter wild-type and mutant cell lines.

A-G, Establishment of *GABPB1L* edited isogenic monoclonal cell lines through scarless genome editing. Genotyping analysis of monoclonal cell lines edited at the *GABPB1L* locus with the indicated pairs of sgRNAs. KO, knockout status (FKO, full knockout; &, heterozygous knockout and/or indels; NA, no detected genotyping bands). *, yellow asterisks highlight clones picked for expansion and further analysis.

A, HEK293T, *TERT*-promoter wild-type.

B, HAP1, *TERT*-promoter wild-type.

C, NHA-S2, *TERT*-promoter wild-type.

D, LN-18, *TERT*-promoter wild-type.

E, U-251, G228A *TERT*-promoter mutant.

F, LN-229, G228A *TERT*-promoter mutant.

G, T98G, G250A *TERT*-promoter mutant.

H, Numbers and statistics of monoclonal GABPB1L full knockout cell lines generated. No large indels, clones where no excisions were observed (these clones might still contain small indels at the sgRNA target sites). Het KO, GABPB1L heterozygous knockout (i.e. exon 9 of some but not all *GABPB1L* alleles are excised). FKO, GABPB1L full knockout (i.e. exon 9 of all *GABPB1L* alleles are excised). *, qRT-PCR and western blot analysis revealed that this clone was not a full knockout. Likely, the genome editing removed one or both of the genotyping PCR primer sites.

I, GABPB1L knockout cell lines that were validated and used for analysis. Genotyping analysis of *GABPB1L* edited monoclonal *TERT*p WT HEK293T, HAP1, NHA-S2 and LN-18 cell lines. LN-18 is a GBM line. Superscripts indicate the sgRNA pair used for editing. WT, parental wild-type cell line. C, PCR no template control.

J, Genotyping analysis of *GABPB1L* edited monoclonal *TERT*p mut U-251, LN-229 and T98G GBM cell lines.

Supplementary Fig. 4 | Sequencing-based analysis of monoclonal GABPB1L knockout clones.

A, Summary of targeted control sequencing of GABPB1L edited monoclonal cell lines. HEK293T, HAP1, NHA-S2 and LN-18 are *TERT*p wild-type cell lines.

B, U-251, LN-229 and T98G are *TERT*p mutant cell lines.

C, Genome editing outcome of select full-knockout monoclonal cell lines. sgRNA targeting sites are highlighted in blue. Protospacer adjacent motifs (PAMs) are underlined. Note, most paired-excision cuts are at the expected position between nucleotide -3 and -4 from the PAM.

Supplementary Fig. 5 | Characterization of inducible shRNAs *in vitro* and *in vivo*.

A, *RPA1* mRNA expression measured via qRT-PCR in U-251 cells expressing either one of two shRNAs targeting RPA1 (shRPA1.844, shRPA1.393), a negative control (shOR2B6.910), or a non-targeting control (shRen.713). Data are plotted relative to shRen.713. All cells were incubated with doxycycline for 6 days prior to analysis. Data represent mean +/- SEM.

B, Competitive proliferation assay in U-251 cells inducibly expressing shRNAs targeting GABPB1L, RPA1 (positive control), OR2B6 (negative control), or Renilla luciferase (non-targeting control). Cell fitness was assayed via changes in percentage of normalized GFP-positive cells over three time-points following doxycycline-mediated shRNA induction. Data represent mean +/- standard deviation of triplicates.

C,D, Representative IVIS bioluminescence images (**C**) and Kaplan-Meier survival curve (**D**) for mice injected with U-251 cells expressing a control shRNA (shRen.713) or RPA1-targeting shRNA (shRPA1.844). Mice were either kept on control chow or put on doxycycline chow to induce shRNA expression (+dox) starting at day 7 post-injection. Doxycycline chow alone had no effect on animal survival. (**D)** *=p-value<0.05 relative to shRen.713 (Kaplan-Meyer log-rank test). n=4-6 mice per condition.

E, Percentage GFP positive inducible shRNA-expressing U-251 cells measured via flow cytometry analysis as assessed following 3 days of doxycycline incubation in culture (*in vitro*) and following *ex vivo* dissection and dissociation of tumor cells from mice maintained on control chow or doxycycline chow (*post mortem*).

F, Change in median fluorescence intensity (MFI) of induced GFP-tagged shRNAs measured via flow cytometry analysis between *in vitro* and *post mortem* settings. Fluorescence intensity of control shRNA (shRen.713) is mostly unchanged, while intensity of GABPB1L-targeting shRNAs is reduced post xenograft.

G, Percentage GFP positive U-251 cells expressing inducible shRNAs towards GABPB1L or shRen.713 (non-targeting control). GFP was measured via flow cytometry analysis as assessed

following three days of doxycycline incubation in culture (*in vitro*) and following *ex vivo* dissection and dissociation of tumor cells from mice maintained on doxycycline chow (*post mortem*).

H, Kaplan-Meier survival curve displaying median and range of survival for mice injected with U-251 cells expressing an shRNA targeting GABPB1L (shGB1L.699) compared to non-targeting control shRen.713 (dashed line; data also presented in Fig 3f). n=6 mice per condition for shGABPB1L.699.

E-G, Data represent mean +/- standard deviation. Statistical analysis by student's *t* test.

Supplementary Fig. 6 | Characterization of GABPB1L knockout cell lines.

A,B, (Top) Analysis of *GABPB1L* mRNA expression measured via qRT-PCR in GABPB1L knockout clones, plotted relative to control (wild-type) cells. (Bottom) Representative immunoblots of GABPB1 expression in GABPB1L knockout clones compared to control (wild-type) cells. The lower band represents GABPB1S, the upper band represents GABPB1L. Experiments were done in *TERT*p mutant (**A**) and *TERT*p wild-type (**B**) cell lines.

C,D, Total *GABPB1* mRNA expression measured via qRT-PCR in GABPB1L knockout clones, plotted relative to control (wild-type) cells, in *TERT*p mutant (**C**) and *TERT*p wild-type (**D**) cell lines.

E,F, *GABPB1S* mRNA expression measured via qRT-PCR in GABPB1L knockout clones, plotted relative to control (wild-type) cells, in *TERT*p mutant (**E**) and *TERT*p wild-type (**F**) cell lines.

G,H, *TERT* expression measured via qRT-PCR in GABPB1L knockout clones, plotted relative to control (wild-type) cells, in *TERT*p mutant (**G**) and *TERT*p wild-type (**H**) cell lines.

A-G Data represent mean +/- SEM.

I,J, Representative gels (**I**) and quantification (**J**) of telomerase activity in GABPB1L knockout clones versus control (wild-type) cells, measured via TRAP assay in 2 *TERT*p mutant cell lines

(U-251, LN-229) and 1 *TERT*p WT line (LN-18). Telomerase banding intensity is normalized to internal control band and plotted relative to wild-type. p, p-value (unpaired, two-tailed student's t-test). Data represent mean +/- standard deviation.

Supplementary Fig. 7 | Effect of *GABPB1L* editing on GABPB2.

A, MUSCLE alignment of GABPB1L, GABPB1S, and GABPB2 protein sequences (SnapGene, version 5.0.7). Matching amino acids are highlighted in yellow. Sequence conservation is shown in colored blocks at the top.

