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Signatures of TOP1 transcription-associated mutagenesis in cancer and germline

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Abstract

The mutational landscape is shaped by many processes, with genic regions vulnerable to mutation but preferentially protected by transcription-coupled repair[1]. In microbes, transcription has been demonstrated to be mutagenic[2,3]; however, the impact of transcription-associated mutagenesis remains to be established in higher eukaryotes[4]. Here we show that ID4, an indel cancer signature of unknown aetiology[5] characterised by short deletions (2-5 bp), is due to a transcription-associated mutagenesis process. We demonstrate defective ribonucleotide excision repair in mammals to be associated with the ID4 signature, with mutations occurring at a TNT sequence motif, implicating Topoisomerase 1 (TOP1) activity at sites of genome-embedded ribonucleotides as a mechanistic basis. Such TOP1-mediated deletions occur somatically in cancer, and the ID-TOP1 signature is also found in physiological settings, contributing to genic *de novo* indel mutations in the germline. Hence, while topoisomerases protect against genome instability by releasing topological stress[6], their activity may also be an important source of mutations in the human genome.

Introduction

Eukaryotic cells employ many strategies to ensure integrity of their genomes, with high-fidelity DNA replication[7] and DNA repair processes countering exogenous and endogenous DNA lesions[8]. The process of transcription targets DNA repair machinery to expressed genes, preferentially reducing their mutation rate following DNA damage[1]. Despite this targeted repair, in micro-organisms the process of transcription itself is mutagenic; a phenomenon referred to as transcription-associated mutagenesis (TAM)[2,3]. In yeast, Topoisomerase 1 (Top1) activity is a major source of TAM and results in a distinctive transcription-dependent signature of 2-5 bp deletions at tandem repeat sequences[9-11]. Genome-embedded ribonucleotides have been established as a cause of Top1-TAM deletions in yeast[12]. Such ribonucleotides are frequently incorporated by DNA polymerases during replication, and represent the most prevalent aberrant nucleotides in the eukaryotic genome[13,14]. These genome-embedded ribonucleotides are normally removed by ribonucleotide excision repair (RER), a process initiated by the heterotrimeric Ribonuclease H2 enzyme[15]. However, when Top1 cleaves at embedded ribonucleotides instead of RNase H2 this can result in small deletions[16,17].

In the last decade, widespread use of genome sequencing has enabled unbiased sampling of human mutations, substantially advancing understanding of mutagenesis in the germline[18] and in neoplasia[19]. Multiple mutational processes act during cancer evolution, and mathematical methods have been developed to define signatures that may correspond to individual mutagenic mechanisms, through decomposition of tumour mutational profiles[19]. This has successfully defined cell-intrinsic, environmental and treatment-related origins for many base substitution signatures in cancer[20-22]. However, the origin of a substantial number of signatures remains unknown, and some may be artefactual. Recently, cancer signature analysis has been extended to indels[5], small (1-49 bp) insertions and deletions. Such indels are an important class of mutations that contribute substantially to disease-causing germline variants (>20%) and human variation[23] .

Here we investigate an indel signature of unknown cause, ID4. We show experimentally that ID4 deletions are increased in RNase H2 deficient cell lines and cancers and delineate a human TOP1-mediated TAM signature (ID-TOP1) relevant to both somatic and germline mutagenesis.

Results

ID4, a distinct cancer indel signature

The ID4 cancer signature, as categorised by COSMIC[24], comprises 2-5 bp deletions, often with loss of a single repeat unit at short repeat sequences[5]. Most commonly these occur where the deleted sequence is repeated one, two or three times in tandem (Fig. 1a). Hereafter we use the term SSTRs (short-short tandem repeats) to distinguish such short tandem repeats (STRs) with less than 5 repeats (i.e. <6 repeat units) from microsatellite STRs with many repeats. In addition to these SSTR deletions, ID4 is characterised by small deletions at sequences with microhomology (MH), in particular 2 bp deletions with single nucleotide microhomology (SNMH). Both features are distinct from cancer deletion signatures resulting from other well-recognised mechanisms like replication slippage and non-homologous/microhomology-mediated end joining (NHEJ/MMEJ) (Extended Data Fig. 1a,b). In support of a distinct aetiology, SSTR and SNMH deletions are not apparent in cancer associated with homologous recombination (HR) or mismatch repair (MMR) deficiency, which are expected to have higher levels of MMEJ and replication slippage mutagenesis, respectively (Extended Data Fig. 1c,d).

ID4 resembles a yeast mutation signature

Noting similarities to a Top1-induced transcription-associated mutagenesis (Top1-TAM) in *Saccharomyces cerevisiae*, we re-analysed published genome-wide mutation accumulation experiments performed with *rnh201Δ pol2-M644G* yeast[25]. This strain is particularly susceptible to

Top1-TAM as it accumulates genome-embedded ribonucleotides at high levels due to RNase H2/RER deficiency and enhanced ribonucleotide incorporation by a steric-gate mutation in the catalytic site of the replicative polymerase Pol ε[26]. Similarities to the ID4 signature were apparent with a comparable pattern of small deletions at SSTRs, although mutational events at sites of SNMH were not evident in the yeast data (Fig. 1b). As over one million ribonucleotides are incorporated by DNA polymerases per replicating mouse cell[14], we reasoned that genome-embedded ribonucleotides might cause similar mutational events in mammalian cells. To experimentally address whether TAM contributes to indel formation in human RER-deficient cells, we developed a novel reporter to enable sensitive and specific detection of mutational events arising from TOP1 activity in both yeast and mammals.

Top1-dependent deletions in yeast

Mutation rates are routinely measured in *S. cerevisiae* using well characterised but species-specific selectable markers (LYS2, URA3, CAN1). Therefore, to establish a system that could be transferred between yeast and mammalian cells, we used an approach inspired by the Traffic Light reporter assay[27], incorporating both positive and negative selection cassettes in a single transcriptional unit (Fig. 1c). The hygromycin resistance gene (HygroR) served both as the mutational target and negative selection marker. Indels causing a 2 bp frameshift within HygroR, including 2 bp deletions, result in translation of an otherwise out-of-frame P2A self-cleaving peptide and the neomycin resistance (NeoR) gene, permitting positive selection of mutated colonies with neomycin (Extended Data Fig. 2a). To enrich the target for 2 bp tandem repeats, *in silico* re-design incorporated synonymous substitutions such that SSTRs accounted for >50% of the HygroR open reading frame.

For validation, the reporter was inserted into the *S. cerevisiae* genome and fluctuation assays used to assess mutation rates in strains deficient for RER and/or Top1. A 37-fold increase in mutation rate was seen for the *rnh201Δ* (RNase H2 null) strain compared to wild type (Fig. 1d), with a mutation rate of 6.1×10^{-9} per bp per generation (95% CI, $5.4\text{-}6.9 \times 10^{-9}$), whereas the increased mutation rate was abolished in the *rnh201Δ top1Δ* double mutant strain, in keeping with Top1-dependent mutagenesis at genome-embedded ribonucleotides[12,28]. Notably, there was a 10-fold decrease in the mutation rate for *top1Δ* compared to the wild-type strain, and a 35-fold decrease in 2 bp SSTR deletions (Extended Data Fig. 2b), consistent with previous reports[10,11]. In addition, the observed mutational spectrum was most similar for wild-type and *rnh201Δ* strains, but substantially different compared to *top1Δ* and *rnh201Δ top1Δ* strains (Fig. 1e; Extended Data Fig. 2c-f). Taken together, we conclude that the same Top1-mediated mutations occur, albeit at different frequencies, in wild-type cells when RER is functional and in RNase H2 deficient strains when elevated levels of ribonucleotides are present in the genome.

TOP1-mediated mutations in human cells

Having validated the reporter in yeast, the same 2 bp repeat-enriched HygroR sequence was used to address whether TOP1-mediated mutagenesis at embedded ribonucleotides is conserved in human cells (Fig. 2; Extended Data Fig. 2g). NeoR was replaced by the puromycin resistance (PuroR) gene, with reporter expression driven from the mammalian ubiquitous CAG promoter, permitting rapid antibiotic selection in mammalian cells. This modified reporter was inserted at the *AAVS1* safe harbour locus in HeLa cells (Fig. 2a; Extended Data Fig. 3a-e). CRISPR/Cas9-mediated genome editing, targeting the catalytic site of *RNASEH2A*, was then used to generate two independent knockout (KO) reporter clones, alongside a control clone that had also been taken through editing and clonal selection steps (Fig 2b,c; Extended Data Fig. 3). The control clone retained RNase H2 activity, while there was complete loss of cellular RNase H2 activity in KO clones, accompanied by high levels of ribonucleotides in genomic DNA (Fig. 2b,c; Extended Data Fig. 3f,g).

In fluctuation assays, RNase H2 null clones demonstrated a significant 3.1-fold increase in mutation rate (Fig. 2d) and 5.2-fold more 2 bp SSTR deletions (Extended Data Fig. 3h) compared with RNase

H2 proficient cells (RNASEH2A+), consistent with conservation of TOP1-directed mutagenesis in human cells. As in yeast (Fig. 1e), the overall mutational profile of reporter mutations was similar between RNase H2 proficient and null HeLa cells (cosine similarity 0.89, $P < 10^{-4}$), predominantly comprised of 2 bp SSTR deletions (Fig. 2e).

The mutation rate for RNase H2 null HeLa cells (8.0×10^{-9} per bp per generation; 95% CI, $6.7\text{-}9.5 \times 10^{-9}$) was similar to that seen for *rnh201Δ* yeast (Fig. 1d), whereas the rate was substantially higher for RNASEH2A+ control cells compared to wild-type yeast. However, the increased mutation rate in RNase H2 null HeLa cells likely underestimates the true impact of RER deficiency in human cells, as despite the control RNASEH2A+ HeLa reporter cells retaining protein expression (Fig. 2b), the clone had also acquired mutations at the CRISPR editing site that reduced enzymatic activity (Fig 2c), causing a moderate increase in genomic ribonucleotide content (Extended Data Fig. 3f,g).

To confirm these findings we used a complementary approach to establish the relevance of such mutational events genome-wide, performing mutation accumulation experiments using hTERT-RPE1 (*TP53*$^{-/-}$) diploid cell lines. Ancestral populations for RNase H2 wild-type and null cells (RNASEH2A-KO or RNASEH2B-KO; Extended Data Fig. 4a-d) were established after initial single cell sorting, and clones then grown for approximately 100 generations. Single cell sorting was performed every 25 generations, creating bottlenecks to capture accumulating mutations (Fig. 3a). Combined variant calling on whole genome sequencing (WGS) from paired ancestral and endpoint cultures identified a total of 1,698 acquired high confidence indel mutations, captured by at least 3 out of 4 variant callers. Consistent with TOP1-mediated mutagenesis, among all indel categories, only 2-5 bp deletions were found to be substantially (7.4-fold) and significantly enriched in RNase H2 null RPE1 cells compared to wild-type (Fig. 3b; Extended Data Fig. 4e,f), with an estimated rate of 1.1×10^{-10} 2-5 bp deletions per generation per bp for KO and 1.4×10^{-11} for WT. Of these deletions in RNase H2 null cells, 82% were 2 bp deletions, of which 48% were at SSTRs (Extended Data Fig. 4g). Furthermore, signature decomposition using SigProfilerExtractor[5] reported a 21% ID4 contribution in RNase H2 null cells, that increased to 61% when subtraction of background mutation patterns was performed to identify RER-deficiency specific mutation signatures (Fig. 3c,d; Extended Data Fig. 5). The ID4 signature was substantially enriched in transcribed genomic regions (Extended Data Fig. 5e). ID5, a clock-like signature[5], was also enriched in KO cells, likely due to slower growth and longer culture time needed to achieve the same number of doublings for RNase H2 null cells[14].

MH deletions specific to mammalian TOP1

Small deletions at sequences with microhomology are an additional feature of ID4 (Fig. 1a), not observed in *rnh201Δ pol2-M644G* yeast (Fig. 1b). However, consistent with a ribonucleotide-induced mutational origin in mammalian cells, they are observed frequently in RNase H2-deficient RPE1 cells, in which SNMH sites account for 31% of 2 bp deletions, indicating that in humans they share the same aetiology as those occurring at SSTRs. Taken together, our reporter and mutation accumulation experiments demonstrate that genome-embedded ribonucleotides cause a similar mutational signature in yeast and mammalian cells. Therefore Topoisomerase 1-mediated mutagenesis likely also occurs in humans and is associated with 2-5 bp deletions at SSTR and SNMH sequences.

ID4 mutations in a murine cancer model

To determine if TOP1-induced mutations resulting in the ID4 signature can be detected *in vivo*, we next studied an RER-deficient murine cancer model in which Villin-Cre conditional deletion of *Rnaseh2b* and *Tp53* results in intestinal malignancy[29]. Whole genome sequencing of paired tumour-normal tissue samples from 6 mice, identified a total of 989 high-confidence tumour-specific somatic indels. Analysis of the resulting mutational signature established that ID4 substantially contributed in all tumours (Fig. 4a,b and Extended Data Fig. 6a), accounting for 32% of acquired indels. Consistent with a transcription-associated process, the ID4 signature was again most evident in transcribed genomic regions (Fig. 4b). Commonly occurring cancer signatures[5] ID1, ID2 and ID5 were also seen, in line with expectations of multiple mutational processes active in neoplasia.

The observed ID4 mutation spectrum corresponded closely to that seen in the RPE1 mutation accumulation experiment: 28% of indels were at 2-5 bp deletions, of which the majority were again 2 bp deletions (82%) predominantly at SSTRs (51%) and sites of SNMH (34%) (Extended Data Fig. 6b,c). This is consistent with the occurrence of TOP1-induced somatic mutations at genome-embedded ribonucleotides *in vivo*, conserved across different tissue and cellular contexts, and shows that it can be detected in a cancer setting.

A sequence motif for ID4 mutations

While COSMIC defines the ID4 signature on the basis of indel size and repeat/microhomology context (Fig. 1a), the number of indels in the murine RER-deficient tumour model permitted us to further investigate the characteristics of mammalian Topoisomerase 1-induced mutations. We focussed our analysis on 2 bp deletions, as such events represented 81% of >1 bp deletions in the context of tandem repeats and 85% of deletions in sequences with microhomology.

First, we classified all 2 bp deletions at STR/SNMH sequences into 6 non-redundant dinucleotide classes, grouping together complementary sequences (Fig. 4c). We noted that the deleted sequences substantially deviated from genome-wide frequencies, with a complete absence of CC/GG and CG/GC deletions, as well as an overrepresentation of the CT category (containing CT, TC, GA and AG deletions). All observed deletions therefore included at least one thymidine (T), which functionally could be accounted for by the very strong preference of mammalian Topoisomerase 1 to cleave at a phosphodiester bond with a T immediately upstream[30].

Next, to investigate the wider sequence context, we aligned sequences containing all 228 two bp deletions (Extended Data Fig. 6e), which indicated that deletions preferentially occur when T nucleotides are spaced at a 2-base interval. Indeed, this TNT motif was present in 100% of SNMH (*n*=77) and STR sites (*n*=124), providing a common unifying sequence context for both deletion types (Fig. 4d), a finding replicated in both our RPE1 (Extended Data Fig. 6e) and yeast datasets (Extended Data Fig. 7). We found TNT to be substantially overrepresented at deletions sites compared to the genome-wide null expectation. Furthermore, while the TNT motif is common at tandem repeat sequences, 2 bp deletions at this motif are still significantly enriched when considering the occurrence of 2 bp STR and SNMH sequences in mouse and human genomes (Fig. 4e; Extended Data Fig. 6f), and STR sequences in the yeast genome (Extended Data Fig. 7).

To account for thymidines spaced at a 2-base interval and the occurrence of mammalian SNMH deletions, we developed a revised model based on the established strand realignment model for yeast Top1-mediated mutagenesis[12,16,17]. In this "TNT model", TOP1 cleaves preferentially 3' of an embedded ribouridine, with nucleophilic attack by the 2'-OH of the ribose ring resulting in TOP1 release and formation of a non-ligatable nick with a terminal 2',3'-cyclic phosphate (Fig. 4f, i-iii). This then provides a substrate for TOP1 cleavage 2 bp or more upstream[17], preferentially at a thymidine[30]. When this second cleavage event happens at a base identical to that of the first cleaved nucleotide, an event more likely at STR and microhomology sequences, strand realignment can then occur, resulting in a nick permissive to religation and TOP1cc reversal (Fig. 4f, iv-vi). An alternative mechanism of sequential Top1 cleavage, in which double-strand breaks occur due to nicking of opposite strands[31] could not be reconciled with our TNT model, but may account for deletions occurring at non-STR/SNHM sites. Within the TNT motif, deletions were most common at CT and GT dinucleotides in both mammals and yeast (Fig. 4c; Extended Data Fig. 6 and 7b,e), which may be explained, at least in part, by preferential incorporation of ribouridine at CT and GT dinucleotides (Extended Data Fig. 7f and [32]).

Implicating TOP1-TAM as the cause of the ID4 signature permits us to include additional features in the definition of this COSMIC signature, namely preference for a TNT sequence motif at 2 bp deletion sites and enrichment in transcribed genes. Hereafter, we refer to this extended definition as ID-TOP1. To establish the relevance of the ID-TOP1 signature for human disease and genetic variation, we next examined publicly available datasets.

ID-TOP1 in human cancer

RNASEH2B is frequently deleted in human cancer, in particular in chronic lymphocytic leukaemia (CLL) given its proximity to a tumour suppressor locus, the *DLEU2-mir-15-16* microRNA cluster[33]. Such RNase H2 deficient human cancers should therefore be enriched for the ID4/ID-TOP1 signature. We analysed whole genome sequencing data for 348 CLL patients from two independent cohorts[34,35], stratified on *RNASEH2B* deletion status. Somatic variant calling identified a significant increase in 2-5 bp deletions in RNase H2 null tumours (Fig. 5a), while other indels were equally represented across wild-type, heterozygous and null categories (Extended Data Fig. 8a). Of the 2-5 bp deletions in tumours with biallelic *RNASEH2B* loss more than half (57%) were 2 bp deletions, which were predominantly at STR and SNMH sequences and substantially enriched for the TNT motif (Extended Data Fig. 8b,c), consistent with the ID-TOP1 mutational signature. Furthermore, mutational signature decomposition for RNase H2 null CLL cases confirmed the presence of the ID4 signature, most apparent in genic regions (Extended Data Fig. 8d). We therefore conclude that the ID-TOP1 signature is present in human cancer and enriched in tumours that are RNase H2 deficient.

Topoisomerase 1 also causes mutations in RER proficient cells (Fig. 1d-f and [10,11]), and therefore is likely to cause mutations in other cancers, with deletions expected to occur most frequently in highly transcribed genes[4]. Accordingly, analysis of WGS data across cancer types (ICGC/PCAWG) demonstrated that the 2-5 bp deletion rate correlates with expression levels of ubiquitously expressed genes (Pearson's r = 0.86; P = 0.0014), with deletions markedly elevated in the most highly expressed genes (Fig. 5b), in line with previous reports of such deletions in certain cancer genes[36,37]. Examination of 2 bp deletions (42% of 2-5 bp deletions) across cancer types also demonstrated them to be predominantly in STR and SNMH contexts (Extended Data Fig. 8f) and enriched for the TNT sequence motif (Fig. 5c). Furthermore, using a dataset of TOP1 cleavage events captured by TOP1-seq[38], we found 2-5 bp deletions increase in frequency with TOP1 enzymatic activity, with such deletions more prevalent in regions of high TOP1 activity (Fig. 5d). Likewise, TOP1-ID deletion rates also corresponded to TOP1 activity and transcription level, in contrast to all other deletions (Extended Data Fig. 8g,h). Taken together, this establishes a significant role for TOP1-mediated mutagenesis in the generation of somatic deletions.