B, *GABPB2* expression via qRT-PCR in GABPB1L knockout clones plotted relative to control (wild-type) cells in *TERT*p WT cell lines. Data represent mean +/- SEM.

Supplementary Fig. 8 | GABPB1L heterozygous knockout slows *in vivo* growth and sensitizes tumors to treatment with TMZ

A, TERT mRNA expression levels measured via qRT-PCR in U-251 cells with doxycycline-induced shRNAs targeting TERT (shTERT.3795, shTERT.3592) compared to negative control (olfactory receptor OR2B6, shOR2B6.910) and non-targeting (renilla luciferase, shRen.713) shRNAs. Cells were incubated with doxycycline for 6 days prior to harvest. Data represent mean +/- SEM.

B, Quantification of yH2AX immunoblots in U-251 cells expressing doxycycline-induced shRNAs targeting TERT (shTERT.3795, shTERT.3592) or GABPB1L (shGB1L.968, shGB1L.1202) compared to a non-targeting (renilla luciferase, shRen.713) shRNA. Data normalized to GAPDH and plotted relative to 0 uM TMZ within in condition. *=p-value<0.05 (unpaired, two-tailed student's t-test). Data represent mean +/- standard deviation.

C,**D**, Representative immunoblots of wild-type and GABPB1L knockout U-251 (**C**) and LN-229 (**D**) cells treated with 50 uM TMZ for 20 hours prior to harvest.

E,G, Quantification of G2/G1 ratio from FACS-based cell cycle analysis of U-251 (**E**) and LN-229 (**G**) cells treated with a dose titration of TMZ for 20 hours prior to harvest.

F, Representative images from FACS-based cell cycle analysis of LN-229 wild-type and GABPB1L knockout cells treated with specified concentrations of TMZ 60 hours post-treatment.

H, *MGMT* mRNA expression in U-251 and LN-229 wild-type cells and GABPB1L heterozygous knockout clones, measured via qRT-PCR. MGMT-expressing T98G wild-type cells were used as a positive control. Data plotted relative to T98G wild-type. Data represent mean +/- SEM.

I,**J**, Representative IVIS bioluminescence images (**I**) and Kaplan-Meier survival curves (**J**) of mice injected with wild-type U-251 cells or one of three GABPB1L heterozygous knockout U-251 clones. n=9 mice per condition. *=p-value<0.01 compared to wild-type (Kaplan-Meyer log-rank test).

K,L, Representative IVIS bioluminescence images (**K**) and Kaplan-Meier survival curve (**L**) for mice injected with either wild-type LN-229 cells or one of three GABPB1L knockout LN-229 clones. Note that heterozygous knockout of GABPB1L slowed growth of LN-229 tumors *in vivo*, while *in vivo* growth of the single FKO clone exhibiting GABPB2 upregulation and no change in *TERT* was unaffected. n=8-9 mice per condition. *=p<0.01 relative to wild-type (Kaplan-Meyer log-rank test).

M,N, Representative IVIS bioluminescence images (**M**) and average log-transformed radiance readings +/- SEM over 22 days of tumor growth (**N**) for mice injected with either wild-type U-251 cells or one of two GABPB1L heterozygous knockout U-251 clones and p.o. dosed with either TMZ or a vehicle control as described in Fig. 4B. **N**: p, p-value (unpaired, two-tailed student's t-test).

O,**P,** Representative IVIS bioluminescence images (**O**) and average log-transformed radiance readings +/- SEM over 21 days of tumor growth (**P**) for mice injected with either wild-type LN-229 cells, one of two GABPB1L heterozygous knockout LN-229 clones (C10, C26), or a full

GABPB1L knockout clone (C19), and p.o. dosed with either TMZ or a vehicle control as described in Fig. 4B. **P**: p, p-value (unpaired, two-tailed student's t-test).

Fig. 1

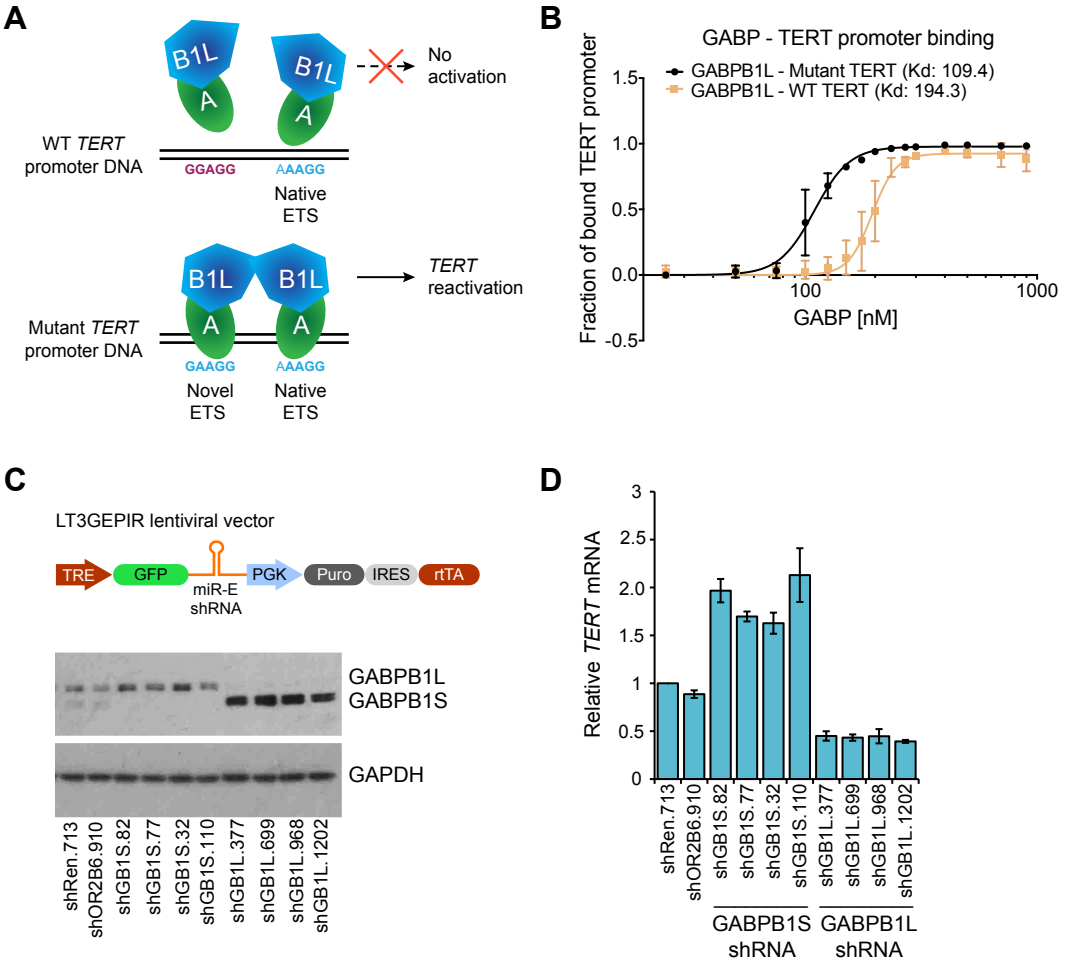

A
B1L
A
No activation
WT TERT promoter DNA
GGAGG
AAAGG
Native ETS
TERT reactivation
Mutant TERT promoter DNA
GAAGG
Novel ETS
B
GABP - TERT promoter binding
GABPB1L - Mutant TERT (Kd: 109.4)
GABPB1L - WT TERT (Kd: 194.3)
Fraction of bound TERT promoter
GABP [nM]
C
LT3GEPIR lentiviral vector
TRE
GFP
miR-E shRNA
PGK
Puro
IRES
rtTA
GABPB1L
GABPB1S
GAPDH
shRen.713
shOR2B6.910
shGB1S.82
shGB1S.77
shGB1S.32
shGB1S.110
shGB1L.377
shGB1L.699
shGB1L.968
shGB1L.1202
D
Relative TERT mRNA
GABPB1S shRNA
GABPB1L shRNA

Fig. 2

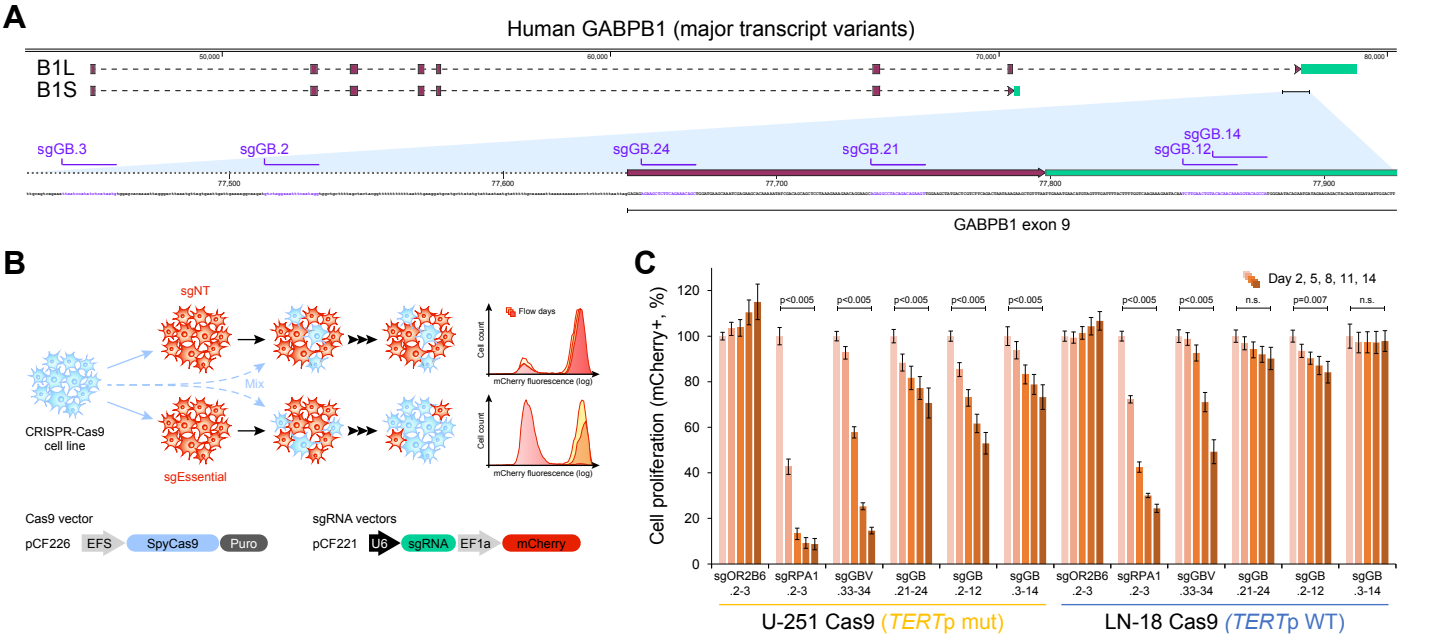
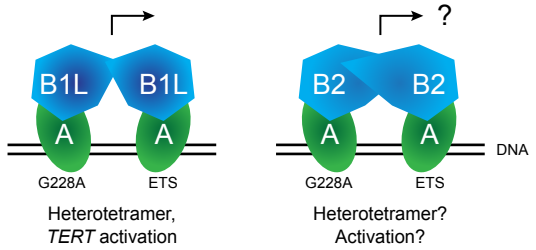

A
Human GABPB1 (major transcript variants)
B1L
B1S
sgGB.3
sgGB.2
sgGB.24
sgGB.21
sgGB.14
sgGB.12
GABPB1 exon 9
B
sgNT
sgEssential
CRISPR-Cas9 cell line
Mix
Flow days
Cell count
mCherry fluorescence (log)
Cas9 vector
pCF226
EFS
SpyCas9
Puro
sgRNA vectors
pCF221
U6
sgRNA
EF1a
mCherry
C
Day 2, 5, 8, 11, 14
Cell proliferation (mCherry+, %)
p<0.005
n.s.
p=0.007
sgOR2B6 .2-3
sgRPA1 .2-3
sgGBV .33-34
sgGB .21-24
sgGB .2-12
sgGB .3-14
U-251 Cas9 (TERTp mut)
LN-18 Cas9 (TERTp WT)
D
TERTp wild-type
TERTp mutant
GABPB1L edited clones (%)
n.s.
p=0.02
Retained
Het KOs
Full KOs
E
Start dox
Day 4 7 13 20 28 36 46 54
U251 shRen.713
U251 shGB1L.968
U251 shGB1L.1202
x10^7
Units: Radiance (photons/sec/cm2/sr)
F
shRen (M=27)
shGB1L.968 (M=52)
shGB1L.1202 (M=46)
Survival (%)
Days post xenograft

Fig. 3

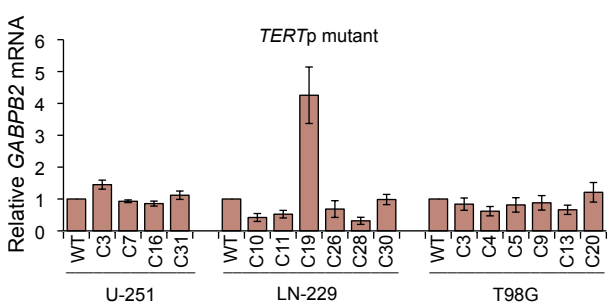
A

GABPB protein alignment

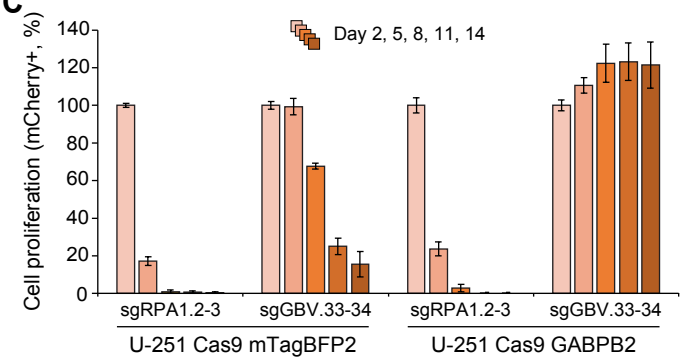
```
GABPB1L   ...ESAEIEEREALQKQLDEANREAQKYRQQLLKKEQEAEAYRQKLEAMTRLQTNKEAV*
GABPB1S   ...ESAEIEVRSLLP-----------------------------GVLCRSHPK*
GABPB2    ...ESKEGNERELLQQQLQEANRRAQEYRHQLLKKEQEAEQYRLKLEAIARQQPNGVDFTMVEEVAEVDAVVVTEGELEERETKVTGSAGTTEPHTRVSMATVSS*
```