To further explore the role of transcription in deletion mutagenesis of cancer genomes, we identified genes that are highly expressed, but only in certain tissues. For prostate adenocarcinoma, highly expressed prostate-restricted genes were significantly enriched for 2-5 bp deletion mutations compared to other genes in this cancer type, as well as the same genes in other cancers (two-tailed Fisher's exact test, OR 3.5, $P = 2.5 \times 10^{-8}$ after Bonferroni correction; Extended Data Fig. 8i). Importantly, this analysis considers the same sets of genes between cancer types and therefore rules out sequence composition biases as a confounder for elevated ID-TOP1 mutagenesis in highly expressed genes. Extending this approach in an all-versus-all comparison between 8 cancer types and 17 tissues demonstrated specificity between high expression in a tissue of origin and enrichment for 2-5 bp deletions (Fig. 5e). These results extend the relevance of TOP1-mediated mutagenesis to other cancers, confirms the ID-TOP1 mutational signature to be transcription-associated, and supports the occurrence of TAM in humans.

TOP1-mediated deletions in the germline

TOP1 is ubiquitously expressed, so we reasoned that it could cause germline as well as somatic mutations. To investigate this possibility we examined mutations from parent-child trio WGS studies in the Gene4Denovo database[39]. *De novo* mutations identified in such datasets represent germline events, as they occur in germ cells or during early embryonic cell divisions. Strikingly, 2-5 bp deletions were the largest category identified, accounting for 33% of the 40,936 *de novo* indels (Fig. 5f), and the majority of these were compatible with the ID-TOP1 signature. Analysis of 2 bp deletions (41% of 2-5 bp deletions) demonstrated that most occur at SSTR or MH sites (Extended Data Fig. 9a,b), with enrichment of the TNT sequence motif both genome wide and in the context of STR/SNMH sites (Fig. 5g; Extended Data Fig. 9c). Likewise for 3 and 4 bp deletions, TNNT and TNNNT motifs respectively were significantly overrepresented compared to genome-wide expectation (Extended Data Fig. 9d), in support of sequential TOP1 cleavage and strand realignment as the underlying cause. Consistent with TOP1-TAM aetiology, 2-5 bp deletion and ID-TOP1 deletion frequency correlated with transcript expression in male germ cells (Fig. 5h and Extended Data Fig. 9e). We therefore conclude that the ID-TOP1 mutational signature also occurs in the human germline, implicating TOP1-induced strand realignment mutagenesis as an important mutational process in mammalian cells.

Discussion

Here we establish a biological basis for the ID4 cancer signature[5], experimentally demonstrating it to occur in RNase H2 deficient cells both *in vitro* and *in vivo*. This implicates TOP1-mediated cleavage at genome-embedded ribonucleotides as its cause. TOP1 is cell-essential in mammals, and it is therefore not possible to similarly confirm a genetic dependency on TOP1 in human cells, as has been done in yeast[12]. However, conservation of this mechanism across eukaryotes is supported by us finding a Topoisomerase 1 dependent TNT deletion motif present in both yeast and humans, and demonstrating that deletion frequency is dependent on human TOP1 activity levels. Previously published work also provides evidence for TOP1-mutagenesis at ribonucleotide sites in humans. The reversible transesterification reaction of Type 1 Topoisomerases is conserved from yeast to humans[6], and human TOP1 has site-specific activity for ribonucleotides[40], causing DNA breaks in mammalian RNase H2 deficient cells[33]. Furthermore, generation of 2 bp deletions through sequential TOP1 cleavage at embedded ribonucleotides has been biochemically reconstituted with both human and yeast enzymes[17,31].

We define additional features of this ID-TOP1 mutational signature, with deletions strongly enriched at TNT motifs in both yeast and mammals, a sequence context specific to Topoisomerase 1 (Extended Data Fig. 7g,h), and deletions most frequent in highly transcribed regions. Consequently, we show that a transcription-associated mutagenesis process first identified in yeast[10,12,41] is relevant to higher eukaryotes, establishing TOP1-induced mutagenesis as an important process for human variation and disease. Additional signatures associated with topoisomerases or indeed RNase H2 may be identified in future, particularly given that ID17 has been recently been linked to TOP2A^{K743N} cancers[42].

The substantial contribution of ID-TOP1 deletions to germline mutagenesis has particular significance given that such deletions will be disproportionately disruptive, particularly in transcribed regions. Notably, such deletions occur in the context of normal RER function, consistent with the mutagenic potential of Topoisomerase 1 in physiological wild-type settings (Fig. 1d and [10,11]). Given that genome-embedded ribonucleotides are the most common endogenous lesion in replicating mammalian cells[14], they are the most likely sites of TOP1-TAM mutagenesis, where TOP1 could cleave before their removal by RNase H2-dependent RER. Processing of TOP1cc may be an alternative, less frequent source of 2-5 bp deletions[41], but we did not detect ID4 in Topoisomerase 1 inhibitor treated cancers (Extended Data Fig. 8j). TOP1 canonical function is to relieve DNA topological stress, arising during both transcription and replication[6] (Extended Data Fig. 10). Hence TOP1-mediated deletions are not restricted to transcribed regions of the genome, with deletions also evident in non-genic regions with high TOP1 activity (Extended Data Fig. 8k). However, overall, enhanced TOP1 activity associated with transcription accounts for more frequent mutagenesis within genes.

Given the essential nature of topoisomerase activity across tissues and cell states, TOP1-mediated mutagenesis is likely to occur in many contexts. Frequent TOP1-mediated human germline mutations (Fig. 5i-k) and identification of ID4 at early embryonic stages[43] suggest developmental vulnerability to TOP1-TAM. In addition, 2-5 bp somatic deletions at SSTRs are also observed at high frequency in non-dividing neurons[36], and ID4 has been identified in multiple tumour types[5]. As such, this mutational process is likely to be significant not only in cancers with RER deficiency, but also those with high TOP1 activity and tumours with defects in relevant repair mechanisms, such as enzymes that process TOP1cc[6] or non-ligatable TOP1-induced nicks[44-46]. In addition, alternative RER pathways may exist[47] that could reduce TOP1-mutagenesis. The ID-TOP1 signature may provide a useful biomarker with potential future diagnostic and therapeutic utility[48], for instance as an indicator of TOP1-induced genome instability targetable by PARP or ATR inhibitors[33,49].

In conclusion, alongside its essential role in relieving DNA torsional stress, TOP1 also drives mutagenesis in somatic and germline contexts, relevant to neoplasia, inherited disease and human variation.

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Figure legends

Fig. 1 | Top1-dependent deletions in *S. cerevisiae* resemble ID4, a cancer mutational signature of unknown aetiology. a, The ID4 signature comprises small deletions (typically 2, 3 or 4 bp in size) of one repeat unit at short short tandem repeat (SSTR) and microhomology (MH) sites. i-vi; repeated sequence in bold colour; deletions in red; SNMH, single nucleotide MH. **b,** Indel mutations similar to those detected in ID4 accumulate genome-wide in yeast with high levels of genome-embedded ribonucleotides. Reanalysis of WGS for *rnh201Δ pol2-M644G* yeast[25]. **c,** Schematic of novel frameshift mutation reporter containing many 2 bp SSTRs. Frameshift mutations in HygroR result in neomycin resistant yeast colonies. P_{TEF}, TEF promoter; HygroR/NeoR, hygromycin/neomycin resistance genes; P2A, self-cleaving peptide. **d,e,** Fluctuation assays demonstrate that Top1-mediated 2 bp SSTR mutations occur in wild-type and RNase H2 deficient (*rnh201Δ*) backgrounds. Mutation rates, median ± 95% confidence intervals for *n*=16 independent cultures per strain (**d**). WT and *rnh201Δ* have similar indel mutation spectra, and differ from *top1Δ* strains. Spectra of neomycin resistant colonies. n, number of independent indels detected (**e**). Cosine similarity P-values empirically determined, Extended Data Fig. 2e,f.

Fig. 2 | Two bp SSTR deletions are increased in RNase H2 null HeLa cells. a, Schematic of reporter targeting to *AAVS1* safe harbour locus to generate reporter cells. PuroR, puromycin resistance; P_{CAG}, CAG promoter; HA, homology arm (L, left; R, right). Also see Extended Data Fig. 3. **b,c,** Validation of *RNASEH2A* knockout reporter clones. Immunoblot of cell lysates detecting the three RNase H2 subunits. GAPDH, loading control. For gel source data, see Supplementary Fig. 1 (**b**). Cellular RNase

H2 enzyme activity. Bars, mean; error bars, s.d.; *n*=3 technical replicates. HeLa, no modification; Parental, HeLa with reporter (grey); RNASEH2A+, CRISPR-edited reporter clone retaining RNase H2 activity (green); KO1, KO2, CRISPR-mediated *RNASEH2A* knockout clones (red) (**c**). **d,** Fluctuation assays establish a significantly increased mutation rate in RNase H2 null (KO) cells. Median ± 95% confidence intervals. Data points, rates for independent cultures (RNase H2 proficient, RNASEH2A+, *n*=9; knockout, KO1, open circles, *n*=10; KO2, open squares, *n*=6). **e,** 2 bp SSTR and SNMH deletions are frequent in both RNASEH2A+ and KO cells. Indel mutation spectra. n, number of indels identified by sequencing colonies from independent cultures.

Fig. 3 | ID4 SSTR and MH mutations are increased genome-wide in RNase H2 deficient RPE1 cells. **a,** Schematic of mutation accumulation experiment. Long-term culture of hTERT-RPE1 *TP53*$^{-/-}$ RNase H2 wildtype (WT) and null cell lines (AKO, BKO: RNASEH2A, RNASEH2B knockout respectively) bottlenecked every 25 doublings by single cell sorting. **b,** Mutations acquired during long-term culture were significantly enriched for 2-5 bp deletions in RNase H2 null cells, but not other mutation categories (also see Extended Data Fig 4e). Mean ± s.d.; P-value, two-sided Fisher's exact test with Bonferroni correction, WT (*n*=3 independent clones) vs KO (*n*=2 independent clones) for 2-5 bp deletions vs all other indel types. **c,d,** ID4 occurs in RNase H2 null cells (**c**), and is the major signature once background mutations observed in wildtype cells are subtracted (**d**).

Fig. 4 | RER-deficient tumours have an ID4 signature, associated with transcription and a TNT sequence motif. **a,** ID4 contributes substantially to the mutational spectrum of Rnaseh2b-KO murine intestinal tumours (WGS, paired tumour-normal samples from *n*=6 mice). **b,** ID4 contribution is greater in transcribed regions of the genome. Two-sided Fisher's exact test, ID4 vs other indels. *n*=969 indels from 6 biologically independent tumours. **c,** 2 bp STR/SNMH deletions have biased sequence composition. Genome, frequency of dinucleotides in STR/SNMH sequences in the mappable genome. Deletions (bold red), right aligned. **d,e,** A TNT sequence motif is present at all 2 bp STR and SNMH deletions. Sequence logo: Two-bit representation of the sequence context of 2 bp deletions at STR and SNMH sequences (**d**). Deletion sites are significantly enriched for the TNT sequence motif compared to genome-wide occurrence, for all genome sequence, as well as STR and SNMH sites. P-values, Two-sided Fisher's exact test, observed vs expected. *n*=228 (all; *P*=1.7x10^{-28}), 124 (STR; *P*=0.0008), 77 (SNMH; *P*=1.4x10^{-8}) deletions in 6 biologically independent tumours (**e**). **f,** Model for TOP1-mediated mutations at TNT sequences containing embedded ribonucleotides, in which strand realignment results in 2 nt deletion (description in main text).

Fig. 5 | TOP1-mediated deletions in human cancer and germline. a, 2-5 bp deletions are significantly increased in CLL with biallelic *RNASEH2B* deletions (null). Box, 25-75%; line, median; whiskers 5-95%; data points, values outside range. WT, *n*=116, 85; het (heterozygous), *n*=72, 59; null, *n*=10, 6 tumours (GEL, ICGC respectively). Multiple-testing corrected q-values, 2-sided Mann-Whitney. **b-d,** ID-TOP1 deletions are frequent somatic mutations in cancer. Indels per expression stratum of ubiquitously expressed genes (defined in Extended Data Fig. 8e). Dotted line, genome-wide rate (**b**). Two bp deletions preferentially occur at TNT motifs. P-values, two-sided Fisher's exact test, observed vs expected. *n*=11,853 (all; *P*<10^{-200}), 6,699 (STR; *P*=1.9x10^{-60}), 2,872 (SNMH; *P*=1.5x10^{-51}) deletions (**c**). 2-5 bp deletions increase with TOP1 cleavage activity in ID4-positive PCAWG tumours (**d**). Solid lines, relative deletion rate. Shading, 95% confidence intervals from 100 (**b**) or 1,000 bootstrap replicates (**d**), n= 11,853 biologically independent tumours[50] (**b**-**d**)**. e,** 2-5 bp deletions are enriched at tissue-specific highly transcribed genes in associated cancers. Heatmap of significant Odds Ratio scores (2-5 bp deletions in top 10% tissue-restricted genes vs deletions in other genes, relative to expected frequency from all other tissues) for tissue-tumour pairs. Two-sided Fisher's exact test. **f-h,** ID-TOP1 deletions are frequent human *de novo* mutations enriched in highly transcribed germ cell genes. 2-5 bp deletions are the most common indels in the human germline. Gene4Denovo WGS data ([39] ; *n*=40,936 indels) (**f**). TNT sequence motif is significantly enriched in *de novo* 2 bp deletions (**g**). P-values, two-sided Fisher's exact test, observed vs expected; *n*=5,569 two bp deletions (P<10^{-200}), at STR (*n*=3,294; P=5.2x10^{-47}) and SNMH sequences (*n*=1,093; P=2.9x10^{-26}). 2-5 bp deletion frequency correlates with gene transcription level in germ cells (**h**). Solid lines, Gene4Denovo indel mutations per individual per Mbp. Shading, 95% confidence intervals, 100 bootstrap replicates.

Methods

Plasmids

A description of all plasmids used in this work can be found in Supplementary Table 1. The *S. cerevisiae* reporter was generated by DNA synthesis (GeneArt Gene Synthesis, Thermo Fisher Scientific; gBlocks Gene Fragments, IDT) and conventional cloning (restriction, ligation and Quikchange site-directed mutagenesis). The final construct (pTCW12) was used for *S. cerevisiae* reporter strain construction and fluctuation assays. A Gateway compatible reporter construct for mammalian cells (pTCW14) was similarly generated using a combination of DNA synthesis and conventional cloning strategies. Gateway cloning was then used to move the reporter cassette into pAAVS-Nst-CAG-Dest (a gift from Knut Woltjen; Addgene plasmid # 80489; [51]) to generate pTCW15 for targetting it to the human AAVS1 locus.

In silico re-design of the Hygromycin resistance gene

To increase the frequency of 2-bp tandem repeats, synonymous substitutions were introduced in the 1 kbp *hph* coding sequence, the *Klebsiella pneumoniae* hygromycin resistance gene (HygroR)[52]. Using Python, a 5-codon (15-base) sliding window was moved one codon at a time, to identify all possible synonymous permutations. Permutations were ranked on the basis of tandem dinucleotide repeat sequence length, with the highest ranking sequences used to replace whole codons, prioritising dinucleotide couplet repeats over mononucleotide repeats. Edited codons were then censored from subsequent permutation. Subsequently, to eliminate stop codons that would arise after a 2 bp deletion or equivalent frameshift mutations, further synonymous changes were made, where possible preserving tandem repeat sequences.

Yeast strains and growth conditions

All *S. cerevisiae* strains used in this work (Supplementary Table 2) are isogenic with BY4741 [53] and were grown at 30°C. *TOP1* and *RNH201* open reading frames (ORFs) were deleted using 1-step allele replacement using PCR products generated from plasmid templates with selection cassettes (Supplementary Table 2) and primers containing 60 nt homology directly up and downstream of the ORF. Gene deletions were confirmed by PCR. The 2 bp deletion reporter was inserted at the *AGP1* locus using a PCR product amplified from pTCW12 using primers AGP1-MX6-F and AGP1-MX6-R (Supplementary Table 3). Correct reporter insertion was confirmed by PCR and Sanger sequencing. Growth under selection was on YPD (10 g/l yeast extract, 20 g/l bactopeptone, 20 g/l dextrose, 20 g/l agar) supplemented with hygromycin B (300 mg/l), nourseothricin (100 mg/l) and/or G418 (1 g/l), or on Synthetic Defined medium (6.7 g/l yeast nitrogen base without amino acids, complete supplement single dropout mixture (Formedium), 20 g/l dextrose, 20 g/l agar).

Fluctuation assays (yeast)

Fluctuation assays were performed as previously described[54]. Yeast was grown overnight in YPD with hygromycin B (300 mg/l), plated on YPD and grown at 30°C to obtain individual colonies derived from a single cell without HygroR mutations. For each strain, 16 independent colonies were then used to inoculate 5 ml YPD, and grown for 3 days at 30°C with shaking at 250 rpm. Cells were pelleted by centrifugation and resuspended in 1 ml of H_2O. Undiluted suspensions for each culture were plated (100 µl per plate) on 2 YPD plates supplemented with 1 g/l G418, with the exception of *rnh201Δ* for which a 10^{-2} dilution was used. In addition, each suspension was serially diluted to 10^{-6} of which 100 µl per plate was spread on 2 YPD plates to estimate the total number of viable cells per culture. Plates were incubated at 30°C for 2-3 days, and colonies counted. Mutation rates were determined in Microsoft Excel 2016 for each individual culture, and an overall rate for each strain calculated using the Lea Coulson method of the median[55]. The number of mutants for each culture were ranked, and those ranked 4rd and 13th used to calculate the rates that define the lower and upper limits of the 95% confidence interval[56]. A single G418-resistant colony for each independent culture was used to determine the spectrum of frame shift mutations. A 1.3 kbp region including HygroR was amplified in two overlapping amplicons (primers S297F and S1113R; S752 and S1658R) using FastStart PCR Master Mix (Roche) and direct colony PCR (5 min 95˚C; 35 cycles 30 s 95˚C, 30 s 58˚C, 45 s 72˚C; 45 s 72˚C). Each amplicon was Sanger sequenced using primers described in Supplementary Table 3, and analysed using Sequencher 5.4.6 (Gene Codes Corporation) and/or Mutation Surveyor V3.30 (SoftGenetics). Mutation rates (per bp) were calculated for 1,032 bp of sequence in which productive frameshift mutations can occur.

Cell lines

Human cell lines used in this work are summarised in Supplementary Table 4. All cells were grown at 37°C and 5% CO_2, authenticated using STR DNA profiling in the labs of origin and shown to be mycoplasma negative through routine testing. HeLa cells (a gift from G. Stewart, University of Birmingham, UK; originally purchased from ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco/Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. hTERT-RPE1 cells (a gift from D. Durocher, University of Toronto, Canada; originally purchased from ATCC) were grown in DMEM/F12 medium mixture

(Gibco/Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The 2-bp deletion reporter was integrated at the *AAVS1* safe harbour locus in HeLa cells using a published CRISPR/Cas9 targeting protocol [51]. HeLa cells were transfected with pXAT2 and pTCW15 in Opti-MEM reduced-serum medium using Invitrogen Lipofectamine 2000 (Thermo Fisher Scientific). After 48 h cells were re-plated in medium containing 500 µg/ml G418, and after another 48 h and a second round of re-plating in selective medium, single cells were sorted into 96-well plates using a BD FACSJazz instrument (BD Biosciences). Resulting G418-resistant clones were screened by PCR for reporter integration at the correct locus, retention of integration-free *AAVS1* and Sanger sequencing of resulting PCR products. Single-locus integration was confirmed by FISH as previously described[57], using pTCW16 to generate a fluorescently labelled probe. The full reporter sequence of selected clones was checked, with amplification of a 1.9 kbp fragment using Prime Star Max PCR Master Mix (Takara Bio) with primers HygroR_up and PuroR_rev (40 cycles 10 s 98˚C, 15 s 70˚C, 2 min 72˚C), followed by Sanger sequencing with additional primers (Supplementary Table 3). To generate RNASEH2A-KO reporter cells, the selected parental HeLa reporter clone was transfected with pMAR526 and pMAR527 (Supplementary Table 1), using Lipofectamine 2000. Forty-eight hours after transfection, single EGFP-expressing cells were sorted into 96-well plates and grown until colonies formed. Initial screening was based on PCR amplification (primers RNASEH2A-ex1F and RNASEH2A-ex1R) of the CRISPR/Cas9-targeted region of *RNASEH2A* with mutations present in selected clones determined by Sanger sequencing. The cellular RNase H2 status was then confirmed by immunoblotting, RNase H2 enzymatic activity assay, and alkaline gel electrophoresis to determine ribonucleotide content of genomic DNA (detailed methods below).