B



C



D

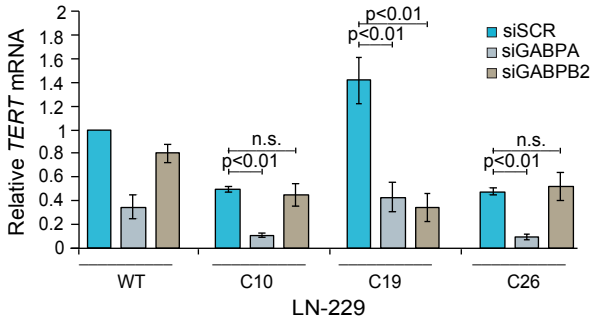
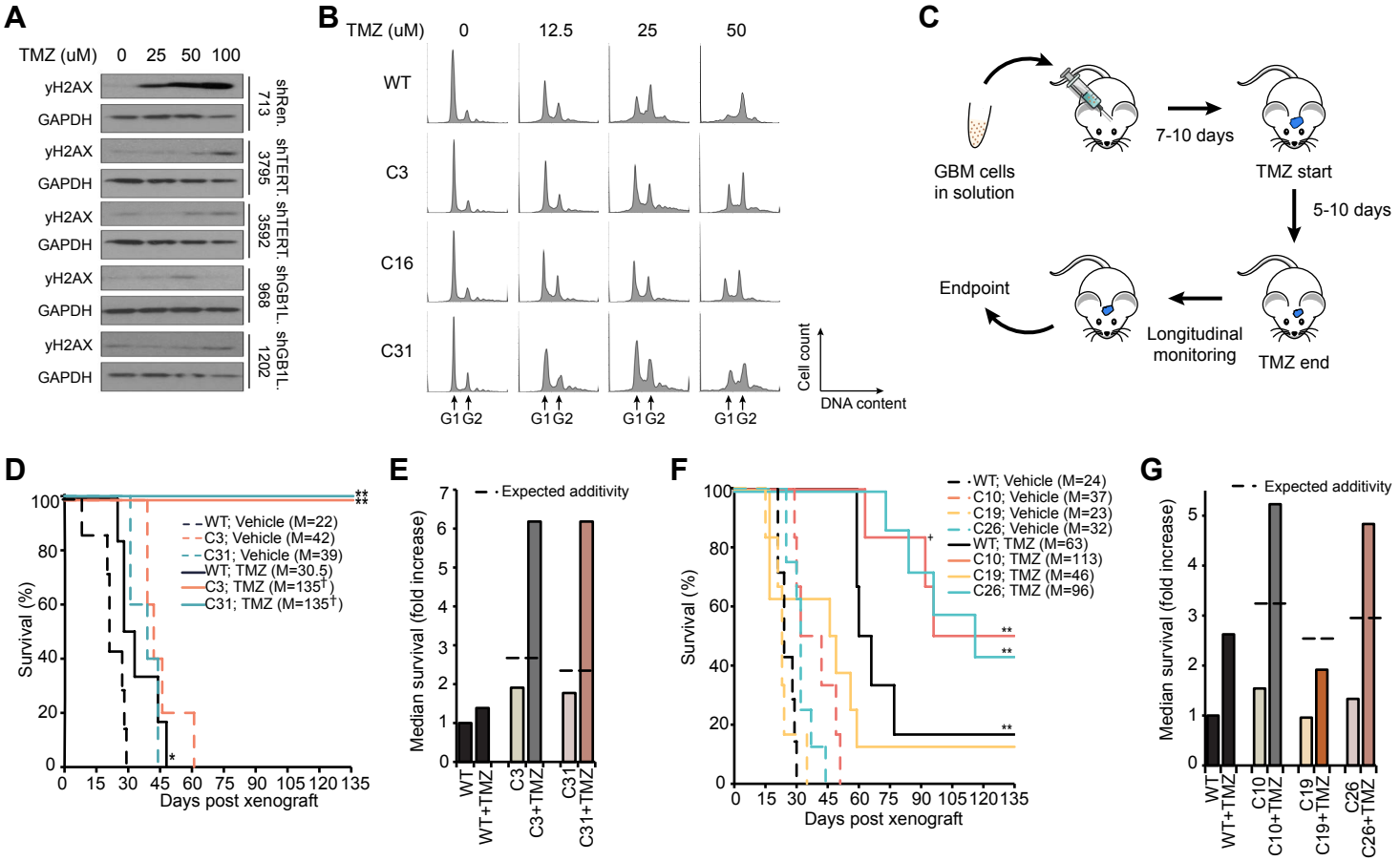
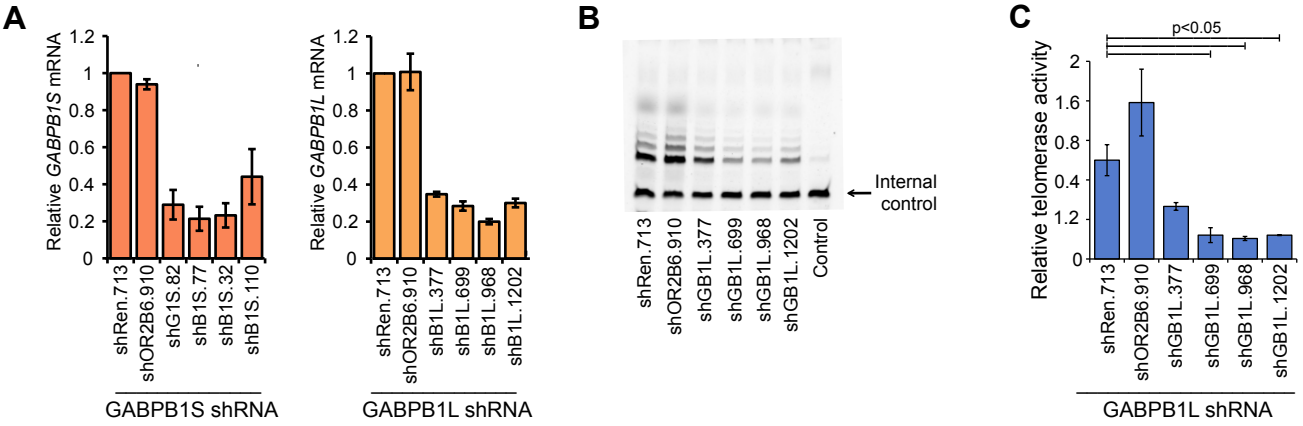


Fig. 4

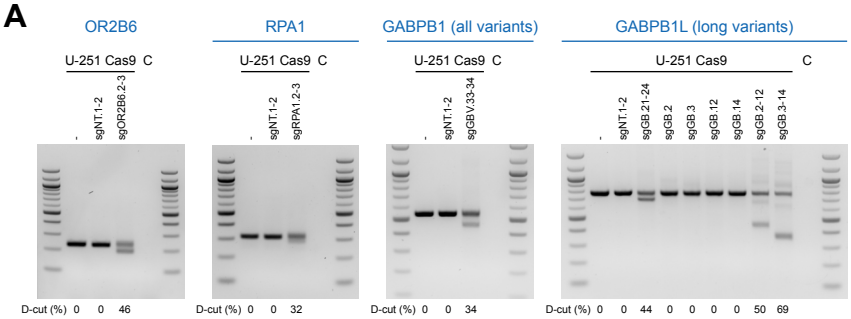

A
TMZ (uM) 0 25 50 100
γH2AX
GAPDH
shRen. 713
shTERT. 3795
shTERT. 3592
shGB1L. 968
shGB1L. 1202
B
TMZ (uM) 0 12.5 25 50
WT
C3
C16
C31
G1 G2
Cell count
DNA content
C
GBM cells in solution
7-10 days
TMZ start
5-10 days
TMZ end
Longitudinal monitoring
Endpoint
D
Survival (%)
Days post xenograft
WT; Vehicle (M=22)
C3; Vehicle (M=42)
C31; Vehicle (M=39)
WT; TMZ (M=30.5)
C3; TMZ (M=135†)
C31; TMZ (M=135†)
E
Expected additivity
Median survival (fold increase)
WT
WT+TMZ
C3
C3+TMZ
C31
C31+TMZ
F
WT; Vehicle (M=24)
C10; Vehicle (M=37)
C19; Vehicle (M=23)
C26; Vehicle (M=32)
WT; TMZ (M=63)
C10; TMZ (M=113)
C19; TMZ (M=46)
C26; TMZ (M=96)
G
Expected additivity
Median survival (fold increase)
WT
WT+TMZ
C10
C10+TMZ
C19
C19+TMZ
C26
C26+TMZ

Supplementary Fig. 1

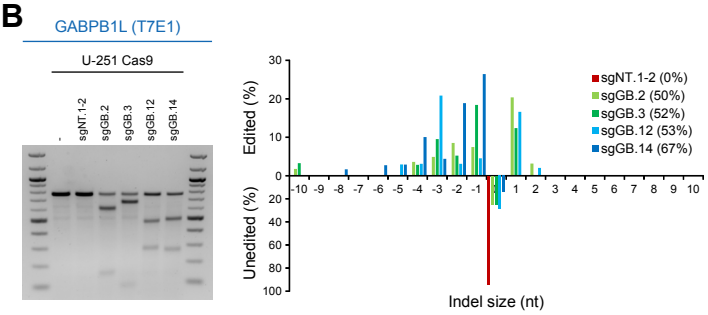


Supplementary Fig. 2

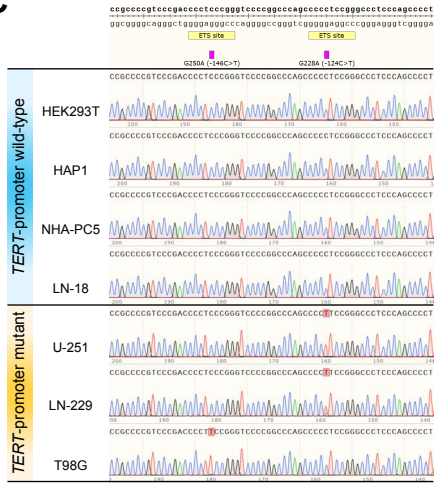
A



B



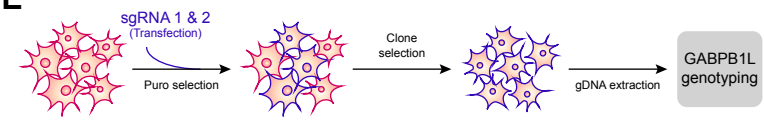
C



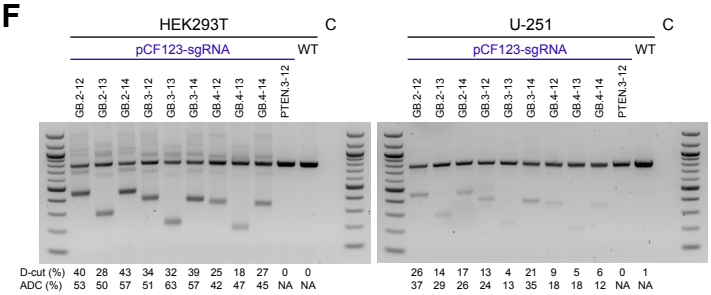
D

Cell line	*TERT-promoter mutations*	*SNPs (germline)*
HEK293T	228-wt (-124), 250-wt (-146)	wt
HAP1	228-wt (-124), 250-wt (-146)	-245T>C hom
NHA-PC5	228-wt (-124), 250-wt (-146)	-245T>C het
LN-18	228-wt (-124), 250-wt (-146)	-245T>C hom
U-251	G228A (-124C>T, hom), 250-wt (-146)	wt
LN-229	G228A (-124C>T, het), 250-wt (-146)	-245T>C het, -269G>A het
T98G	228-wt (-124), G250A (-146C>T, het)	-245T>C het