Fluctuation assays (human)

Hygromycin resistant HeLa reporter cells (400 µg/ml hygromycin B) were recovered from frozen stocks in the absence of selection. The following day, 10 wells of a 96-well plate were seeded with

2,000 cells per well for each line. The experiment was performed with the operator blinded to the identity of the cell lines. Cells were cultured under non-selective conditions and re-plated subsequently in 24-well, 6-well plates and ultimately T75 flasks, in which they were grown to confluence. Cells were then dissociated using Gibco TrypLE (Thermo Fisher Scientific) and cells counted using a Moxi Z automated cell counter. After serial dilution 1,000 cells were plated into two 10-cm plates for each culture and grown for 14 days to determine plating efficiency. All other cells were plated into two 10-cm plates, 0.5 µg/ml puromycin added after 4 h, with medium subsequently changed every 2-3 days for 14 days to remove dead cells and maintain a puromycin concentration of 0.5 µg/ml.

To establish mutation spectra, colonies were removed by scraping and then cultured in a 96-well plate. When confluent, cells were lysed with 75 µl DirectPCR Lysis Reagent (Viagen Biotech) and 0.4 mg/ml PCR-grade Proteinase K (Roche), heating overnight at 55 °C followed by 45 min at 85 °C. Only one sample per independent culture was used for PCR amplification and Sanger sequencing to determine the nature of mutations in the HygroR coding sequence. A 1.24 kbp region including HygroR was amplified with Prime Star Max PCR Master Mix (Takara Bio), HygroR_up and H1327R primers (40 cycles 10 s 98˚C, 15 s 70˚C, 2 min 72˚C). Sanger sequencing was then performed with additional primers (Supplementary Table 3) and mutations identified using Mutation Surveyor V3.30 (SoftGenetics). All mutants showed double traces of equal height from the point of indel mutations, consistent with the presence of two copies of the reporter in all reporter lines. As FISH indicated presence of the reporter at a single AAVS1 locus, we inferred that two copies of the reporter were inserted in tandem at this locus. As a 2 bp deletion or equivalent frameshift mutation in either HygroR copy would bring the associated PuroR coding sequence into the translated reading frame, we corrected mutation rate calculations (per bp) for the presence of 2 copies.

To determine colony numbers, plates were washed with PBS, fixed with 2% formaldehyde in PBS for 10 min, rinsed with water, and colonies stained with 0.1% crystal violet solution for 10 min. Plates were then washed with water and left to dry before counting colonies. After counting the experiment was unblinded. Mutation rates were determined for each individual culture in Microsoft Excel 2016, and an overall rate for WT and KO strains calculated using the Lea Coulson method of the median. The number of mutants for each culture were ranked, and appropriate ranks[56] used to calculate the rates that define the lower and upper limits of the 95% confidence interval.

Immunoblotting

Whole-cell extracts (WCE) to determine protein levels of RNase H2 subunits by immunoblotting and for RNase H2 activity assays were prepared as previously described[58]. Equal amounts of protein from WCE were separated by SDS-PAGE on 4-12% NuPAGE gels and transferred to PVDF. Membranes were probed in 5% milk (w/v; Marvel Original Dried Skimmed), TBS+0.2% Tween-20 (v/v) with the following antibodies: sheep anti-RNase H2 (raised against human recombinant RNase H2, 1:1,000)[14]; mouse anti-RNASEH2A G-10 (Santa Cruz Biotechnologies sc-515475, lot #A1416, 1:1,000); rabbit anti-GAPDH (Abcam ab9485, 1:2,000, lot #GR3380498-1). For detection we Rabbit Anti-Sheep Immunoglobulins/HRP (Dako, P04163, lot #00047199, 1:2,000); Goat Anti-Mouse Immunoglobulins/HRP (Dako, P0447, lot #20039214, 1:10,000); Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technologies, 7074S, lot #29, 1:10,000); Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) and an ImageQuantLAS4000 device, or IRDye secondary antibodies and an Odyssey CLx Imaging System (LI-COR Biosciences). Uncropped immunoblots are presented in Supplementary Fig. 1.

RNase H2 activity assays

To assess cellular RNase H2 activity, a FRET-based fluorescent substrate release assay was performed as previously described[14]. Briefly, RNase H2-specific activity was determined by measuring the cleavage of double-stranded DNA substrate containing a single embedded ribonucleotide. Activity against a DNA-only substrate of the same sequence was used to correct for background activity. Substrates were formed by annealing a 3'-fluorescein-labeled oligonucleotide (GATCTGAGCCTGGGaGCT or GATCTGAGCCTGGGAGCT; uppercase DNA, lowercase RNA) to a complementary 5'-DABCYL-labelled DNA oligonucleotide (Eurogentec). Reactions were performed in 100 µl reaction buffer (60 mM KCl, 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 0.01% BSA, 0.01% Triton X-100) with 250 nM substrate in black 96-well flat-bottomed plates (Costar) at 24°C. WCE was prepared as described above, protein concentrations determined using a Bio-Rad Bradford Protein Assay, and the final protein concentration per reaction was 50 ng/µl. Fluorescence was read (100 ms) every 5 min for up to 90 min using a VICTOR2 1420 multilabel counter (Perkin Elmer), with a 480-nm excitation filter and a 535-nm emission filter. Initial substrate conversion after background subtraction was used to calculate RNase H2 enzyme activity.

Alkaline gel electrophoresis

To determine the presence of excess genome-embedded ribonucleotides in nuclear DNA, alkaline gel electrophoresis of RNase H2 treated genomic DNA was performed as previously described[58]. Briefly, total nucleic acids were isolated from pellets from ~1 million cells by incubation in ice-cold buffer (20 mM Tris–HCl pH 7.5, 75 mM NaCl, 50 mM EDTA) with 200 µg/ml proteinase K (Roche) for 10 min on ice, followed by addition of N-lauroylsarcosine sodium salt (Sigma) to a final concentration of 1%. Nucleic acids were phenol:chloroform-extracted, isopropanol precipitated and dissolved in nuclease-free water. For alkaline gel electrophoresis, 500 ng of total nucleic acids was incubated with 1 pmol of purified recombinant human RNase H2 (isolated as previously described[59]) and 0.25 µg of DNase-free RNase (Roche) for 30 min at 37°C in 100 µl reaction buffer (60 mM KCl, 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 0.01% Triton X-100). Nucleic acids were ethanol precipitated, dissolved in nuclease-free water and 250 ng separated on 0.7% agarose gels in 50 mM NaOH, 1 mM

EDTA. After overnight electrophoresis, the gel was neutralised in 0.7 M Tris–HCl pH 8.0, 1.5 M NaCl and stained with SYBR Gold (Invitrogen). Imaging was performed on a FLA-5100 imaging system (Fujifilm), and densitometry plots were generated using AIDA Image Analyzer v3.44.035 (Raytest).

Mutation accumulation experiment

TP53-KO hTERT-RPE1 cells without and with loss-of-function mutations in *RNASEH2A* or *RNASEH2B*, introduced by CRISPR/Cas9 genome editing, a gift from D. Durocher (The Lunenfeld–Tanenbaum Research Institute, Toronto), have been previously described[33]. RNase H2 proficient (WT), RNASEH2A-KO and RNASEH2B-KO cells were single cell sorted into 96-well plates using a BD FACSJazz instrument (BD Biosciences). Multiple individual clones for each were expanded to confluent T75 flasks for cryopreservation and genomic DNA isolation of these ancestral populations. In addition, lines were again single cell sorted into 96-well plates to start the mutation accumulation experiment. Cultures were expanded by subsequent growth in 24-well, 6-well plates and T75 flasks until confluent (approximately 25 population doublings), and this process of single cell sorting and expansion was repeated 4 more times providing bottlenecks to capture mutations that occurred since the previous sort. From the first to the last single cell sort a total of approximately 100 population doublings occurred and the final culture was expanded for cryopreservation and genomic DNA isolation of these end-point populations.

Genomic DNA was isolated using phenol extraction as previously described[58], for alkaline gel electrophoresis and whole genome sequencing. Library preparations and sequencing were performed by Edinburgh Genomics. Libraries were prepared using Illumina SeqLab specific TruSeq PCRFree High Throughput library preparation kits as per manufacturer's instructions, with DNA samples sheared to a 450 bp mean insert size. Libraries were sequenced using paired-end reads on an Illumina HiSeqX instrument using v2.5 chemistry to achieve minimum mean genome-wide sequencing depth of 30x per sample.

Mouse whole genome sequencing

Villin-Cre^{+} Trp53$^{fl/fl}$ Rnaseh2b$^{fl/fl}$ mice with epithelial-specific deletion of *Trp53* and *Rnaseh2b* on a C57Bl/6J background have been described previously[29]. Animal experiments were conducted with appropriate permission, in accordance with guidelines for animal care of the Christian-Albrechts-University (Kiel, Germany), in agreement with national and international laws and policies. No randomisation or blinding was performed. Paired tumour-normal DNA was isolated from small intestinal tumours (*Trp53$^{-/-}$ Rnaseh2b$^{-/-}$*) and liver tissue (*Trp53$^{+/+}$ Rnaseh2b$^{+/+}$*) from 52-week old females, using a Qiagen DNeasy Blood & Tissue Kit. Library preparations and sequencing were performed by Edinburgh Genomics using Illumina DNA PCR-Free Library Prep as per manufacturer's instructions. Paired end sequencing was performed by Edinburgh Genomics on a NovaSeq 6000 using v1.5 chemistry. Mean genome-wide sequencing depth of at least 30x for liver samples and 60x for tumour samples was obtained.

***S. cerevisiae* WGS analysis**

Whole genome sequencing SRA files for *rnh201Δ pol2-M644G S. cerevisiae*[25] from the NCBI Sequence read archive (SRA) were converted to FASTQ files using SRA Toolkit v2.5.4-1 (SRA Toolkit Development Team; http://ncbi.github.io/sra-tools/). FASTQ reads were aligned to the GSE56939_L03_ref_v2 reference genome ([60]; Supplementary Table 5) and sorted BAM files created using BWA-MEM 0.7.12[61], and deduplicated with SAMBLASTER v0.1.22[62]. To select high quality indel variants, GATK (v3.6-0) Haplotype Caller (without Base Quality Score Recalibration)[63] variant calling was performed with "Hard Filters" (--filterExpression "QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0") . Filtering for strain-specific variants was performed as previously described[60], with minor modifications. Filters: 1) eliminate variants shared with an ancestral clone; 2) required ≥ 20 reads for variant allele in descendent; 3) exclusion of repetitive sequences as defined in [60]; 4) reference/variant depth ratio 0.4-0.6; < 0.4 if homozygous variant allele .

RPE1 WGS analysis

FASTQs were converted to unaligned BAM format and Illumina adaptors marked using GATK v4.1.9.0 FastqToSam and MarkIlluminaAdapters tools[64]. Reads were aligned to the human genome (hg38, including alt, decoy and HLA sequences) using BWA-MEM v0.7.16 [61] and read metadata merged using GATK's MergeBamAlignment tool. PCR and optical duplicate marking and base quality score recalibration were performed using GATK. Variants from NCBI dbSNP build 151 were used as known sites for base quality score recalibration. Post-processed alignments were genotyped using Mutect2, Strelka2, Platypus and SvABA using somatic calling models for each pair of ancestral and endpoint cultures, as detailed below.

Mouse WGS analysis

FASTQ processing and alignment was performed as for RPE1 WGS analysis, using the GRCm38 mouse genome reference and known variant sites from the Mouse Genomes Project[65] (REL-1807-SNPs_Indels) for base quality score recalibration. Somatic variant calling of post-processed alignments was performed using Mutect2, Strelka2, Platypus and SvABA for each tumour-liver pair, as detailed below. Somalier v0.2.12 (https://github.com/brentp/somalier) was used to confirm each paired tumour and liver sample originated from the same animal.

Human Ethics approval

Data generated from Genomics England 100,000 genomes and ICGC-CLL studies were analysed. In these respective studies, informed consent for participation was obtained. Ethical approval for Genomics England 100,000 genomes project: East of England and South Cambridge Research Ethics Committee; CLL-ICGC: International Cancer Genome Consortium (ICGC) guidelines from the ICGC Ethics and Policy committee were followed and the study was approved by the Research Ethics Committee of the Hospital Clínic of Barcelona.

CLL WGS analysis

Genomics England: CLL tumour-normal pairs (*n*=198) were processed as part of the 100,000 Genomes Project (pilot and main programme v8). Samples were sequenced using the Illumina HiSeq X System with 150 bp paired-end reads at a minimum of 75x coverage for tumours and 30x coverage for germline samples. Reads were mapped to GRCh38 using ISAAC aligner v03.16.02.19 [66]. SNVs and indels were called using Strelka v2.4.7 using somatic calling mode. Structural and copy number variants were called using Manta v0.28.0 and Canvas v1.3.1 [67], respectively. Samples with a tumour purity estimate from Canvas of less than 50% were excluded from analysis. *RNASEH2B* copy number was determined using a combination of Canvas, Manta, read depth counts with samtools v1.9 and confirmed by manual inspection using IGV (v2.5.0)[68].

ICGC: WGS from the ICGC-CLL cohort[35] (*n*=150) was re-analysed. Raw reads were mapped to the human reference genome (GRCh37) using BWA-MEM (v0.7.15)[61]. BAM files were generated, sorted, indexed and optical or PCR duplicates flagged using biobambam2 (https://gitlab.com/german.tischler/biobambam2, v2.0.65). Copy number alterations were called from WGS data using Battenberg (cgpBattenberg, v3.2.2)[69], ASCAT (ascatNgs, v4.1.0)[70], and Genome-wide Human SNP Array 6.0 (Thermo Fisher Scientific) data[35] re-analysed using Nexus 9.0 Biodiscovery software (Biodiscovery). *RNASEH2B* copy number was established by combining the three analyses and manual review with IGV.

Colorectal Cancer WGS analysis

Irinotecan-treated (*n*=39) and irinotecan-untreated (*n*=78) colorectal cancers from the 100,000 Genomes Project Colorectal Cancer Domain were 1:2 matched using a multivariate greedy matching algorithm without replacement, implemented in the Matching R-package[71]. Matching was conducted considering sex, age at sampling, whether a primary tumour or metastasis had been sequenced, microsatellite instability status, and whether the individual have previously received radiotherapy, oxaliplatin, capecitabine or fluorouracil treatment.

Somatic Variant Calling

Somatic variant calling was performed in parallel using four distinct methods: Mutect2 (as part of GATK v4.1.9.0)[72,73], Strelka2 (v2.1.9.10)[74], SvABA (v1.1.3)[75] and Platypus (v0.8.1)[76]. High-confidence indel calls were defined as the intersected output of these four tools, where variants passed all filters for ≥3 of 4 callers. The intersection was performed using the bcftools (v1.10.2)[77] isec function after normalising variant calls and left-aligning ambiguous alignment gaps using the bcftools norm function. For Platypus (v0.8.1)[76], joint calling all samples in each cohort was performed before filtering for somatic variants; the other variant callers were run in paired tumour-normal mode. For the RPE1 mutation accumulation experiment the endpoint and ancestral cultures were defined as “tumour” and “normal" samples respectively. Variant filtering strategies were optimised to both available information on segregating genetic variation for humans and mice, and the functionality of each calling method as detailed below.

Mutect2: unfiltered genotypes for all normal samples were combined to filter germline variants. Somatic calls were obtained using GATK’s FilterMutectCalls command. Human polymorphism data and allele frequencies from, gnomAD[78] were provided to Mutect2 for the filtering of germline variants.

SvABA: Germline indel and structural variants were filtered using --dbsnp-vcf and --germline-sv-database options. Mouse indels were obtained from Mouse Genomes Project version 5 SNP and (ftp://ftp-mouse.sanger.ac.uk/REL-1505-SNPs_Indels/mgp.v5.merged.indels.dbSNP142.normed.vcf.gz); structural variants from SV release version 5 (ftp://ftp-mouse.sanger.ac.uk/REL-1606-SV/mgpv5.SV_insertions.bed.gz and ftp://ftp-mouse.sanger.ac.uk/REL-1606-SV/mgpv5.SV_deletions.bed.gz). Human indels were extracted from NCBI dbSNP build 151 and common structural variants from dbVAR (https://hgdownload.soe.ucsc.edu/gbdb/hg38/bbi/dbVar/).

Strelka2: candidate small indels for each pair were first generated by Manta (v1.6.0)[79] in somatic calling mode. Strelka2 was then executed in somatic calling mode for each pair with Manta's candidate small indels output provided to the --indelCandidates option.

Platypus: Germline variants were filtered on the basis of any "normal" sample with ≥ 2 variant allele reads. Somatic variant calls for each sample pair were retained if "tumour"/endpoint sample > 2 variant reads; site depth > 9; and "normal" sample read depth ≥ 20, <2 variant reads. Additionally, a >10x ratio of tumour to normal for variant/total depth was required.

For Genomics England CLL tumour-normal pairs, pre-existing Strelka2 calls from the 100,000 Genomes Project pipeline were used, while variant calling with Mutect2, Platypus and SvABA was performed as above. Colorectal cancer tumour-normal pairs from Genomics England were processed as for Genomics England CLL but without Mutect2 analysis. For ICGC CLL, somatic indels were called using Mutect2 (GATK v4.0.2.0)[72,73], Strelka2 (v2.8.2)[74], SvABA (v1.1.0)[75], and Platypus (v0.8.1)[76]. Candidate small indels generated by Manta (v1.2)[79] were used as input for Strelka2. Mutect2, Strelka2 and SvABA were run in paired tumour-normal mode. somaticMutationDetector.py (https://github.com/andyrimmer/Platypus/tree/master/extensions/Cancer) was used to identify somatic indels called by Platypus with a minimum posterior of 1. SNVs called by Platypus were considered somatic if they had at least 2 alternative reads in the tumour, fewer than 2 alternative reads in the normal, a minimum tumour VAF of 10x the control VAF, and a minimum depth of 10.

Germline mutation analysis

De novo WGS variants were downloaded from the Gene4Denovo database (Supplementary Table 5). Reference assembly conversion errors were removed by discarding variants where the reference allele did not match the genome reference at the given position or where the variant position was greater than the length of the reference chromosome. In addition, individuals with total *de novo* variants below the 10th (*n*=33) or above the 90th (*n*=140) percentile were excluded. For germline gene expression we used pre-defined expression groups[80] based on Ensembl release 90 annotation (ftp://ftp.ensembl.org/pub/release-90/gtf/homo_sapiens/Homo_sapiens.GRCh38.90.gtf.gz). Initially stratified as nine expression groups from 1 (=unexpressed) to 9 (=high), we collapsed them into a smaller set of unexpressed (1), low (2, 3, 4), mid (5, 6, 7) and high (8, 9). The annotations were converted to GRCh37 coordinates using liftover (kent source version 417). Genomic segments overlapping multiple distinct expression groups, due to overlapping genes, were assigned to the higher of those expression groups. For each expression group we summed the count (*c*) of *de novo* indels contained within the genomic span of those genes. This was converted to rate estimates by dividing by the union genomic span (*g* nucleotides) of genes in that expression group, and adjusting for the number of mutated genomes considered (*n*); *rate = c/(gn)*. To obtain 95% confidence intervals, gene selection was bootstrapped (sampled to an identical number with replacement) 100 times and the 0.025 and 0.975 quantiles of the bootstrapped rate calculation taken as the 95% confidence interval.