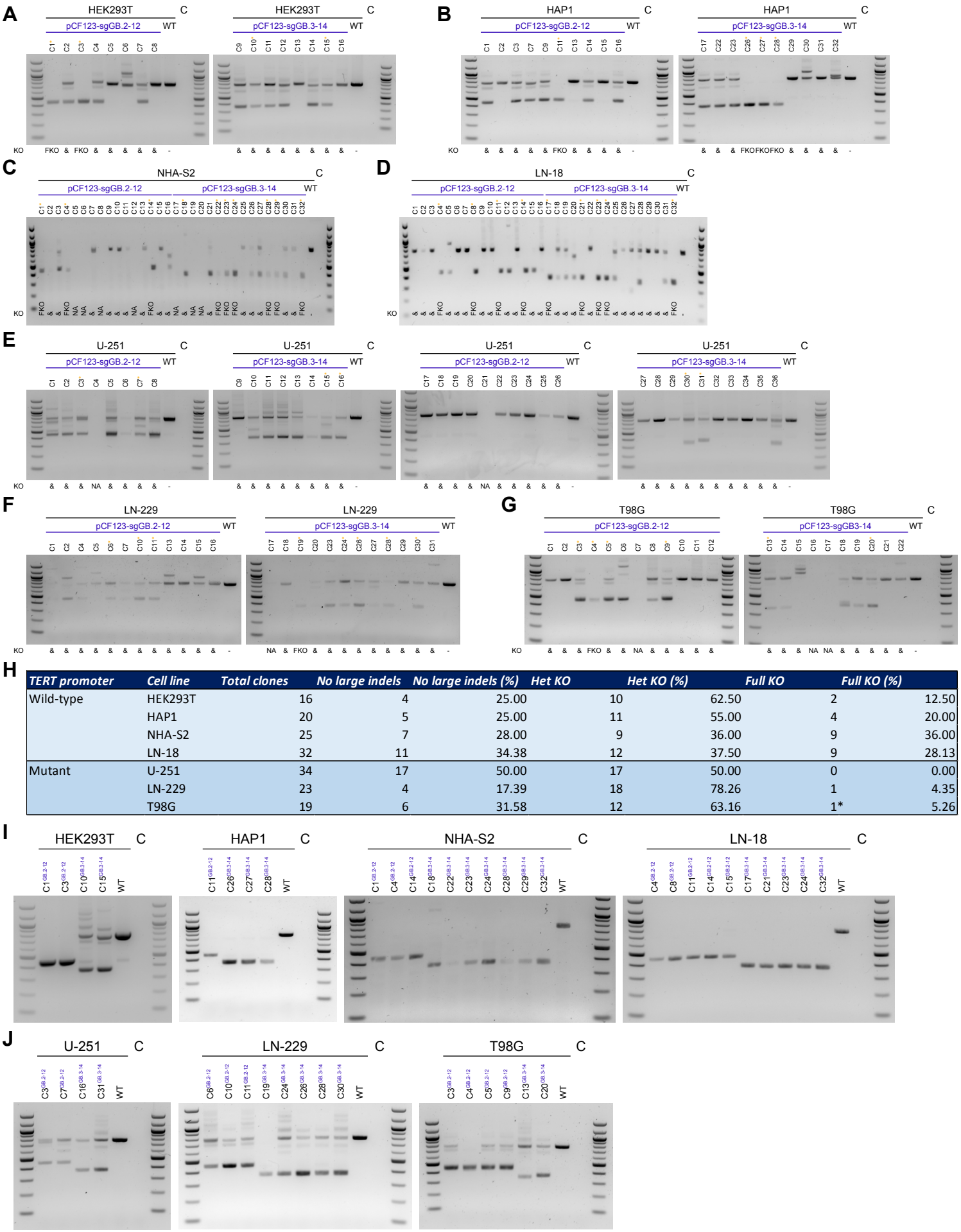
E



F



Supplementary Fig. 3



H

TERT promoter	Cell line	Total clones	No large indels	No large indels (%)	Het KO	Het KO (%)	Full KO	Full KO (%)
Wild-type	HEK293T	16	4	25.00	10	62.50	2	12.50
	HAP1	20	5	25.00	11	55.00	4	20.00
	NHA-S2	25	7	28.00	9	36.00	9	36.00
	LN-18	32	11	34.38	12	37.50	9	28.13
Mutant	U-251	34	17	50.00	17	50.00	0	0.00
	LN-229	23	4	17.39	18	78.26	1	4.35
	T98G	19	6	31.58	12	63.16	1*	5.26

Supplementary Fig. 4

A

Cell line	PCR band	Editing
HEK293T-C1		Clean double-del
HEK293T-C3		Clean double-del with -1/0
HEK293T-C10	Top	Indels at sgGB.3-14
	Bottom	Clean double-del
HEK293T-C15	Top	Indels at sgGB.3-14
	Bottom	Clean double-del
HAP1-C11		Clean double-del
HAP1-C26		Double-cut, multiphasic
HAP1-C27		Clean double-del
HAP1-C28		Clean double-del with 0/+1

Cell line	PCR band	Editing
NHA-S2-C1		Clean double-del
NHA-S2-C4		Clean double-del
NHA-S2-C14		Double-cut, multiphasic
NHA-S2-C18	Bottom	Clean double-del with -1/0
NHA-S2-C22		Double-cut, multiphasic
NHA-S2-C23		Double-cut, multiphasic
NHA-S2-C24		Double-cut, multiphasic
NHA-S2-C28		Clean double-del
NHA-S2-C29		Clean double-del
NHA-S2-C32		Double-cut, multiphasic
LN18-C4		Clean double-del
LN18-C8		Double-cut, multiphasic
LN18-C11		Double-cut, multiphasic
LN18-C14		Clean double-del
LN18-C15	Bottom	Clean double-del
LN18-C17		Double-cut, multiphasic
LN18-C21		Double-cut, multiphasic
LN18-C23		Clean double-del
LN18-C24		Clean double-del with +1/-1
LN18-C32		Clean double-del

B

Cell line	PCR band	Editing
U251-C3	Top	Indels at sgGB.2-12
	Bottom	Clean double-del
U251-C7	Top	Indels at sgGB.2-12
	Middle	Indels at sgGB.2-12
	Bottom	Double-cut, multiphasic
U251-C15	Top	Indels at sgGB.3-14
	Middle	Deletion and SpyCas9 insert
	Bottom	Double-cut, multiphasic
U251-C16	Top	Indels at sgGB.3-14
	Bottom	Double-cut, multiphasic
U251-C30	Top	Indels at sgGB.3-14
	Bottom	Double-cut, multiphasic
U251-C31	Top	Indels at sgGB.3-14
	Bottom	Double-cut, multiphasic

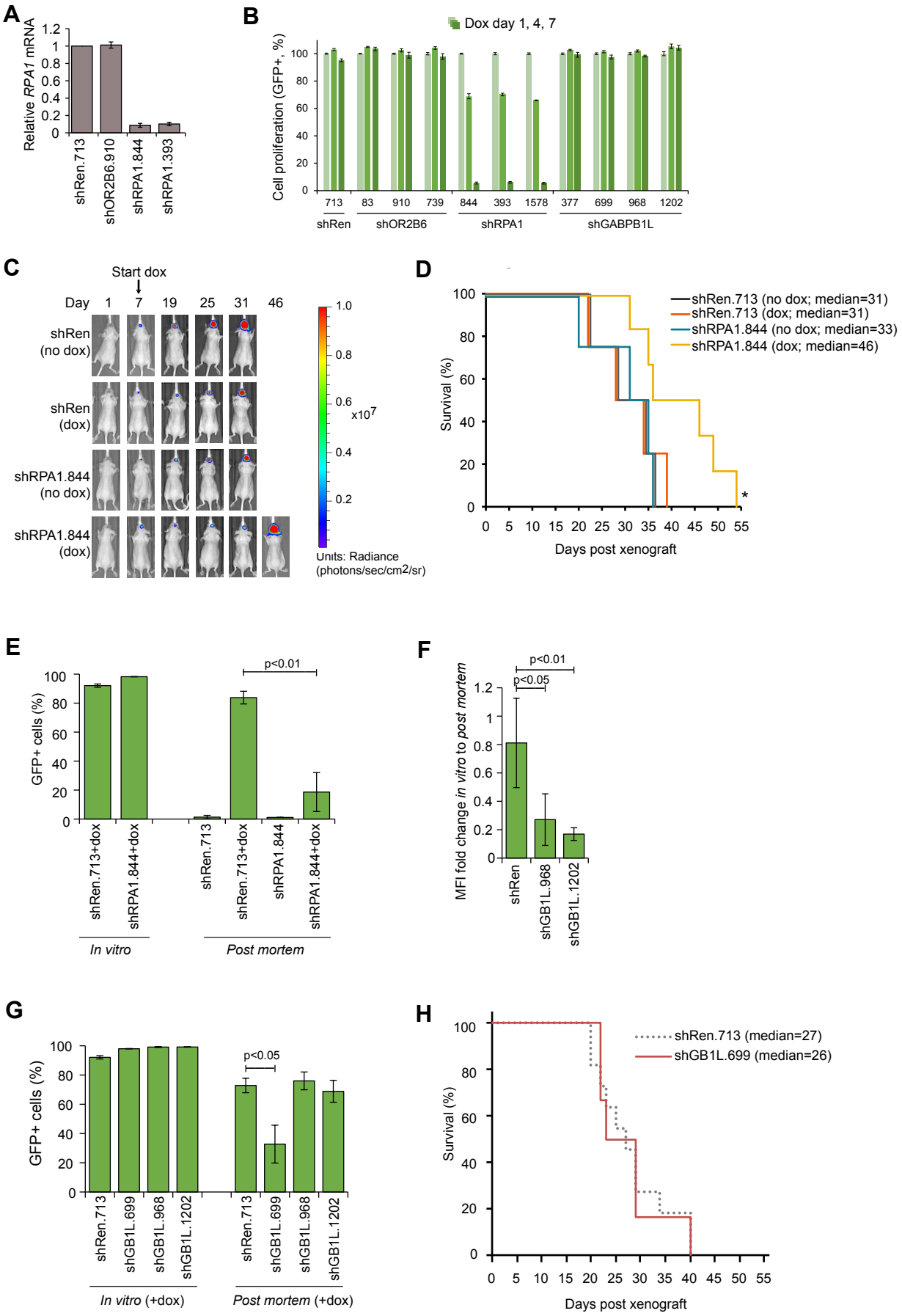
Cell line	PCR band	Editing
LN229-C6	Top	Indels at sgGB.2-12
	Bottom	Clean double-del
LN229-C10	Top	Indels at sgGB.2-12
	Bottom	Double-cut, multiphasic
LN229-C11	Top	Indels at sgGB.2-12
	Bottom	Double-cut, multiphasic
LN229-C19	Bottom	Double-cut, multiphasic
LN229-C24	Top	Indels at sgGB.3-14
	Bottom	Clean double-del with +1/-1
LN229-C26	Top	Indels at sgGB.3-14
	Bottom	Double-cut, multiphasic
LN229-C28	Top	Indels at sgGB.3-14
	Bottom	Double-cut, multiphasic
LN229-C30	Top	Indels at sgGB.3-14
	Bottom	Double-cut, multiphasic
T98G-C3	Bottom	Double-cut, multiphasic
T98G-C4		Double-cut, multiphasic
T98G-C5	Bottom	Double-cut, multiphasic
T98G-C9	Bottom	Double-cut, multiphasic
T98G-C13	Bottom	Clean double-del with -10/+5
T98G-C20	Bottom	Double-cut, multiphasic