ICGC Pan-cancer expression analysis

The ICGC PCAWG somatic mutations[50] (https://dcc.icgc.org/api/v1/download?fn=/PCAWG/consensus_snv_indel/final_consensus_passonly.snv_mnv_indel.icgc.public.maf.gz) and ICGC PCAWG "baseline" gene expression[50] were obtained (ArrayExpress https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5200/). Genomic annotation of gene extents on the GRCh37 reference genome match the Ensembl version 75 annotation (http://ftp.ensembl.org/pub/release-75/gtf/homo_sapiens/Homo_sapiens.GRCh37.75.gtf.gz) of the ICGC gene expression calls. Mean, median and maximal gene expression (transcripts per million, TPM) were calculated for each gene across the 76 ICGC baseline gene expression tissues/samples. Only genes annotated on the main autosomal chromosomes, 1 to 22 and the X chromosome were considered. Overlapping genes were removed, keeping only the most abundantly (highest median, then mean in the case of ties) expressed genes from overlapping pairs. This filtering was applied hierarchically, starting with the most abundant. Following [81] genes with housekeeping-like expression were defined as those with maximal expression of less than ten times median expression. Housekeeping-like genes were decile binned into expression groups based on median expression. Mutations were stratified by type (1 bp deletion, 2-5 bp deletion) or by the "TN*T" motif defined below and counted by intersection with the annotated genomic extents of genes in each expression group.

For the analysis of tissue-biased gene expression, the 76 ICGC baseline samples were grouped by annotated tissue (e.g. breast, prostate, kidney, liver) and matched where possible to the tissue of origin for ICGC cancer types. For each tissue, the median expression (in TPM) of each gene was calculated for (a) within-tissue samples and (b) for all other samples. The 90th quantile of gene expression (q90, top 10%) within a tissue was set as a threshold for "high" level expression. Genes with high expression in a tissue (a) but a median expression of less than q90*0.1 in the other tissues (b) were considered highly expressed but tissue restricted (HETR). For the set of HETR genes from a tissue, we counted the number of 2-5 bp deletions within the annotated genomic extent of the HETR genes in a cancer type of interest. We similarly counted 2-5 bp deletions in all other genes for that cancer type, and counted both the HETR and non-HETR 2-5 bp deletions from all other cancer types within the ICGC cohort. For each cancer:tissue pair this provided 4 sets of counts, analysed as two-tailed Fisher's exact test using the R fisher.test function. A positive odds-ratio indicating enrichment of 2-5 bp deletions in the HETR genes, compared to a background of the remainder of the ICGC cohort in which HETR genes are not highly expressed. For each cancer type considered, this test was repeated for each tissue type (*n*=17). Analyses were carried out for eight of the ICGC cohort cancer types which met the combined criteria of having a well-matched and known tissue of origin amongst the ICGC baseline samples, and requiring the cancer type cohort to have at least *n*=2,500 2-5 bp deletions in aggregate. This represents *n*=17*8=136 statistical tests, adjusted for by Bonferroni correction. Odds ratios (r) for mutation depletion were transformed to their reciprocal (1/r) for display purposes.

ICGC Pan-cancer TOP1-seq analysis

Data corresponding to two replicates of TOP1-seq, a modified ChIP-seq technique to immunoprecipitate only catalytically engaged TOP1[38], were downloaded from the NCBI GEO database (accession code GSE57628, samples GSM1385717 and GSM1385718). Autosomal chromosomes 1 to 22 and the X chromosome were divided into 1 kbp bins and for each bin the amount of mappable sequence was determined using Umap's regions mappable using 36mers[82] to approximate read length of the TOP1-seq data. For each 1 kbp window, the TOP1-seq signal within mappable regions was summed for each replicate and mean signal calculated. This mean was divided by the amount of mappable sequence to calculate the TOP1-seq signal per bp and each 1 kb window was then assigned to decile bins using this value.

Somatic deletion calls from ID4-positive PCAWG samples (as defined in https://dcc.icgc.org/api/v1/download?fn=/PCAWG/mutational_signatures/Signatures_in_Samples/SP_Signatures_in_Samples/PCAWG_SigProfiler_ID_signatures_in_samples.csv) were counted within the same 36-mer mappable regions for each 1kbp window and either stratified by type (1 bp deletion, 2-5 bp deletion) or by the "TN*T" motif defined below. Relative rates of deletions in each category were calculated relative to the first TOP1-seq signal decile.

Mutational signatures

De novo extraction and decomposition of mutational signatures was performed in Python 3.8.5 using SigProfilerExtractor (v1.1.0)[5], along with SigprofilerMatrixGenerator (v1.1.14/1.1.15)[83] and SigprofilerPlotting (v1.1.27). Recommended default settings (including 500 NMF replicates) were applied (https://github.com/AlexandrovLab/SigProfilerExtractor). Subtraction of mutations in RPE1 wildtype cells from those detected in RNase H2 null cells was performed as follows. The average number of indels per line for each of the 83 categories was determined for the three wildtype lines. Counts per category for AKO and BKO lines were subtracted using these averages, with negative values set to 0. SigProfilerExtractor was then performed on the resulting WT-subtracted AKO and BKO ID-83 matrices for both *de novo* signature detection and decomposition analysis.

Indel sequence context analysis

WGS indels were categorized based on repeat sequence context. Genome-wide occurrence of short repeats and regions of microhomology were identified and filtered to include only the mappable genome, defined by Umap's regions mappable using 100mers [82]. For both WGS-identified indel variants and genome-wide occurrence, scoring of 2-bp deletions compliant with the "TNT" motif at MH/SSTR sites required the deleted bases to match the sequence NT with a T immediately 5' of the deleted dinucleotides. More generally, for varying sized deletions these were considered to fit a "TN*T" motif if the deletion lay within an SSTR or region of microhomology containing the motif $TN_{(d-1)}T$ where d = the length of the deletion. Genome-wide occurrences were estimated from 100,000 randomly generated deletions of given lengths within the mappable genome. For SSTRs and MH regions, all regions containing the respective motifs $(TN_{(r-1)})_n$ or $TN_{(r-1)}T$ were identified (where r = the length of the repeat unit and $n > 1$), and the fraction of SSTR/MH sequence containing TNT motifs determined against total SSTR/MH sequence in the mappable genome.

To derive a null expectation for *de novo* deletions matching the TNT, TNNT and TNNNT motif for 2, 3, and 4 bp deletions respectively, deletions at repeats from the Gene4Denovo database were first classified by deletion length, repeat type (STR or MH) and repeat length. Bootstrap samples of corresponding repeats from the genome were generated with 1,000 replicates. That is, for each deletion category an equal number of repeats of matching repeat type, repeat unit length and total repeat length were randomly drawn from the genome for each bootstrap sample.

Sequence logos

Genomic sequences containing 2 bp deletions were reversed and complemented when the deleted dinucleotide contained an adenosine (A), except when the dinucleotide was AT or TA. For SNMH and STR deletions, the position of the deleted dinucleotide cannot be unequivocally assigned, and therefore the deleted sequence was right aligned in the repeat/microhomology region, either to the most 3’ T, where present, or otherwise to the limit of the repeat/microhomology region. Sequences were converted to bit score matrices and logos drawn using Logomaker v0.8[84].

Embedded ribonucleotide sequence context analysis

EmRiboSeq data from *rnh201Δ* yeast prepared during mid-log phase growth[85] was obtained (Supplementary Table 5) and aligned to the sacCer3 reference genome as previously described to identify the genomic coordinates of genome embedded ribonucleotides[86]. Bedtools (version v2.30.0, [87]) utilities groupby, slop and getfasta were used to extract and count the sequence context of genome embedded ribonucleotides with downstream analysis and plotting implemented in R (version 4.0.5). Genome sequence composition adjusted relative rates were calculated as previously described[32] such that under the null expectation of no sequence bias in ribonucleotide incorporation, all sequence contexts have an expected relative rate of 1/n where n is the number of contexts considered.

Statistical methods

Statistical testing was performed using GraphPad Prism v9.1.1, Python v3.8.5 or R v3.3.1. Two-side non-parametric Mann-Whitney tests were performed for quantitative measurements; multiple testing correction, FDR set at 0.05; and for categorical data Fisher's exact tests were performed in Python using stats.fisher_exact from scipy v1.6.3. Calculation of cosine similarities: Mutations for each strain were converted into a vector, with ordered values representing different mutation categories as a proportion of total mutations. These were then compared in a pairwise fashion. Given two vectors A and B, the cosine similarity (cos(θ)) was calculated as:

$$\cos(\theta) = \frac{\sum_{i=1}^{n} \quad A_i B_i}{\sqrt{\sum_{i=1}^{n}(A_i)^2}\sqrt{\sum_{i=1}^{n}(B_i)^2}}$$

Hierachical clustering used the hclust function of R (version 4.1.0) with complete linkage clustering of pairwise cosine distances (1 - cosine similarity) between ID-83 mutation spectra, with 41 categories of productive reporter frameshift mutations. For bootstrap support, *n*=1,000 bootstrap datasets were generated by sampling with replacement the mutations observed with a strain, for each strain; then calculating the cosine distance and hierarchical clustering for each bootstrap dataset. Reported bootstrap scores are the percentage of bootstrap replicates hierarchical clustering of which supports the clustering to the right of the indicated position.

To test significance of cosine similarities, we used a null model based on the Dirichlet-multinomial distribution. Briefly, when comparing two mutation count vectors, with total mutations m_1 and m_2, over n mutation classes, we constructed a distribution of cosine values by comparing 10,000 simulated pairs of random vectors generated as follows. For each simulated pair, we sampled from a Dirichlet-multinomial distribution with the concentration parameters as a vector of ones of dimension n, and number of trials as m_1 for the first vector in the pair, and m_2 for the second vector. The null distribution was obtained by computing the cosine similarity of the 10,000 pairs of mutation count vectors.

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Author Contributions

M.A.M.R, T.C.W., M.S.T. and A.P.J. conceived the project and designed the experiments. T.C.W. and M.A.M.R, with help from P.C., performed fluctuation assays and sequencing experiments. M.A.M.R., with help from P.C., performed the RPE1 mutation accumulation experiment. S.B. performed FISH experiments. M.A.M.R., T.C.W. and D.O.R.S. performed all other molecular biology experiments. H.X. and K.A. provided mouse tumour and control tissue samples. D.A.P., T.C.W., M.D.N. and M.S.T. designed and implemented computational analyses. D.A.P., T.C.W. and M.S.T analysed yeast, mouse, RPE1 and Gene4Denovo WGS data. D.A.P. and M.S.T. performed pan-cancer analyses. T.G.E.R.C., K.R and A.S. provided CLL WGS data. A.J.C. provided CRC data. D.A.P., F.N., R.L.H., R.R. and C.P. analysed

CLL data. D.A.P. analysed CRC data. M.A.M.R, C.P., T.S., E.C, M.S.T. and A.P.J. supervised the work. T.C.W., F.N., E.C., T.S., M.S.T. and A.P.J. funded the work. M.A.M.R. and A.P.J. wrote the manuscript. All authors had the opportunity to edit the manuscript. All authors approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information is available for this paper.

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Data Availability

RPE1 mutation accumulation experiment and mouse tumour WGS data are available from European Nucleotide Archive accession PRJEB48753 (https://www.ebi.ac.uk/ena/browser/view/PRJEB48753). All other data were previously published and sources are cited in Supplementary Table 5.

Code Availability

Code documented in Methods is available at https://git.ecdf.ed.ac.uk/ID-TOP1

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Colorectal Cancer Domain UK 100,000 Genomes Project

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Extended data figure legends

Extended Data Fig. 1 | ID4 is distinct from small deletion signatures of known aetiology. a,b, The mechanistic basis for many COSMIC indel signatures is unknown, with only 9 out of 18 having a proposed aetiology. ID2 (**a**) is attributed to DNA polymerase slippage[88,89] and ID6 (**b**) to microhomology mediated end-joining (MMEJ) activity, associated with HR deficiency[5,90]. **c,d,** Mechanism for these signatures supported by: impaired MMR promoting replication slippage mutagenesis in MLH1-/- colonic organoids resulting in ID2 (and ID1) signatures (**c**); ID6 contributing substantially (along with ID8) to the indel signature in ovarian cancer, in which HR deficiency is common (**d**). Analysis of data from [91] in **c**; data for 73 ovarian adenocarcinomas with ID6 contribution from ICGC[5,50] in **d**.

Extended Data Fig. 2 | Yeast and human frameshift mutation reporters detect indels at tandem repeats. a, Yeast reporter. Synonymous substitutions were made in the hygromycin resistance gene

(HygroR), such that it contained many short 2 bp tandem repeats (SSTRs). Expression from the TEF promoter (P_{TEF}) ensures a constitutive high level of transcription. Mutations within HygroR that result in a frameshift simultaneously put the HygroR coding sequence out of frame and the downstream neomycin resistance (NeoR) sequence in frame, allowing antibiotic selection of cells with such mutations. **b,** Top1-dependent 2 bp SSTR deletions occur in both WT and *rnh201Δ* (RNase H2 null) yeast, with the highest mutation rate for *rnh201Δ* (related to Fig. 1d). **c-e,** WT and *rnh201Δ* have similar spectra, and differ from *top1Δ* strains. Mutation spectra of neomycin resistant colonies. n, number of independent colonies sequenced. Other: complex indels, missense mutations or mutation not characterised (**c**). Tree for pairwise clustering with percent bootstrap support to the right of the indicated position, based on cosine scores calculated for mutation spectra (Fig. 1e) of the 41 mutation categories that give productive reporter frameshift mutations (**d**). Matrix of pairwise cosine similarities and P-values between reporter mutation spectra in different yeast strains. Darker blue indicates greater similarity; darker grey greater significance. Test statistic is the cosine similarity value for 41 mutation categories and the null hypothesis is that that the cosine value will be distributed according to the Dirichlet-multinomial model, as described in Methods. The test is one-sided and no adjustments were made for multiple comparisons (**e**). **f,** Null distribution for cosine pairwise vector comparisons for 41 and 83 mutation categories. Plots, cosine values for 10,000 randomly generated pairs of vectors of mutation spectra. Each vector contained 100 randomly assigned mutations (see Methods for further details). Cosine value thresholds indicated for $P < 0.05$ and $P < 0.01$. **g,** The human reporter is expressed from the ubiquitous CAG promoter (P_{CAG}) , and NeoR is replaced with the puromycin resistance gene (PuroR) to allow more rapid antibiotic selection in mammalian cell culture.

Extended Data Fig. 3 | Validation and characterisation of RNASEH2A+ and KO HeLa reporter cells. **a-c,** Reporter integration at the *AAVS1* locus and retention of a reporter-free locus with a 200 bp deletion at the target site was confirmed by PCR and Sanger sequencing. Green arrow head, specific PCR product. Representative of at least 2 independent experiments. **d,e,** FISH shows integration of the reporter (**d**) at a single *AAVS1* locus (**e**). Representative image of approximately one hundred mitotic chromosome spreads in 3 independent experiments. SA, splice acceptor; T2A, self-cleaving peptide; pA, polyadenylation site; also see Fig. 2a. **f,g,** Alkaline gel electrophoresis of RNase H2 treated genomic DNA (**f**) shows a small increase in fragmentation for the RNASEH2A+ control clone and a more substantial increase in two independent RNASEH2A-KO clones (representative of 4 independent experiments), indicating the presence of more genome-embedded ribonucleotides compared to HeLa and parental reporter cells (**g**). "Control KO" cells were reported previously[33,58]. RFU, relative fluorescence units. **h,** 2 bp SSTR deletions are frequent in both RNASEH2A+ and KO cells. Mutation spectra, quantitation of indel type. Relative area of pie charts scaled to mutation rate. n, number of colonies sequenced from independent cultures. Other: complex indels or missense mutations.

Extended Data Fig. 4 | RPE1 RNase H2 null cells accumulate embedded ribonucleotides and 2-5 bp deletions across the genome. a,b, *RNASEH2A* and *RNASEH2B* KO cells (AKO, BKO, respectively) have substantially reduced cellular levels of RNase H2 subunits (**a**) and are deficient for RNase H2 enzyme activity (**b**) at the outset (ancestral) and at the end of the mutation accumulation experiment (end point). Individual data points, *n*=3 technical replicates; mean ± s.d. For gel source data, see Supplementary Fig. 1. **c,d,** Alkaline gel electrophoresis of RNase H2 treated genomic DNA (**c**) shows a substantial increase in fragmentation for *RNASEH2A* and *RNASEH2B* KO clones (representative of 3 independent experiments), indicating the presence of more genome-embedded ribonucleotides compared to two WT control clones (**d**). Densitometry plots of **c**. RFU, relative fluorescence units. As RNase H2 deficiency activates the p53 pathway[14,92], experiments were performed in a *TP53* knockout background. **e,** Only 2-5 bp deletions are significantly increased in RNase H2 null cells. Data points for acquired indel mutations in individual cell lines after 100 population doublings. Individual data points, indel counts per cell line; mean ± s.d.; P-values for two-sided Fisher's exact test between WT (*n*=3 independent clones) and KO (*n*=2 independent clones) for one indel type vs all other indel types, after Bonferroni correction. **f,** Proportions of acquired indels in WT and KO RPE cells. After correction for indels occurring in WT, 69% of indels in RNase H2 null cells are 2-5 bp deletions. n, total indel counts. **g,** Quantification of 2 bp deletions by context. n, total number of 2 bp deletions. For **f** and **g**, chart areas scaled to mutation counts per line.

Extended Data Fig. 5 | ID4 occurs in RNase H2 null RPE1 cells, particularly in transcribed regions. a-d, Mutational spectra detected by WGS after 100 population doublings in RPE1 cells demonstrates that SSTR and SNMH/MH deletions are enriched in RNase H2 cells. Spectra for combined RNase H2 null and wildtype cell lines (**a**), and individual cell lines (**b**). Mutational signature analysis confirms ID4 contribution in RNase H2 null (**c**), but not WT cells (**d**). **e,** In RNase H2 null cells, ID4 contributes significantly more to indel mutations in transcribed genomic regions (P=1.3x10^{-29}). Two-sided Fisher's exact test, ID4 indels vs other indels.

Extended Data Fig. 6 | ID4 mutations in RNase H2 null mouse tumours and RPE1 cells occur at a TNT motif, defining ID-TOP1. a, Mutation spectra for individual Rnaseh2b-KO murine intestinal tumours (WGS, paired tumour-normal samples from 6 mice). **b,** Indel classes, detected in murine Rnaseh2b-KO tumours. n, total indel count for 6 tumours. **c,** Most 2 bp deletions in these tumours occur at SSTRs and sites of single nucleotide microhomology (SNMH). n, number of 2 bp deletions. **d,e,** A TNT sequence motif is present at all 2 bp STR and SNMH deletions in RNase H2 null mouse tumours (**d**) and RPE1 cells (**e**). Related to Fig. 4d and Fig. 3, respectively. Sequence logo: 2-bit representation of the sequence context of 2 bp deletions. Top, all deletions, with those sequences containing a deleted adenosine (except AT/TA) reverse complemented, and deletions right-aligned.

Middle, re-aligned on right-hand T. Bottom, aligned on T (STR and SNMH context only). n, number of deletions. **f,** Deletion sites in RNase H2 null RPE1 cells are significantly enriched for the TNT sequence motif compared to genome-wide occurrence, for all genome sequence, as well as SNMH sites. P-values, two-sided Fisher's exact, observed vs expected. *n*=98 (all; *P*=8.3x10^{-14}), 54 (STR; *P*=0.057), 30 (SNMH; *P*=0.0008) deletions.

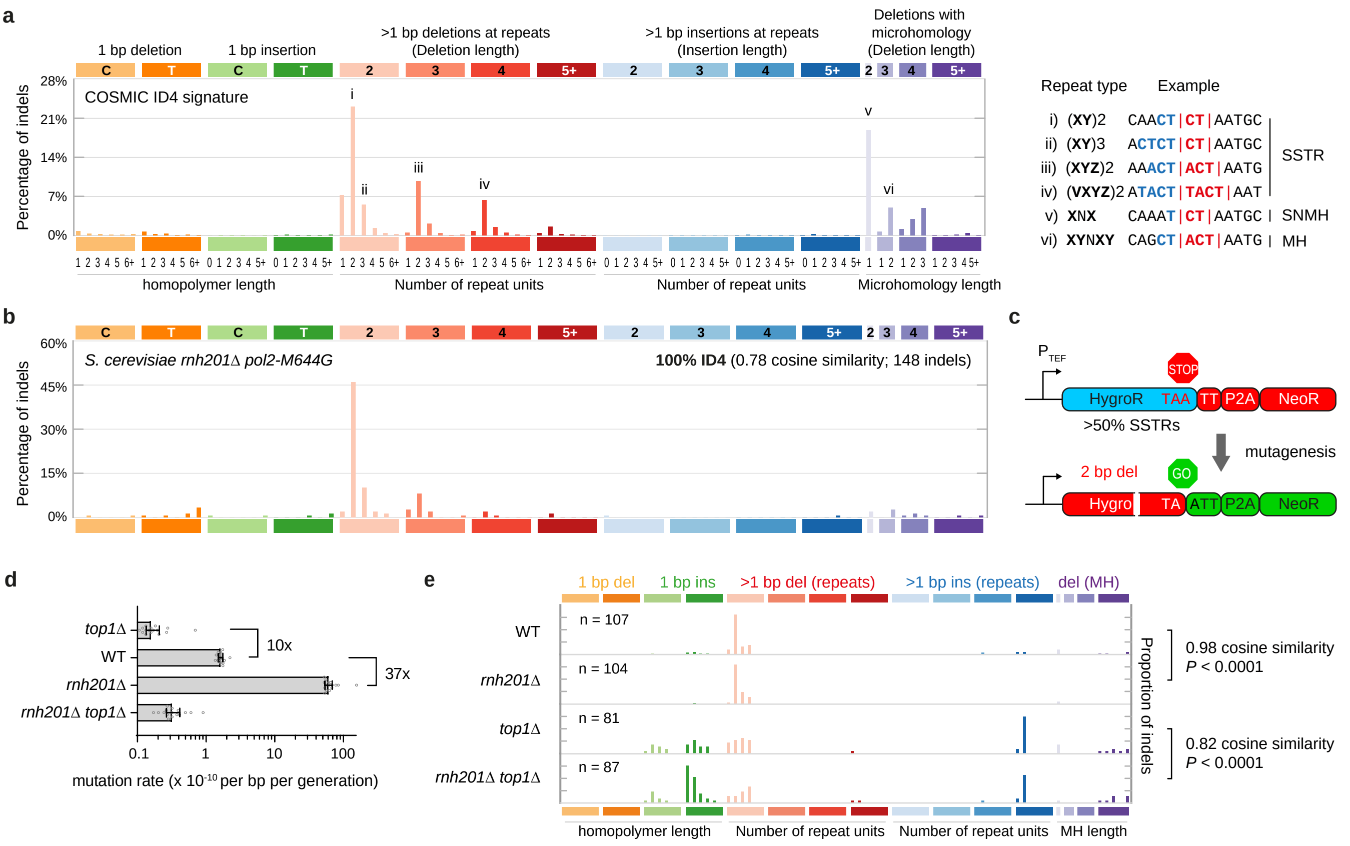
Extended Data Fig. 7 | ID4 deletions in RNase H2 null *S. cerevisiae* occur at a TNT motif in a Top1-dependent manner. a, 2 bp deletion sites in *rnh201Δ pol2-M644G* yeast are significantly enriched for the TNT sequence motif compared to genome-wide occurrence, for all genome sequence, as well as STR sites. P-values, two-sided Fisher's exact, observed vs expected. *n*=94 (all; *P*=1.0x10^{-9}), 91 (STR; *P*=0.029), 3 (SNMH; *P*=1) deletions. **b,** A TNT sequence motif is present at all 2 bp STR and SNMH deletions in *rnh201Δ pol2-M644G* yeast. Sequence logo: 2-bit representation of the sequence context of 2 bp deletions. Top, all deletions, with those sequences containing a deleted adenosine (except AT/TA) reverse complemented, and deletions aligned on right-hand T. Bottom, aligned on T (STR and SNMH context only). n, number of deletions. **c,d,** TN*T motifs extend beyond 2 bp deletions, with enrichment above expectation for 2 bp deletions at TNT, 3 bp deletions at TNNT and 4 bp deletions at TNNNT motifs in *rnh201Δ pol2-M644G* yeast WGS. Null expectations were generated by randomly simulating deletions of 2, 3 and 4 bp (**c**) or 2 bp STR sequences (**d**) genome-wide and scoring those simulated events for TN*T compliance. Each simulated dataset matched the count of observed mutations for the corresponding deletion class and *n*=1,000 replicate simulated datasets were produced. The frequency distribution of TN*T compliance in simulations is plotted as histograms, and comparison to the observed frequency of TN*T compliance (dotted red lines) used to derive a two-tailed empirical P-value. **e,** 2 bp STR deletions have biased sequence composition. Deletion observed in *rnh201Δ pol2-M644G* yeast WGS. Genome, frequency of dinucleotides in STR sequences in mappable genome. **f,** Ribouridine (rU) is more common in a CrU/GrU than in an

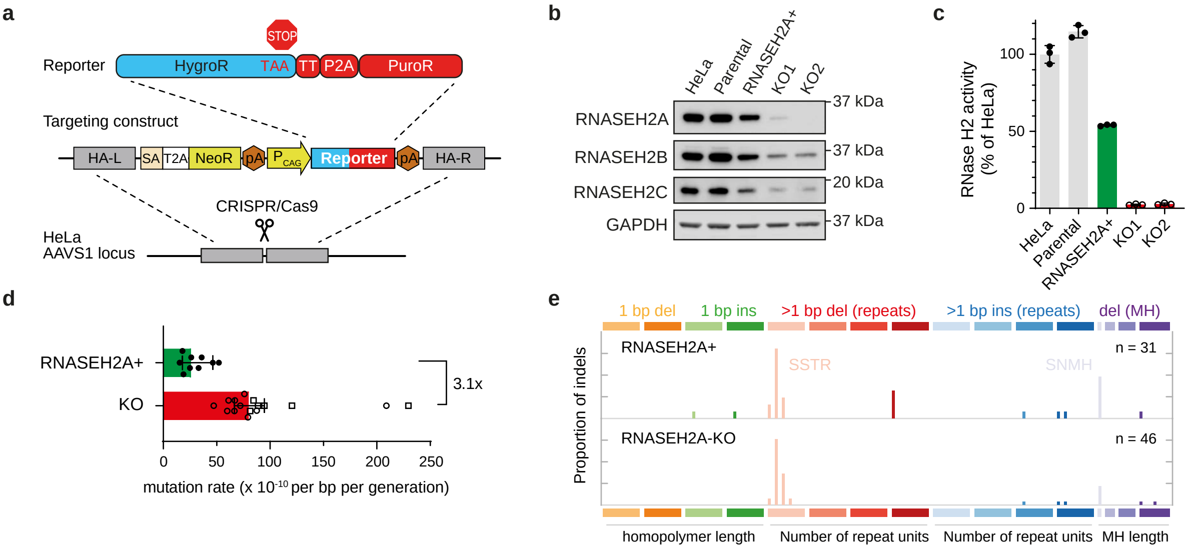
ArU/TrU dinucleotide context. Genome-embedded ribonucleotide frequency determined by emRiboSeq[86]. Dotted line indicates relative rate in absence of bias (=0.25). Horizontal lines, mean; individual data points, values for *n*=4 independent experiments[85]. **g,h,** 2 bp TNT deletions in wildtype and RNase H2 null cells are dependent on Topoisomerase 1. Mutation rates for 2 bp deletions at TNT-compliant SSTRs (**g**). Deletions at TNT motifs are significantly increased above expectation in WT and *rnh201Δ*, but not in *top1Δ* and *rnh201Δ top1Δ* yeast. Horizontal bars, 95% confidence intervals for odds ratio estimates (diamonds). P-values, two-sided Fisher's exact after Bonferroni correction; *n*=86, 28, 103, 19 2-bp deletions, with each deletion from an independent culture, for WT, *top1Δ*, *rnh201Δ*, *rnh201Δ top1Δ*, respectively. Null expectation, random occurrence of mutations in reporter target sequence (**h**).

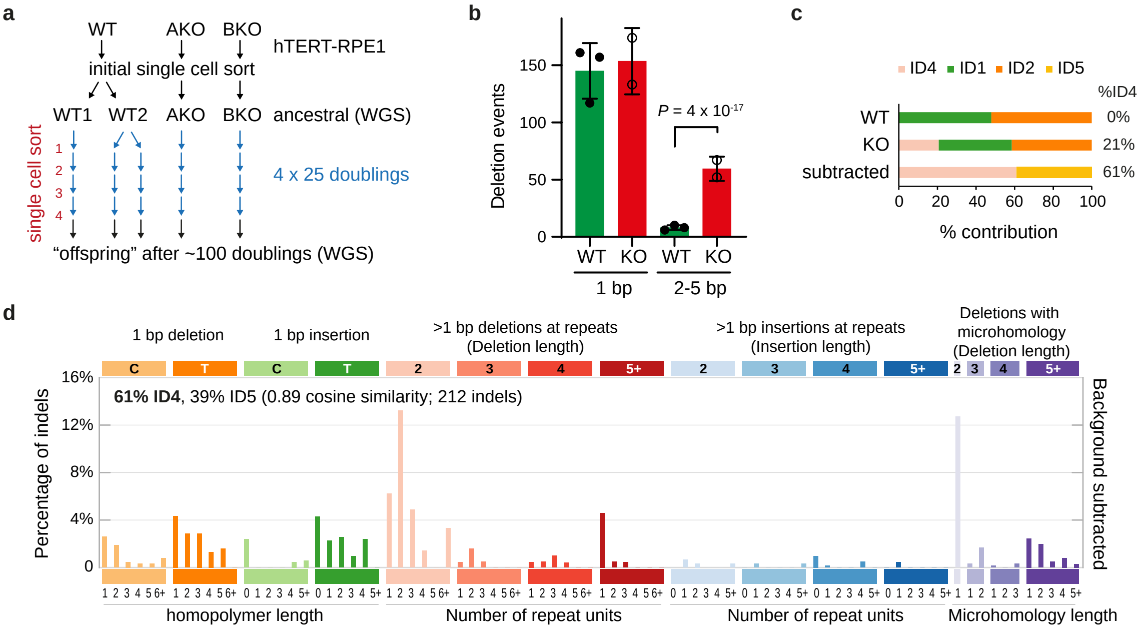
Extended Data Fig. 8 | TOP1-mediated mutagenesis causes increased 2-5 bp deletions in cancer. a, Of all indels, only 2-5 bp deletions are significantly increased in CLL with biallelic *RNASEH2B* loss. Box, 25-75%; line, median; whiskers 5-95% with data points for values outside this range. WT (2 copies), *n*=201; monallelic loss (1 copy), *n*=131; biallelic loss (0 copies), *n*=16 independent tumours. Indels as percentage of all variants per sample (GEL and ICGC data combined). q-values, 2-sided Mann-Whitney test with 5% FDR. **b,c,** In RNase H2 null CLL, 2 bp deletions predominantly occur at STR and SNMH sequences (**b**), and at the TNT sequence motif (**c**), consistent with TOP-mediated mutagenesis. Mean ± s.e.m., percentage of all variants per sample. GEL and ICGC data combined. *n*= 1,711; 1,244; 443 2-bp indels identified in 201, 131, 16 biologically independent tumours, respectively. **d,** ID4 contribution in RNase H2 null CLL is greater in transcribed regions. Two-sided Fisher's exact test, ID4 indels vs other indels (P=9.2x10^{-16}). **e,** Pan-cancer transcript expression data divided into ten expression strata for ubiquitously expressed genes (used in panel **h** and Fig. 5b analysis). Data points, median/maximum expression across cancer types for individual genes. Genes with similar median and maximum TPMs were considered to be ubiquitously expressed and divided into expression groups from low (1) to high (10) expression. **f,** Two bp deletions in cancer preferentially occur at STRs. **g,** ID-TOP1 deletions increase in frequency with TOP1 cleavage activity (measured by TOP1-Seq; [38]). Dotted line, relative rate in lowest TOP1-seq category set to 1. Solid lines, relative deletion rate. ID-TOP1, 2-5 bp MH and SSTR deletions containing the TN*T sequence motif. **h,** ID-TOP1, but not deletions in other sequence contexts, correlate with transcription. **i,** 2-5 bp deletions from prostate adenocarcinoma are most enriched amongst the top 10% of highly expressed prostate 'tissue-restricted' genes. Odds ratio (OR): number of 2-5bp deletions in top 10% tissue restricted genes vs deletions in other genes, relative to expected frequency from all other tissues. **j,** ID4 is not detected in the indel signature of irinotecan-treated colorectal cancers. Untreated (*n*=78), treated (*n*=39). **k,** 2-5 bp deletion frequency in cancer corresponds to TOP1 cleavage activity, in both genic and non-genic regions. Data analysed from PCAWG[50], all tumours in **e**, **h**; ID4 positive tumours in **g**, **k**; Genomics England in **j**. In **g**, **h** and **k**, solid line, relative deletion rate; shading indicates 95% confidence intervals from 1,000 (**g**,**k**) or 100 (**h**) bootstrap replicates.

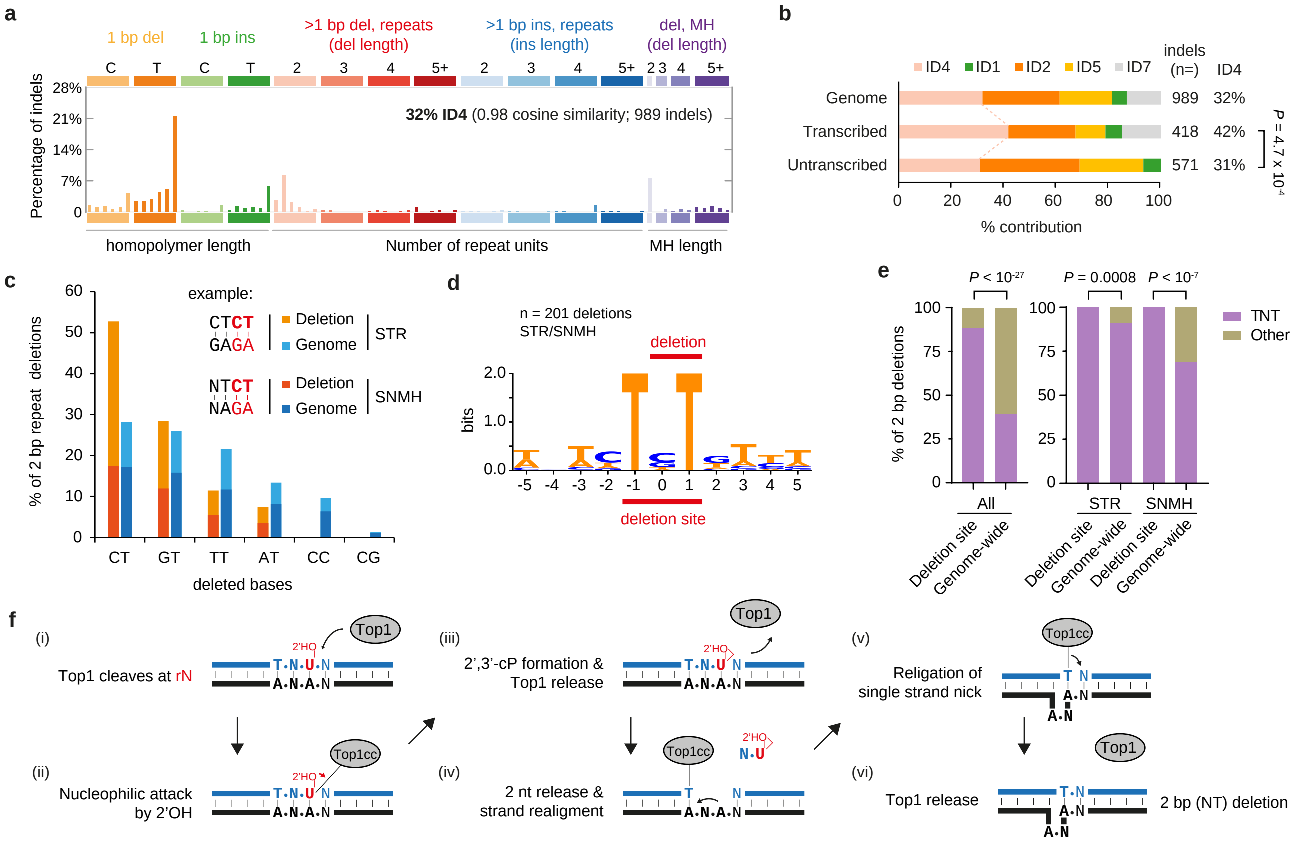
Extended Data Fig. 9 | Human germline *de novo* indels are enriched for ID-TOP1 deletions. a, Most *de novo* 2 bp deletions occur at SSTR, STR and SNMH sequences. **b,c,** A TNT sequence motif is present at the majority of 2 bp STR and SNMH deletions (**b**). Sequence logos: 2-bit representation of the sequence context of 2 bp deletions. Top, all deletions, with those containing A (except AT/TA) reverse complemented, and deletions right-aligned on T (where present). Bottom, STR/SNMH deletions only (**c**). **d,** TN*T motifs extend beyond 2 bp deletions, with enrichment above expectation for 2 bp deletions at TNT, 3 bp deletions at TNNT and 4 bp deletions at TNNNT motifs ($P < 0.001$; two-tailed empirical P-value determined for each category). Bootstrap sampling (*n*=1,000) of 2, 3 and 4 bp STR/MH sequences genome-wide to derive expected frequencies of those matching TN*T motifs. Sampling was performed to match the numbers of deletions at repeats observed in the Gene4Denovo database for each category defined by repeat type, repeat unit length and total repeat length. Histograms, distribution of the number of repeats matching TN*T motifs over these samplings. Solid blue lines, kernel density estimates for these distributions. Dotted red lines, number of deletions observed in Gene4Denovo matching TN*T motifs for each category. **e,** ID-TOP1 correlates with germline expression level. ID-TOP1, defined as 2-5 bp MH and SSTR deletions containing the TN*T sequence motif. Shading, 95% confidence intervals from 100 bootstrap replicates.