C

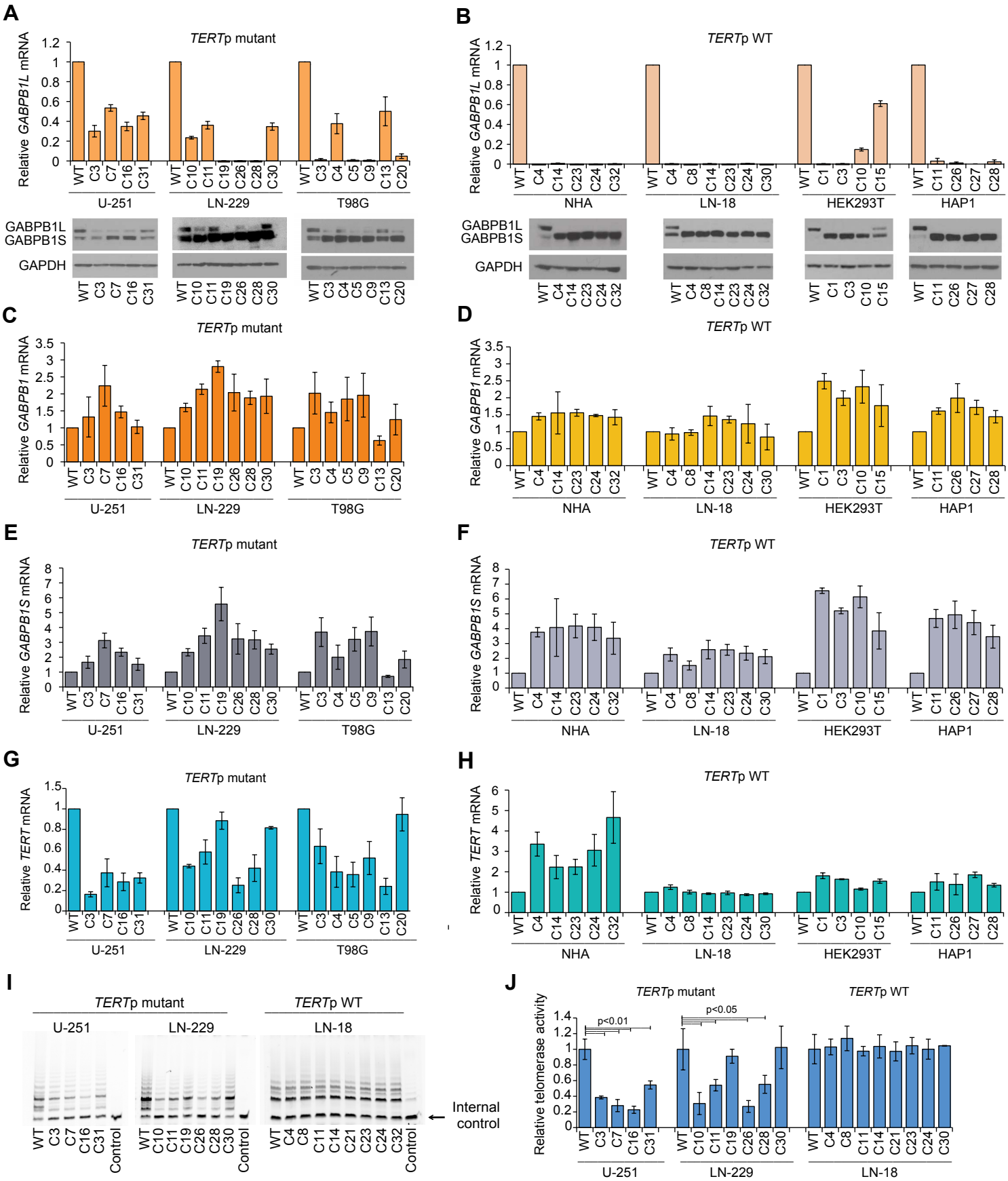
```
sgGB.2-12      ..aaggcaagatgtctaggaaatttcaataggtggctgcttt...AAGAATACAATCTTGAACTGTACACAACAAAGGTACAGCC..
  HEK293T-C1   ..aaggcaagatgtctaggaaatttcaat-------------...---------------------------CAAAGGTACAGCC..
  HEK293T-C3   ..aaggcaagatgtctaggaaatttcaa--------------...---------------------------CAAAGGTACAGCC..
  HAP1-C11     ..aaggcaagatgtctaggaaatttcaat-------------...---------------------------CAAAGGTACAGCC..
  NHA-S2-C1    ..aaggcaagatgtctaggaaatttcaat-------------...---------------------------CAAAGGTACAGCC..
  NHA-S2-C4    ..aaggcaagatgtctaggaaatttcaat-------------...---------------------------CAAAGGTACAGCC..
  LN18-C4      ..aaggcaagatgtctaggaaatttcaat-------------...---------------------------CAAAGGTACAGCC..
  LN18-C14     ..aaggcaagatgtctaggaaatttcaat-------------...---------------------------CAAAGGTACAGCC..

sgGB.3-14      ..cagtcagaaattaatccatatctcataatgtggagcacaa...CTTGAACTGTACACAACAAAGGTACAGCCATGGGAATACA..
  HAP1-C27     ..cagtcagaaattaatccatatctcata-------------...---------------------------CCATGGGAATACA..
  HAP1-C28     ..cagtcagaaattaatccatatctcata-------------...---------------------------CATGGGAATACA..
  NHA-S2-C28   ..cagtcagaaattaatccatatctcata-------------...---------------------------CCATGGGAATACA..
  NHA-S2-C29   ..cagtcagaaattaatccatatctcata-------------...---------------------------CCATGGGAATACA..
  LN18-C23     ..cagtcagaaattaatccatatctcata-------------...---------------------------CCATGGGAATACA..
  LN18-C24     ..cagtcagaaattaatccatatctcataa------------...--------------------------GCCATGGGAATACA..
  LN18-C32     ..cagtcagaaattaatccatatctcata-------------...---------------------------CCATGGGAATACA..
```