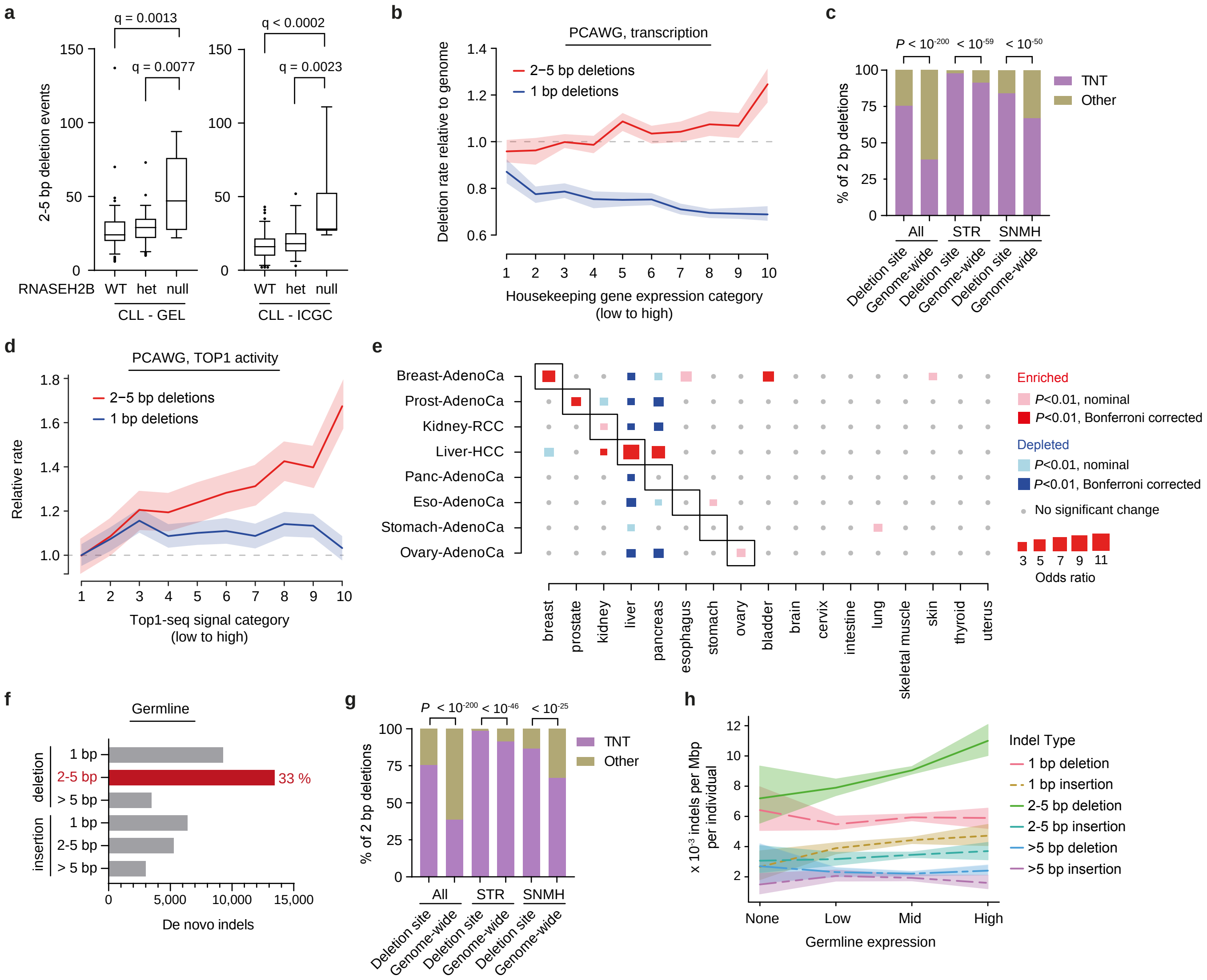
Extended Data Fig. 10 | Topoisomerase 1 causes small deletions while protecting against topological stress. a, The canonical role of Topoisomerase 1 (TOP1) is to relieve torsional stress (sc, supercoiling) during replication and transcription. **b,** TOP1 acts by forming ssDNA nicks to release supercoils and then religates the relaxed DNA. However, TOP1 cleavage at genome-embedded ribonucleotides (frequently incorporated by replicative polymerases such as Pol ε), can lead to short deletions that will be most frequent at sites of torsional stress in the genome, such as occurs at highly transcribed genes. Adapted from [6].

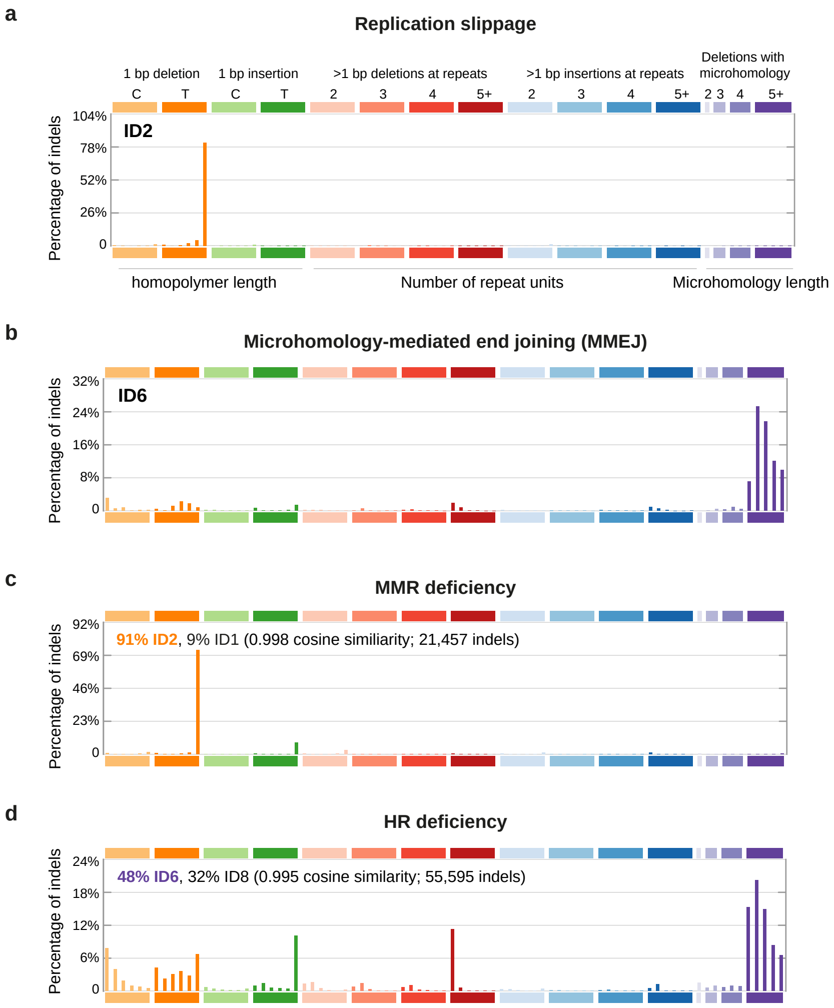

a
1 bp deletion
1 bp insertion
>1 bp deletions at repeats (Deletion length)
>1 bp insertions at repeats (Insertion length)
Deletions with microhomology (Deletion length)
C T C T 2 3 4 5+ 2 3 4 5+ 2 3 4 5+
Percentage of indels
28% 21% 14% 7% 0%
COSMIC ID4 signature
i ii iii iv v vi
1 2 3 4 5 6+ 1 2 3 4 5 6+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 1 2 3 4 5 6+ 1 2 3 4 5 6+ 1 2 3 4 5 6+ 1 2 3 4 5 6+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 1 1 2 1 2 3 1 2 3 4 5+
homopolymer length
Number of repeat units
Number of repeat units
Microhomology length
Repeat type Example
i) (XY)2 CAACT | CT | AATGC
ii) (XY)3 ACTCT | CT | AATGC
iii) (XYZ)2 AAACT | ACT | AATG
iv) (VXYZ)2 ATACT | TACT | AAT
SSTR
v) XNX CAAAT | CT | AATGC
SNMH
vi) XYNXY CAGCT | ACT | AATG
MH
b
C T C T 2 3 4 5+ 2 3 4 5+ 2 3 4 5+
Percentage of indels
60% 45% 30% 15% 0%
S. cerevisiae rnh201Δ pol2-M644G
100% ID4 (0.78 cosine similarity; 148 indels)
c
P_TEF
STOP
HygroR TAA TT P2A NeoR
>50% SSTRs
mutagenesis
2 bp del
GO
Hygro TA ATT P2A NeoR
d
top1Δ
WT
rnh201Δ
rnh201Δ top1Δ
10x
37x
0.1 1 10 100
mutation rate (x 10^-10 per bp per generation)
e
1 bp del
1 bp ins
>1 bp del (repeats)
>1 bp ins (repeats)
del (MH)
WT n = 107
rnh201Δ n = 104
top1Δ n = 81
rnh201Δ top1Δ n = 87
Proportion of indels
0.98 cosine similarity P < 0.0001
0.82 cosine similarity P < 0.0001
homopolymer length
Number of repeat units
Number of repeat units
MH length

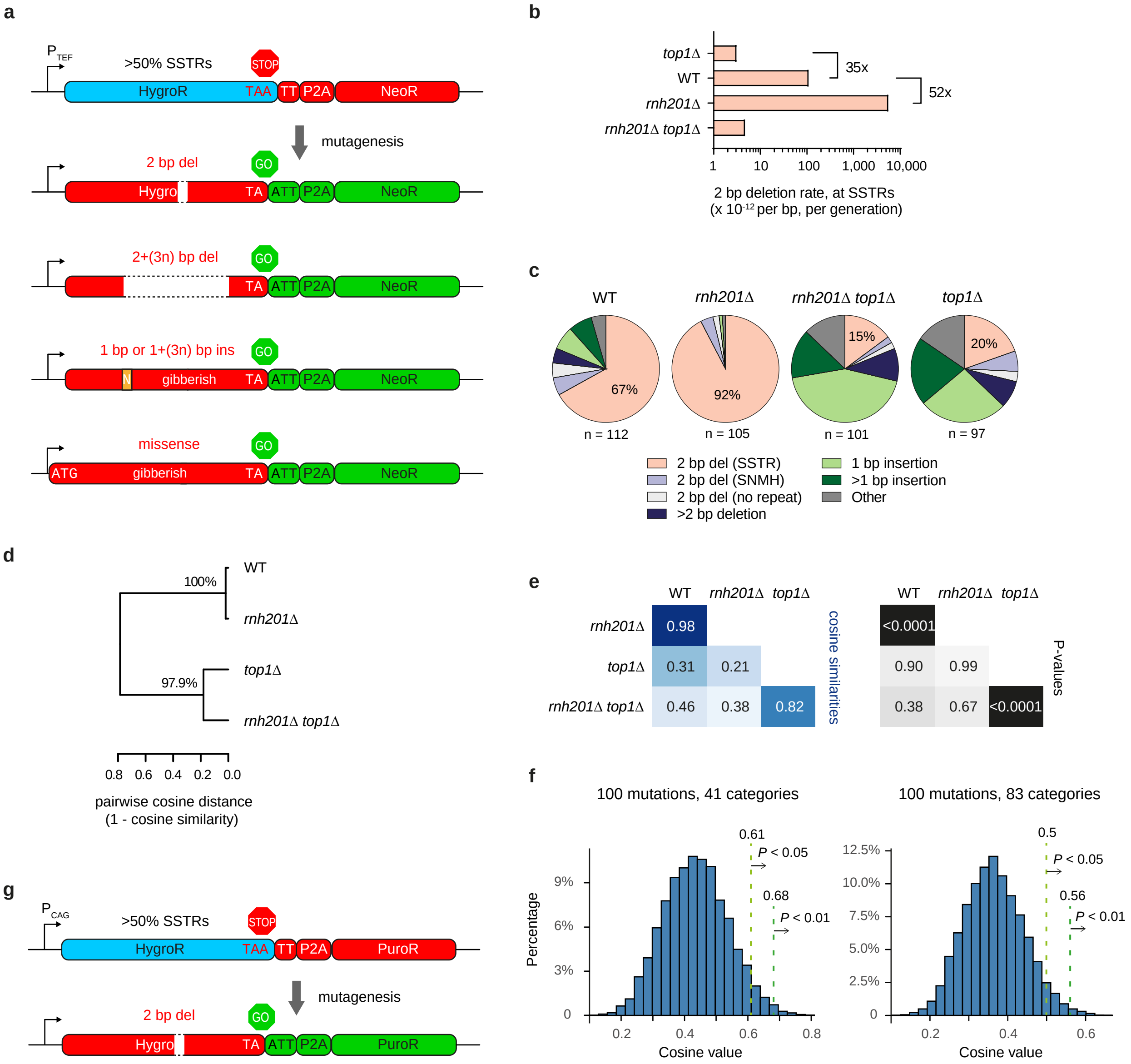

a
STOP
Reporter
HygroR
TAA
TT
P2A
PuroR
Targeting construct
HA-L
SA
T2A
NeoR
pA
P CAG
Reporter
pA
HA-R
CRISPR/Cas9
HeLa
AAVS1 locus
b
HeLa
Parental
RNASEH2A+
KO1
KO2
RNASEH2A
RNASEH2B
RNASEH2C
GAPDH
37 kDa
37 kDa
20 kDa
37 kDa
c
RNase H2 activity
(% of HeLa)
100
50
0
HeLa
Parental
RNASEH2A+
KO1
KO2
d
RNASEH2A+
KO
3.1x
0
50
100
150
200
250
mutation rate (x 10-10 per bp per generation)
e
1 bp del
1 bp ins
>1 bp del (repeats)
>1 bp ins (repeats)
del (MH)
Proportion of indels
RNASEH2A+
n = 31
SSTR
SNMH
RNASEH2A-KO
n = 46
homopolymer length
Number of repeat units
Number of repeat units
MH length


a
WT
AKO
BKO
hTERT-RPE1
initial single cell sort
WT1
WT2
AKO
BKO
ancestral (WGS)
single cell sort
1
2
3
4
4 x 25 doublings
"offspring" after ~100 doublings (WGS)
b
Deletion events
150
100
50
0
P = 4 x 10-17
WT
KO
WT
KO
1 bp
2-5 bp
c
ID4
ID1
ID2
ID5
%ID4
WT
0%
KO
21%
subtracted
61%
0
20
40
60
80
100
% contribution
d
1 bp deletion
1 bp insertion
>1 bp deletions at repeats (Deletion length)
>1 bp insertions at repeats (Insertion length)
Deletions with microhomology (Deletion length)
C
T
C
T
2
3
4
5+
2
3
4
5+
2
3
4
5+
16%
12%
8%
4%
0
Percentage of indels
61% ID4, 39% ID5 (0.89 cosine similarity; 212 indels)
Background subtracted
1 2 3 4 5 6+
1 2 3 4 5 6+
0 1 2 3 4 5+
0 1 2 3 4 5+
1 2 3 4 5 6+
1 2 3 4 5 6+
1 2 3 4 5 6+
1 2 3 4 5 6+
0 1 2 3 4 5+
0 1 2 3 4 5+
0 1 2 3 4 5+
0 1 2 3 4 5+
1
1 2
1 2 3
1 2 3 4 5+
homopolymer length
Number of repeat units
Number of repeat units
Microhomology length


a
1 bp del
1 bp ins
>1 bp del, repeats (del length)
>1 bp ins, repeats (ins length)
del, MH (del length)
C T C T 2 3 4 5+ 2 3 4 5+ 2 3 4 5+
Percentage of indels
28%
21%
14%
7%
0
32% ID4 (0.98 cosine similarity; 989 indels)
homopolymer length
Number of repeat units
MH length
b
ID4 ID1 ID2 ID5 ID7
indels (n=)
ID4
Genome 989 32%
Transcribed 418 42%
Untranscribed 571 31%
P = 4.7 x 10-4
0 20 40 60 80 100
% contribution
c
% of 2 bp repeat deletions
60 50 40 30 20 10 0
example:
CTCT GAGA
Deletion
Genome
STR
NTCT NAGA
Deletion
Genome
SNMH
CT GT TT AT CC CG
deleted bases
d
n = 201 deletions STR/SNMH
deletion
bits
2.0 1.0 0.0
-5 -4 -3 -2 -1 0 1 2 3 4 5
deletion site
e
P < 10-27
P = 0.0008
P < 10-7
% of 2 bp deletions
100 75 50 25 0
TNT
Other
All
STR
SNMH
Deletion site
Genome-wide
f
(i) Top1 cleaves at rN
Top1
2'HO
T·N·U·N
A·N·A·N
(ii) Nucleophilic attack by 2'OH
Top1cc
(iii) 2',3'-cP formation & Top1 release
(iv) 2 nt release & strand realigment
N·U
(v) Religation of single strand nick
(vi) Top1 release
2 bp (NT) deletion
T·N
A·N

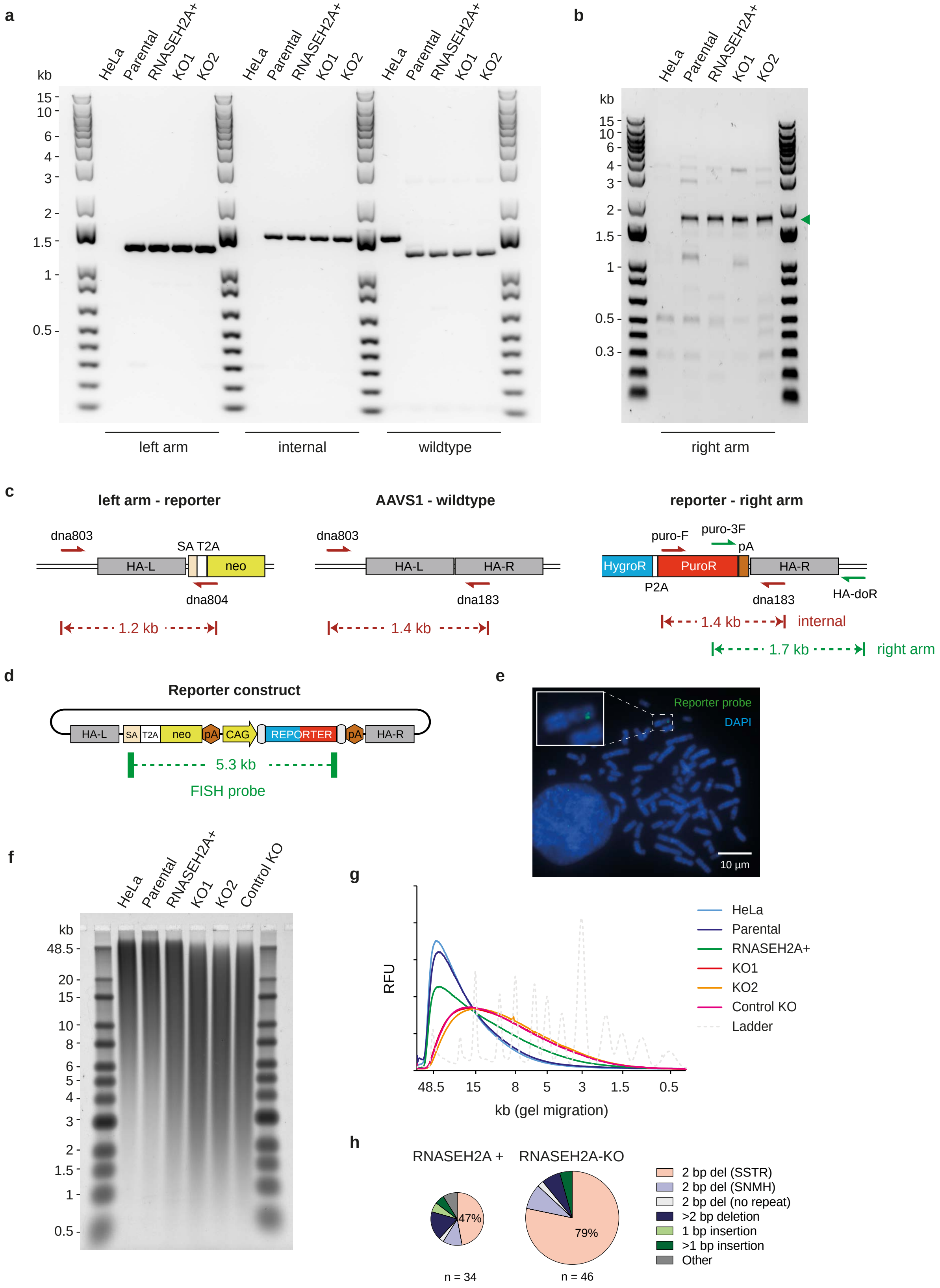

a
q = 0.0013
q = 0.0077
q < 0.0002
q = 0.0023
2-5 bp deletion events
150
100
50
0
RNASEH2B
WT het null
CLL - GEL
CLL - ICGC
b
PCAWG, transcription
2−5 bp deletions
1 bp deletions
Deletion rate relative to genome
1.4
1.2
1.0
0.8
0.6
1 2 3 4 5 6 7 8 9 10
Housekeeping gene expression category
(low to high)
c
P < 10^-200
< 10^-59
< 10^-50
% of 2 bp deletions
100
75
50
25
0
TNT
Other
All
STR
SNMH
Deletion site
Genome-wide
d
PCAWG, TOP1 activity
2−5 bp deletions
1 bp deletions
Relative rate
1.8
1.6
1.4
1.2
1.0
1 2 3 4 5 6 7 8 9 10
Top1-seq signal category
(low to high)
e
Breast-AdenoCa
Prost-AdenoCa
Kidney-RCC
Liver-HCC
Panc-AdenoCa
Eso-AdenoCa
Stomach-AdenoCa
Ovary-AdenoCa
breast
prostate
kidney
liver
pancreas
esophagus
stomach
ovary
bladder
brain
cervix
intestine
lung
skeletal muscle
skin
thyroid
uterus
Enriched
P<0.01, nominal
P<0.01, Bonferroni corrected
Depleted
P<0.01, nominal
P<0.01, Bonferroni corrected
No significant change
3 5 7 9 11
Odds ratio
f
Germline
deletion
insertion
1 bp
2-5 bp
> 5 bp
33 %
0
5,000
10,000
15,000
De novo indels
g
P < 10^-200
< 10^-46
< 10^-25
% of 2 bp deletions
100
75
50
25
0
TNT
Other
All
STR
SNMH
Deletion site
Genome-wide
h
x 10^-3 indels per Mbp per individual
12
10
8
6
4
2
None
Low
Mid
High
Germline expression
Indel Type
1 bp deletion
1 bp insertion
2-5 bp deletion
2-5 bp insertion
>5 bp deletion
>5 bp insertion

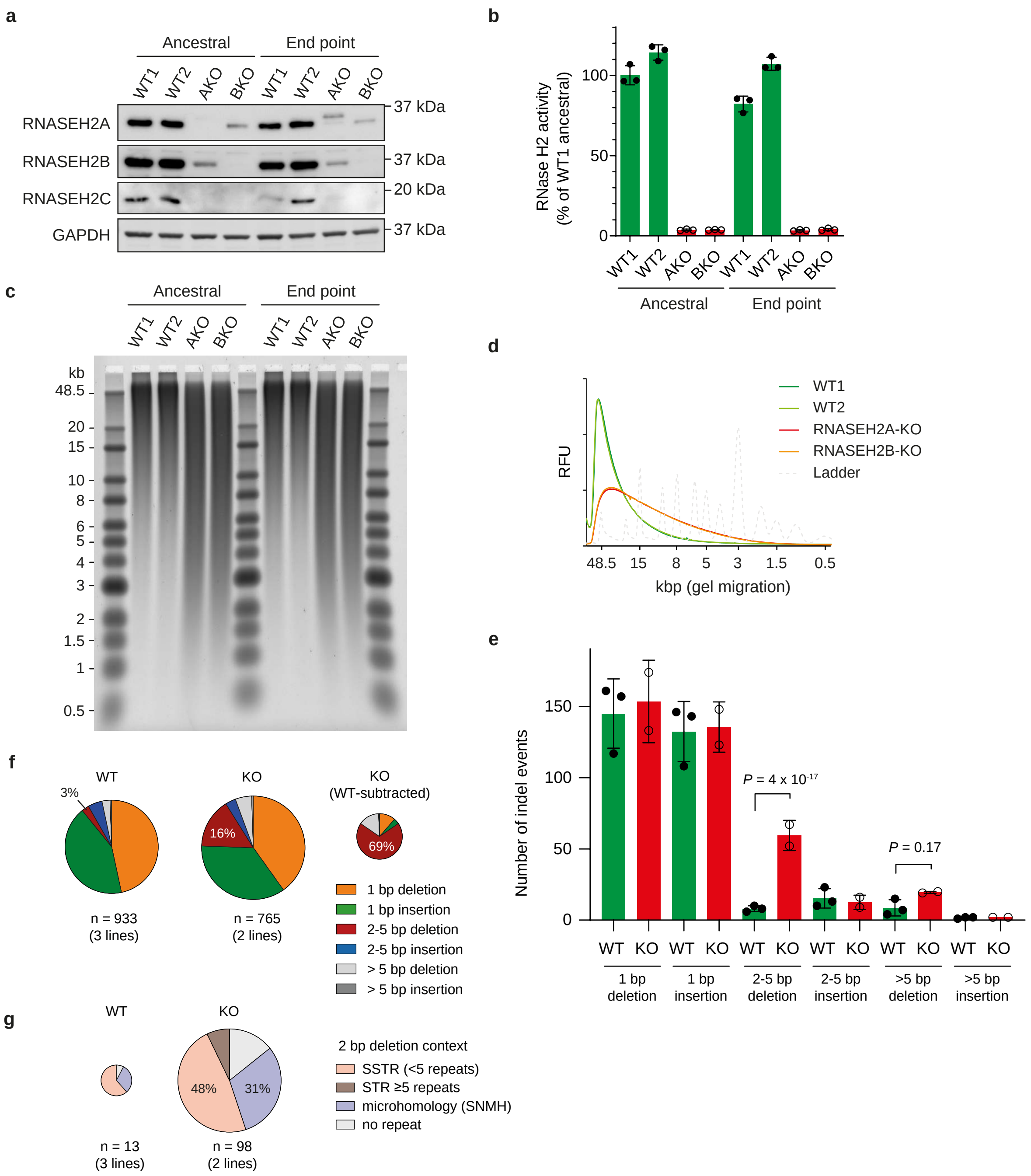

a
Replication slippage
1 bp deletion
1 bp insertion
>1 bp deletions at repeats
>1 bp insertions at repeats
Deletions with microhomology
C
T
C
T
2
3
4
5+
2
3
4
5+
2 3
4
5+
Percentage of indels
104%
78%
52%
26%
0
ID2
homopolymer length
Number of repeat units
Microhomology length
b
Microhomology-mediated end joining (MMEJ)
Percentage of indels
32%
24%
16%
8%
0
ID6
c
MMR deficiency
Percentage of indels
92%
69%
46%
23%
0
91% ID2, 9% ID1 (0.998 cosine similiarity; 21,457 indels)
d
HR deficiency
Percentage of indels
24%
18%
12%
6%
0
48% ID6, 32% ID8 (0.995 cosine similarity; 55,595 indels)

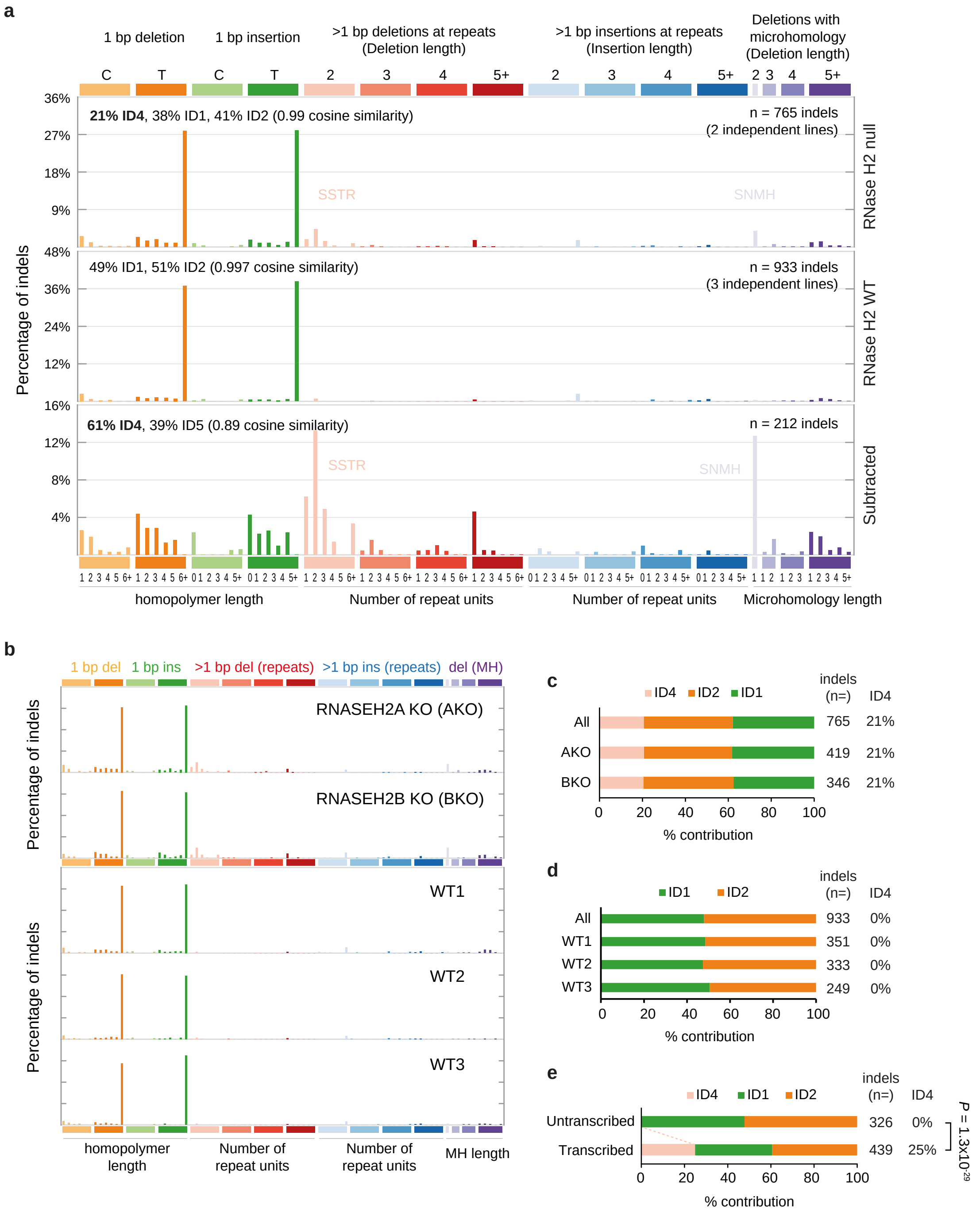


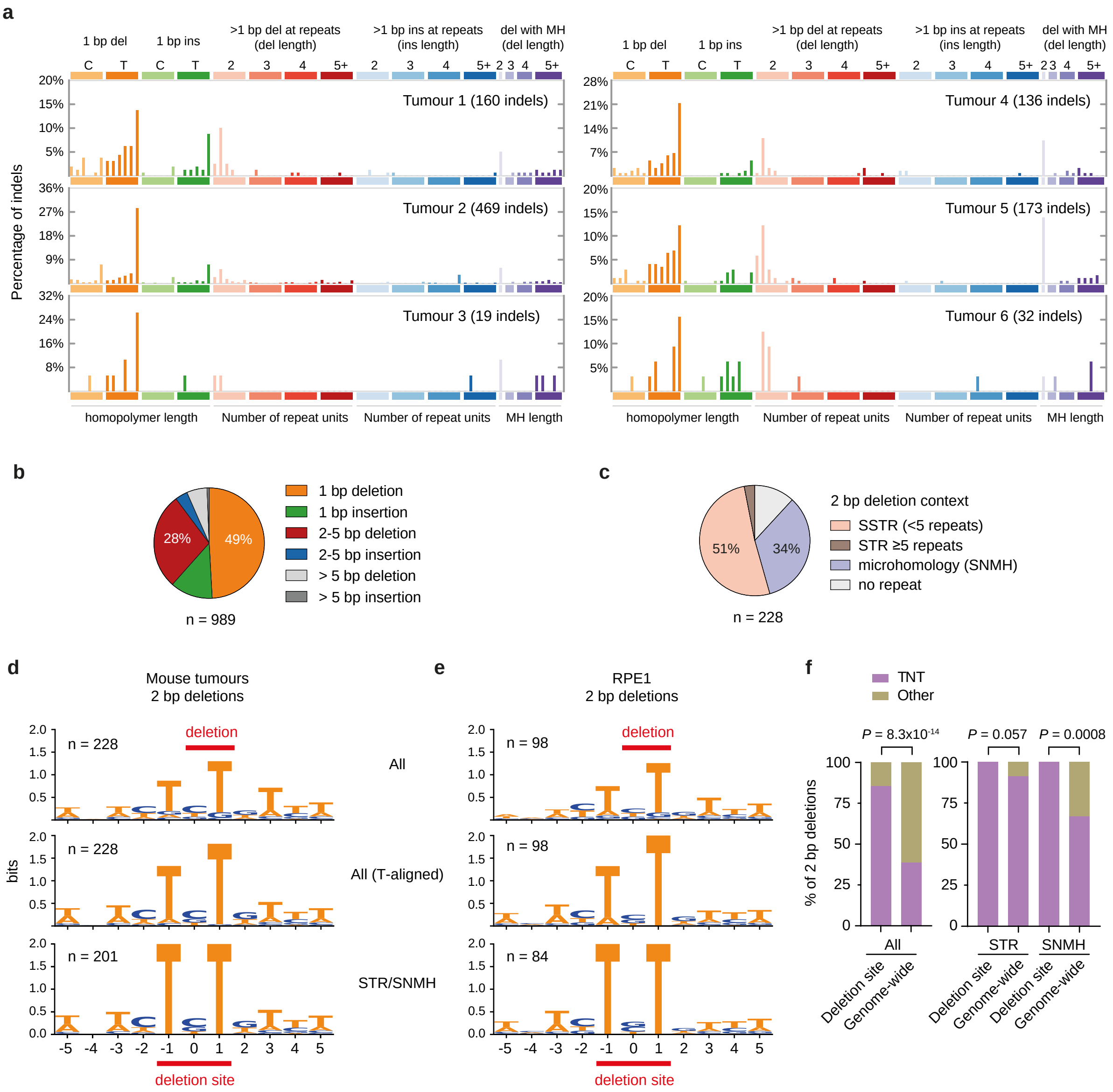
	WT	*rnh201Δ*	*top1Δ*
rnh201Δ	0.98		
top1Δ	0.31	0.21	
rnh201Δ top1Δ	0.46	0.38	0.82

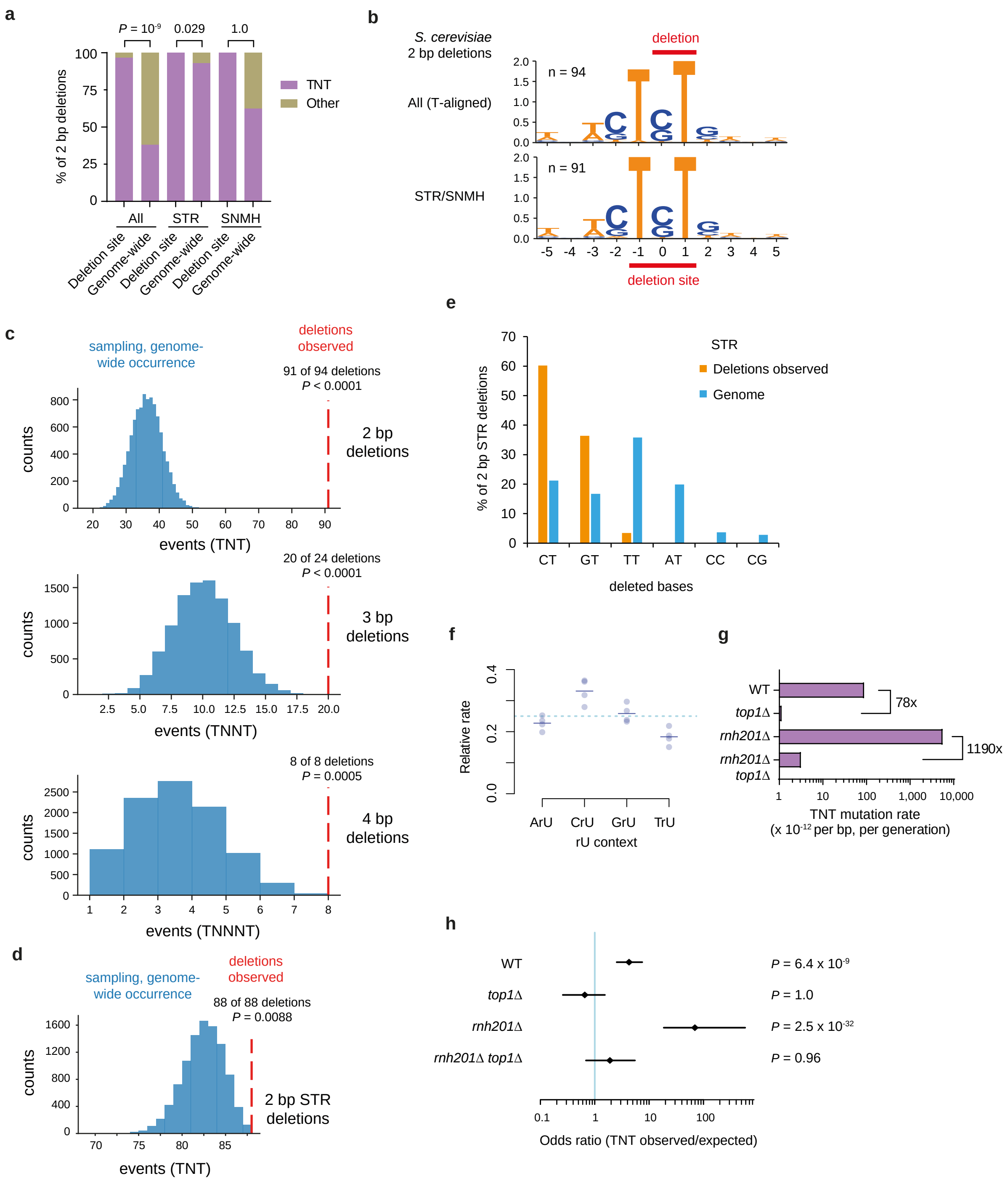
	WT	*rnh201Δ*	*top1Δ*
rnh201Δ	<0.0001		
top1Δ	0.90	0.99	
rnh201Δ top1Δ	0.38	0.67	<0.0001


a
HeLa
Parental
RNASEH2A+
KO1
KO2
kb
15
10
6
4
3
2
1.5
1
0.5
left arm
internal
wildtype
b
kb
15
10
6
4
3
2
1.5
1
0.5
0.3
right arm
c
left arm - reporter
dna803
SA T2A
HA-L
neo
dna804
1.2 kb
AAVS1 - wildtype
dna803
HA-L
HA-R
dna183
1.4 kb
reporter - right arm
puro-F
puro-3F
pA
HygroR
PuroR
HA-R
P2A
dna183
HA-doR
1.4 kb
internal
1.7 kb
right arm
d
Reporter construct
HA-L
SA
T2A
neo
pA
CAG
REPORTER
pA
HA-R
5.3 kb
FISH probe
e
Reporter probe
DAPI
10 µm
f
HeLa
Parental
RNASEH2A+
KO1
KO2
Control KO
kb
48.5
20
15
10
8
6
5
4
3
2
1.5
1
0.5
g
RFU
48.5
15
8
5
3
1.5
0.5
kb (gel migration)
HeLa
Parental
RNASEH2A+
KO1
KO2
Control KO
Ladder
h
RNASEH2A +
RNASEH2A-KO
47%
79%
n = 34
n = 46
2 bp del (SSTR)
2 bp del (SNMH)
2 bp del (no repeat)
>2 bp deletion
1 bp insertion
>1 bp insertion
Other