Supplementary Fig. 5

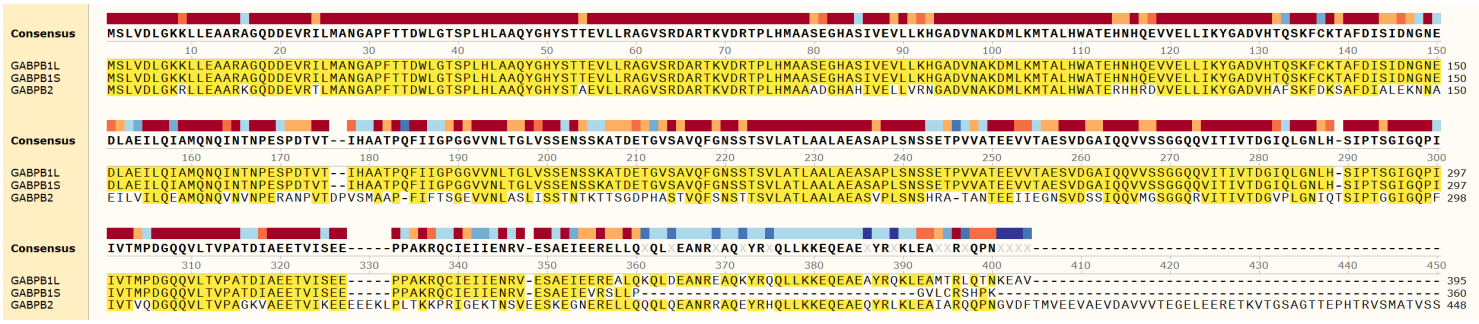


Supplementary Fig. 6

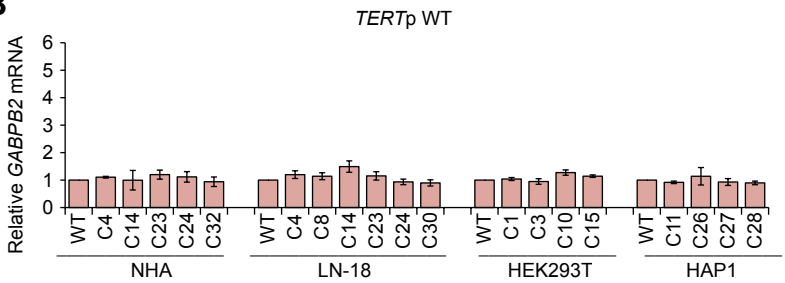
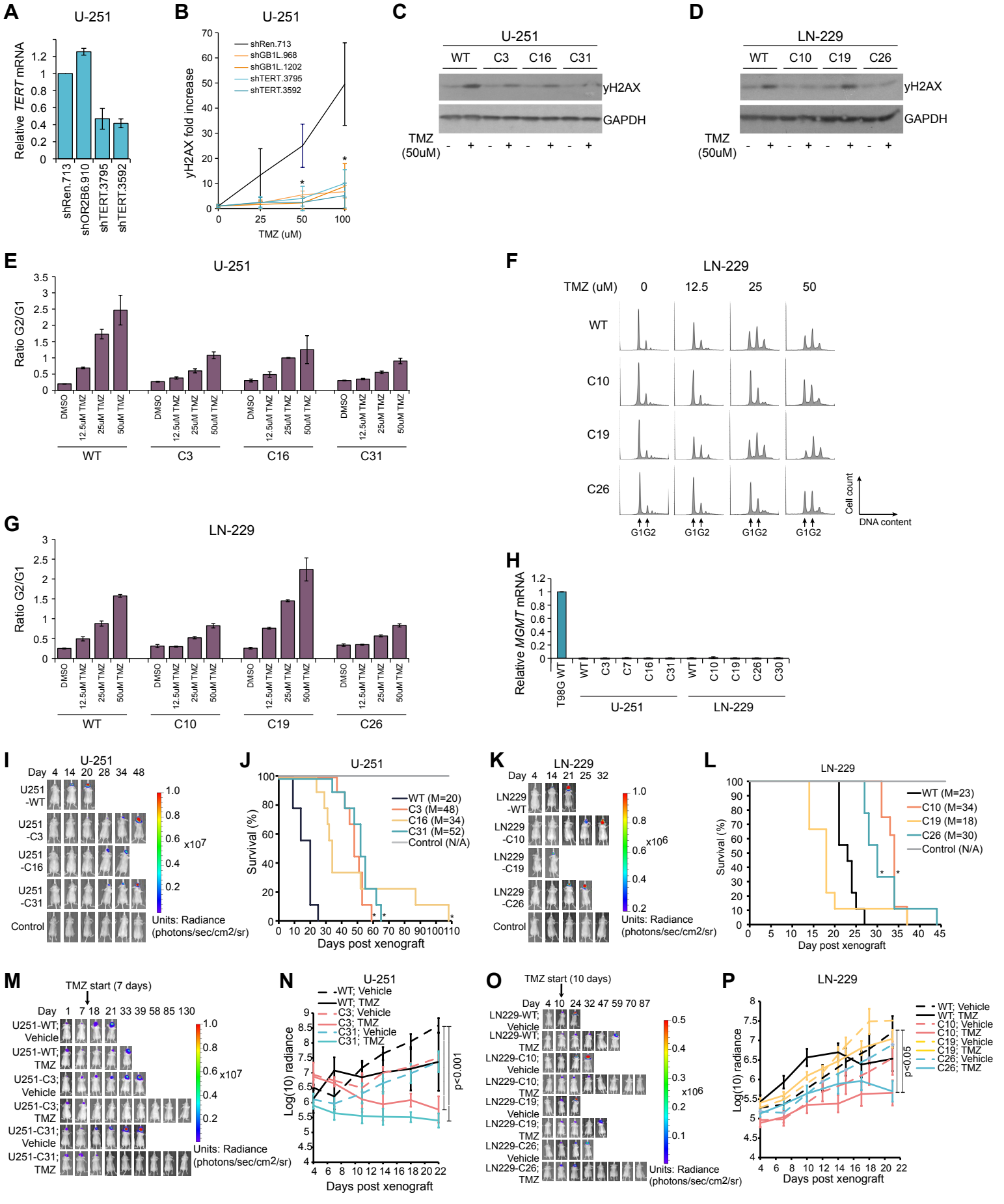


Supplementary Fig. S7

A



B



Supplementary Fig. S8