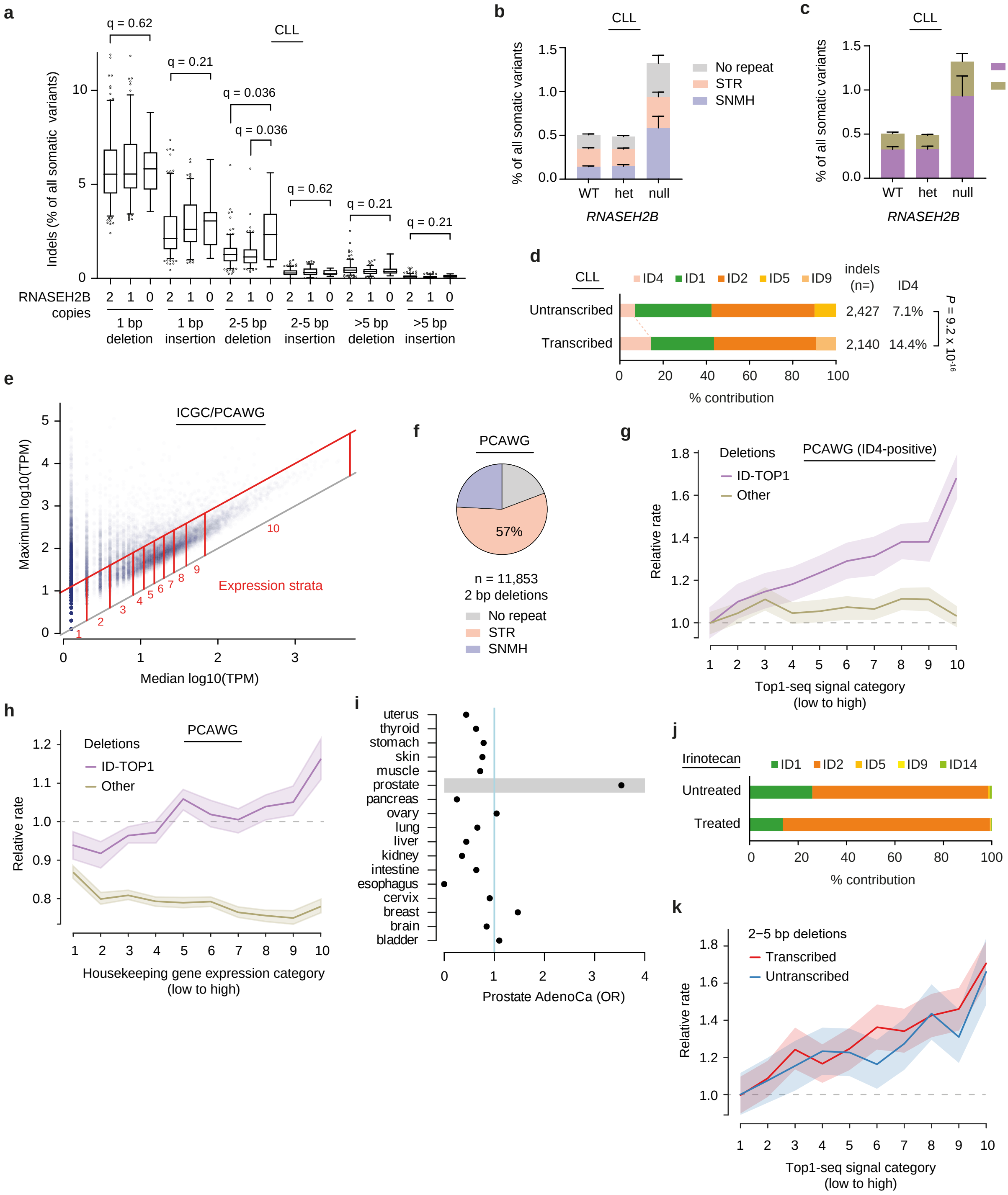

a
Ancestral
End point
WT1
WT2
AKO
BKO
WT1
WT2
AKO
BKO
RNASEH2A
RNASEH2B
RNASEH2C
GAPDH
37 kDa
37 kDa
20 kDa
37 kDa
b
RNase H2 activity
(% of WT1 ancestral)
100
50
0
WT1
WT2
AKO
BKO
WT1
WT2
AKO
BKO
Ancestral
End point
c
Ancestral
End point
WT1
WT2
AKO
BKO
WT1
WT2
AKO
BKO
kb
48.5
20
15
10
8
6
5
4
3
2
1.5
1
0.5
d
RFU
WT1
WT2
RNASEH2A-KO
RNASEH2B-KO
Ladder
48.5
15
8
5
3
1.5
0.5
kbp (gel migration)
e
Number of indel events
150
100
50
0
P = 4 x 10-17
P = 0.17
WT
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT
KO
1 bp
deletion
1 bp
insertion
2-5 bp
deletion
2-5 bp
insertion
>5 bp
deletion
>5 bp
insertion
f
WT
3%
n = 933
(3 lines)
KO
16%
n = 765
(2 lines)
KO
(WT-subtracted)
69%
1 bp deletion
1 bp insertion
2-5 bp deletion
2-5 bp insertion
> 5 bp deletion
> 5 bp insertion
g
WT
n = 13
(3 lines)
KO
48%
31%
n = 98
(2 lines)
2 bp deletion context
SSTR (<5 repeats)
STR ≥5 repeats
microhomology (SNMH)
no repeat

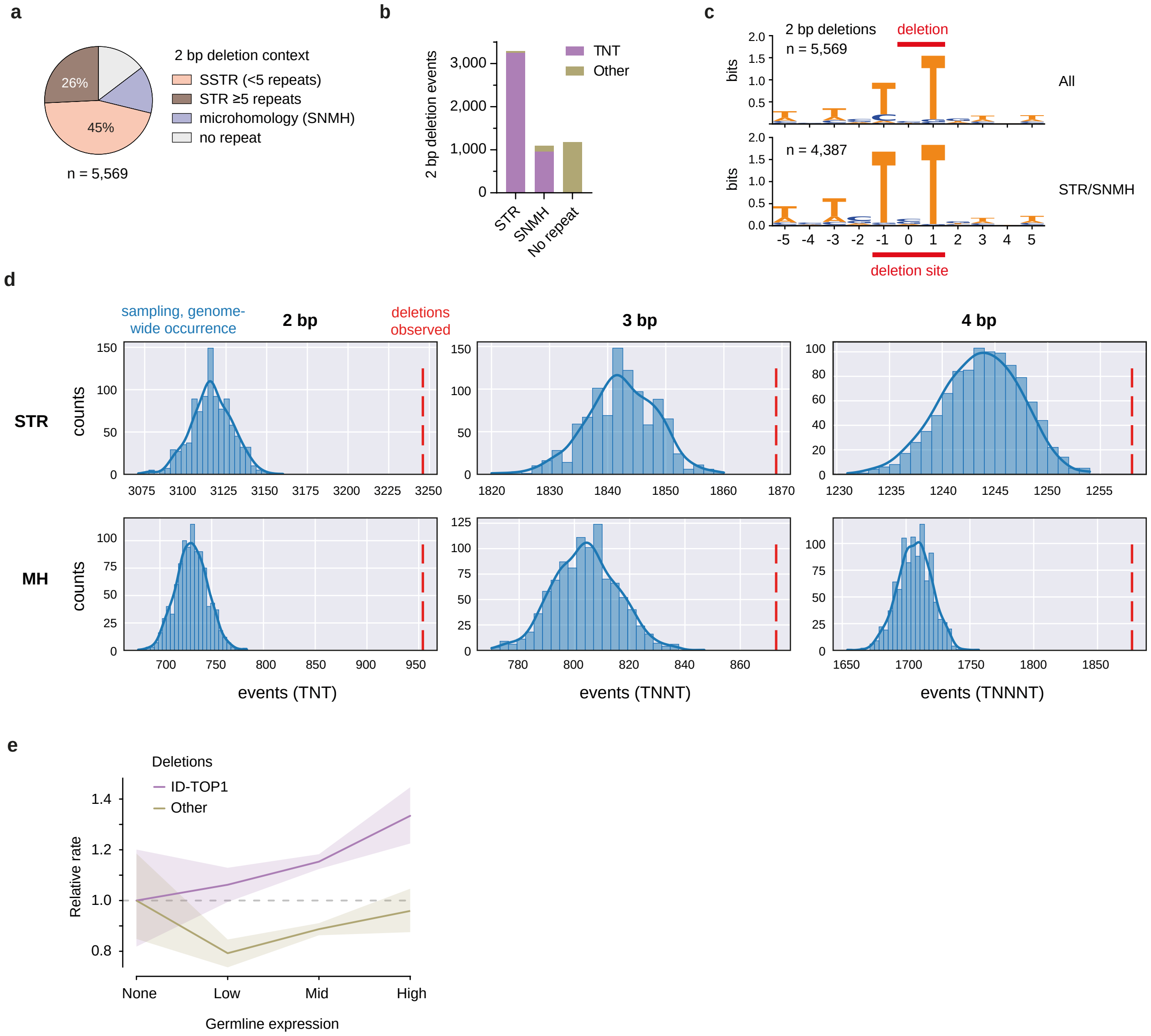

a
1 bp deletion
1 bp insertion
>1 bp deletions at repeats (Deletion length)
>1 bp insertions at repeats (Insertion length)
Deletions with microhomology (Deletion length)
C T C T 2 3 4 5+ 2 3 4 5+ 2 3 4 5+
Percentage of indels
36% 27% 18% 9%
21% ID4, 38% ID1, 41% ID2 (0.99 cosine similarity)
n = 765 indels (2 independent lines)
SSTR
SNMH
RNase H2 null
48% 36% 24% 12%
49% ID1, 51% ID2 (0.997 cosine similarity)
n = 933 indels (3 independent lines)
RNase H2 WT
16% 12% 8% 4%
61% ID4, 39% ID5 (0.89 cosine similarity)
n = 212 indels
SSTR
SNMH
Subtracted
1 2 3 4 5 6+ 1 2 3 4 5 6+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 1 2 3 4 5 6+ 1 2 3 4 5 6+ 1 2 3 4 5 6+ 1 2 3 4 5 6+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 0 1 2 3 4 5+ 1 1 2 1 2 3 1 2 3 4 5+
homopolymer length
Number of repeat units
Number of repeat units
Microhomology length
b
1 bp del 1 bp ins >1 bp del (repeats) >1 bp ins (repeats) del (MH)
Percentage of indels
RNASEH2A KO (AKO)
RNASEH2B KO (BKO)
Percentage of indels
WT1
WT2
WT3
homopolymer length
Number of repeat units
Number of repeat units
MH length
c
ID4 ID2 ID1
indels (n=)
ID4
All 765 21%
AKO 419 21%
BKO 346 21%
0 20 40 60 80 100
% contribution
d
ID1 ID2
indels (n=)
ID4
All 933 0%
WT1 351 0%
WT2 333 0%
WT3 249 0%
0 20 40 60 80 100
% contribution
e
ID4 ID1 ID2
indels (n=)
ID4
Untranscribed 326 0%
Transcribed 439 25%
P = 1.3x10^-29
0 20 40 60 80 100
% contribution

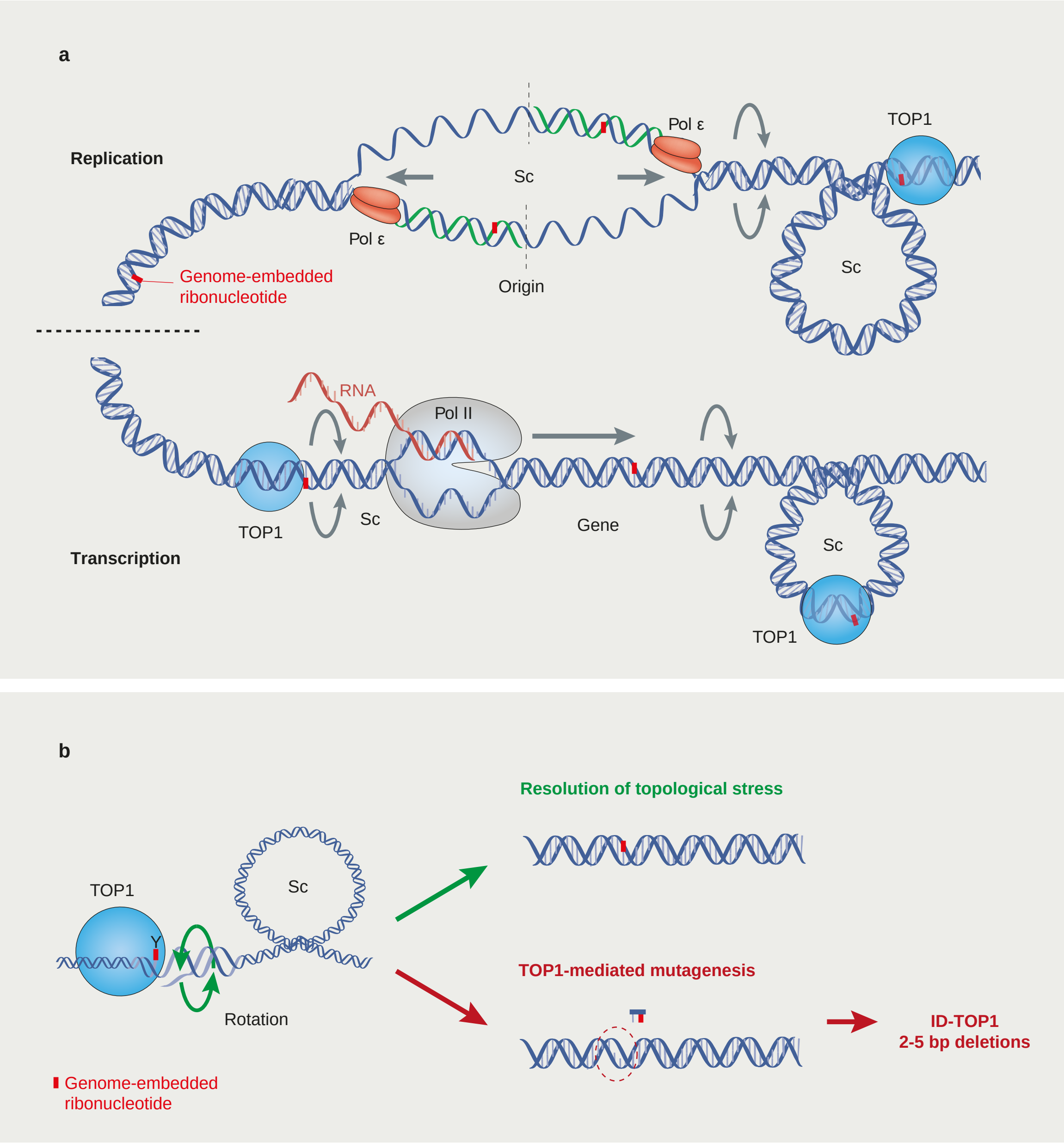

a
1 bp del
1 bp ins
>1 bp del at repeats (del length)
>1 bp ins at repeats (ins length)
del with MH (del length)
C
T
C
T
2
3
4
5+
2
3
4
5+
2
3
4
5+
Percentage of indels
Tumour 1 (160 indels)
20%
15%
10%
5%
Tumour 2 (469 indels)
36%
27%
18%
9%
Tumour 3 (19 indels)
32%
24%
16%
8%
Tumour 4 (136 indels)
28%
21%
14%
7%
Tumour 5 (173 indels)
20%
15%
10%
5%
Tumour 6 (32 indels)
20%
15%
10%
5%
homopolymer length
Number of repeat units
Number of repeat units
MH length
b
28%
49%
1 bp deletion
1 bp insertion
2-5 bp deletion
2-5 bp insertion
> 5 bp deletion
> 5 bp insertion
n = 989
c
51%
34%
2 bp deletion context
SSTR (<5 repeats)
STR ≥5 repeats
microhomology (SNMH)
no repeat
n = 228
d
Mouse tumours
2 bp deletions
e
RPE1
2 bp deletions
deletion
n = 228
n = 228
n = 201
n = 98
n = 98
n = 84
All
All (T-aligned)
STR/SNMH
bits
2.0
1.5
1.0
0.5
0.0
-5
-4
-3
-2
-1
0
1
2
3
4
5
deletion site
f
TNT
Other
P = 8.3x10^-14
P = 0.057
P = 0.0008
% of 2 bp deletions
100
75
50
25
0
All
STR
SNMH
Deletion site
Genome-wide
Deletion site
Genome-wide
Deletion site
Genome-wide



a
P = 10-9
0.029
1.0
100
75
50
25
0
% of 2 bp deletions
TNT
Other
All
STR
SNMH
Deletion site
Genome-wide
Deletion site
Genome-wide
Deletion site
Genome-wide
b
S. cerevisiae
2 bp deletions
All (T-aligned)
STR/SNMH
deletion
n = 94
n = 91
2.0
1.5
1.0
0.5
0.0
-5 -4 -3 -2 -1 0 1 2 3 4 5
deletion site
c
sampling, genome-wide occurrence
deletions observed
91 of 94 deletions
P < 0.0001
counts
800
600
400
200
0
20 30 40 50 60 70 80 90
events (TNT)
2 bp deletions
20 of 24 deletions
P < 0.0001
1500
1000
500
0
2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0
events (TNNT)
3 bp deletions
8 of 8 deletions
P = 0.0005
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8
events (TNNNT)
4 bp deletions
d
sampling, genome-wide occurrence
deletions observed
88 of 88 deletions
P = 0.0088
1600
1200
800
400
0
70 75 80 85
events (TNT)
2 bp STR deletions
e
70
60
50
40
30
20
10
0
% of 2 bp STR deletions
STR
Deletions observed
Genome
CT GT TT AT CC CG
deleted bases
f
0.4
0.2
0.0
Relative rate
ArU CrU GrU TrU
rU context
g
WT
top1Δ
rnh201Δ
rnh201Δ top1Δ
78x
1190x
1 10 100 1,000 10,000
TNT mutation rate
(x 10-12 per bp, per generation)
h
WT
top1Δ
rnh201Δ
rnh201Δ top1Δ
P = 6.4 x 10-9
P = 1.0
P = 2.5 x 10-32
P = 0.96
0.1 1 10 100
Odds ratio (TNT observed/expected)


a
CLL
Indels (% of all somatic variants)
q = 0.62
q = 0.21
q = 0.036
q = 0.036
q = 0.62
q = 0.21
q = 0.21
RNASEH2B copies
2 1 0
1 bp deletion
1 bp insertion
2-5 bp deletion
2-5 bp insertion
>5 bp deletion
>5 bp insertion
b
CLL
% of all somatic variants
No repeat
STR
SNMH
WT het null
RNASEH2B
c
CLL
TNT
Other
WT het null
RNASEH2B
d
CLL
ID4 ID1 ID2 ID5 ID9
indels (n=)
ID4
Untranscribed 2,427 7.1%
Transcribed 2,140 14.4%
P = 9.2 x 10^-16
% contribution
e
ICGC/PCAWG
Maximum log10(TPM)
Median log10(TPM)
Expression strata
f
PCAWG
57%
n = 11,853
2 bp deletions
No repeat
STR
SNMH
g
Deletions
PCAWG (ID4-positive)
ID-TOP1
Other
Relative rate
Top1-seq signal category (low to high)
h
Deletions
PCAWG
ID-TOP1
Other
Relative rate
Housekeeping gene expression category (low to high)
i
uterus
thyroid
stomach
skin
muscle
prostate
pancreas
ovary
lung
liver
kidney
intestine
esophagus
cervix
breast
brain
bladder
Prostate AdenoCa (OR)
j
Irinotecan
ID1 ID2 ID5 ID9 ID14
Untreated
Treated
% contribution
k
2–5 bp deletions
Transcribed
Untranscribed
Relative rate
Top1-seq signal category (low to high)


a
2 bp deletion context
SSTR (<5 repeats)
STR ≥5 repeats
microhomology (SNMH)
no repeat
26%
45%
n = 5,569
b
2 bp deletion events
3,000
2,000
1,000
0
TNT
Other
STR
SNMH
No repeat
c
2 bp deletions
n = 5,569
deletion
bits
2.0
1.5
1.0
0.5
All
n = 4,387
STR/SNMH
0.0
-5 -4 -3 -2 -1 0 1 2 3 4 5
deletion site
d
sampling, genome-wide occurrence
2 bp
deletions observed
3 bp
4 bp
STR
MH
counts
events (TNT)
events (TNNT)
events (TNNNT)
e
Deletions
ID-TOP1
Other
Relative rate
1.4
1.2
1.0
0.8
None
Low
Mid
High
Germline expression


a
Replication
Sc
Pol ε
Pol ε
TOP1
Genome-embedded ribonucleotide
Origin
Sc
RNA
Pol II
Sc
TOP1
Gene
Sc
Transcription
TOP1
b
Resolution of topological stress
TOP1
Sc
Y
Rotation
TOP1-mediated mutagenesis
ID-TOP1
2-5 bp deletions
▮ Genome-embedded ribonucleotide