

Lagging-strand replication shapes the mutational landscape of the genome

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The origin of mutations is central to understanding evolution and of key relevance to health. Variation occurs non-randomly across the genome, and mechanisms for this remain to be defined. Here we report that the 5' ends of Okazaki fragments have significantly increased levels of nucleotide substitution, indicating a replicative origin for such mutations. Using a novel method, emRiboSeq, we map the genome-wide contribution of polymerases, and show that despite Okazaki fragment processing, DNA synthesized by error-prone polymerase- α (Pol- α) is retained *in vivo*, comprising approximately 1.5% of the mature genome. We propose that DNA-binding proteins that rapidly re-associate post-replication act as partial barriers to Pol- δ -mediated displacement of Pol- α -synthesized DNA, resulting in incorporation of such Pol- α tracts and increased mutation rates at specific sites. We observe a mutational cost to chromatin and regulatory protein binding, resulting in mutation hotspots at regulatory elements, with signatures of this process detectable in both yeast and humans.

Mutations occur despite the exquisite fidelity of DNA replication, efficient proofreading and mismatch repair¹, resulting in heritable disease and providing the raw material for evolution. Genome variation is non-uniform², the outcome of diverse mutational processes³, repair mechanisms⁴ and selection pressures^{5,6}. This variability is exemplified by nucleotide substitution rates around nucleosome binding sites, with the highest rates at the nucleosome midpoint (dyad position)^{7–12}.

Bidirectional replication of genomic DNA necessitates discontinuous synthesis of the lagging strand as a series of Okazaki fragments (OFs)^{13,14}, which then undergo processing to form an intact continuous DNA strand^{15,16}. Recently, the genomic locations at which OFs are ligated (Okazaki junctions, OJs) were mapped¹⁷. In this experimental system, OJs occurred at an average rate of 0.6% per nucleotide; however, frequency was strongly influenced by the binding of nucleosomes and transcription factors (TFs). These proteins act as partial blocks to Pol- δ processivity, resulting in the accumulation of OJs at their binding sites. Here, we demonstrate the mutational consequences of such protein binding.

Substitutions correlate with OJs

We were struck by the similarity of the distribution of *Saccharomyces cerevisiae* OJ sites at nucleosomes¹⁷ to that previously reported for nucleotide substitutions^{7,8,10–12}, and set out to investigate the potential reasons for this. We established that nucleotide substitution and OJ distributions are highly correlated (Pearson's correlation coefficient = 0.76, $P = 2.2 \times 10^{-16}$) and essentially identical in pattern (Fig. 1a). Furthermore, differences in OJ distribution by nucleosome type (genic versus non-genic), spacing or consistency of binding were mirrored by the substitution rate distribution (Extended Data Fig. 1a–f). We found similar strong correlation in the regions directly surrounding TF binding sites of Reb1 (Fig. 1b; Pearson's correlation = 0.57, $P = 5.6 \times 10^{-15}$) and Rap1 (Extended Data Fig. 1g), providing further evidence for a direct association. At the sequence-specific binding sites themselves, substitution rates were depressed relative to the OJ, resulting from strong

selection pressure to maintain TF binding, and obscuring any mutational signal at these nucleotides.

Given that both classes of sites (nucleosomes and TFs) are present genome-wide and represent different biological processes, this association was probably the direct consequence of protein binding at these sites. However, to rule out site-specific biases in sequence as a confounding explanation for the observed distributions, we randomly sampled the rest of the genome for trinucleotides of identical sequence compositions and calculated the substitution rate at these sites, on a nucleotide-by-nucleotide position basis (Extended Data Fig. 1h–j). This resulted in loss of the observed patterns, establishing that nucleotide composition bias was not a contributing factor. Furthermore, the observed association was not restricted to polymorphism rates, as yeast inter-species nucleotide substitution patterns at both nucleosome and Reb1 TF binding sites were identical (Extended Data Fig. 1k, l).

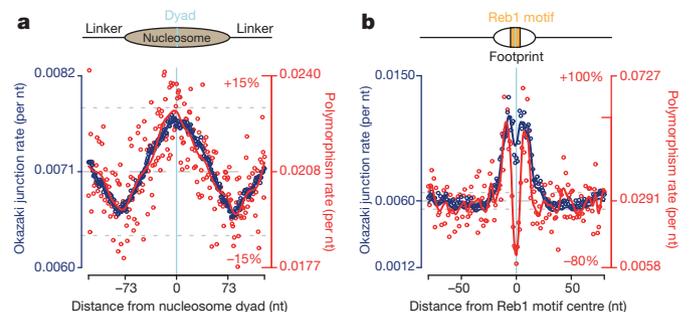


Figure 1 | Increased substitution rates at OJs. **a, b,** Nucleotide (nt) substitution rates (red) closely correlate with increased OJ site frequency (blue) at nucleosome (**a**) and Reb1 (**b**) binding sites. *S. cerevisiae* polymorphism rates per nucleotide computed using sequences from nucleosome ($n = 27,586$) and Reb1 binding sites ($n = 881$). Individual data points, open circles. Solid curves, best-fit splines. Mean, dashed grey line; $\pm 10\%$ dotted grey lines.

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We therefore concluded that OJ frequency and nucleotide substitution rates could be causally related, and set out to investigate the potential mechanism for this association.

Mutations at 5' ends of OFs

The synthesis and processing of OFs is directional. Therefore, substitution rates would be expected to be asymmetrical relative to the direction of synthesis, if a component of this process was the cause. As most of the genome is preferentially replicated with either the forward or reverse strand as the lagging strand, we orientated regions by their dominant direction of lagging-strand synthesis. This revealed substantially increased nucleotide substitution rates immediately downstream of OJs (Fig. 2a), the level of mutational signal correlating with OJ site frequency. Quantification of substitution rates for the five nucleotides immediately upstream and downstream of the OJ (Fig. 2b) demonstrated that high frequency OJ sites (11-fold increased OJ rate relative to baseline; top 99.9th centile of sites) displayed the highest substitution rate ($P < 2.2 \times 10^{-16}$), with significant increases ($P < 2.2 \times 10^{-16}$) for medium frequency sites, (6.1-fold, 99–99.9th centile) but not low frequency sites ($P = 0.3$, 1.7-fold, OJ sites <99th centile). This was not due to site-specific sequence biases, as the increase in substitution rate was lost after a trinucleotide preserving genome shuffle. Therefore, point mutations are enriched at the 5' ends of mature OFs of frequently occurring OJ sites, sites that correspond to protein barriers to Pol- δ processivity¹⁷.

Pol- α DNA retention hypothesis

We next considered which aspect of lagging-strand synthesis might be responsible. OFs are generated by the consecutive actions of Pol- α and Pol- δ (Fig. 2c). When the previously synthesized, downstream OF is encountered, OF processing occurs¹⁸, involving the coordinated action of FEN1 and DNA2 nucleases^{15,16} in conjunction with continuing DNA synthesis by Pol- δ , before final ligation of adjoining DNA fragments. During this process, most if not all of the 10–30-nucleotide-long DNA primer synthesized by Pol- α ^{19,20} has been thought to be removed alongside the RNA primer, and replaced by Pol- δ -synthesized DNA^{16,21,22}.

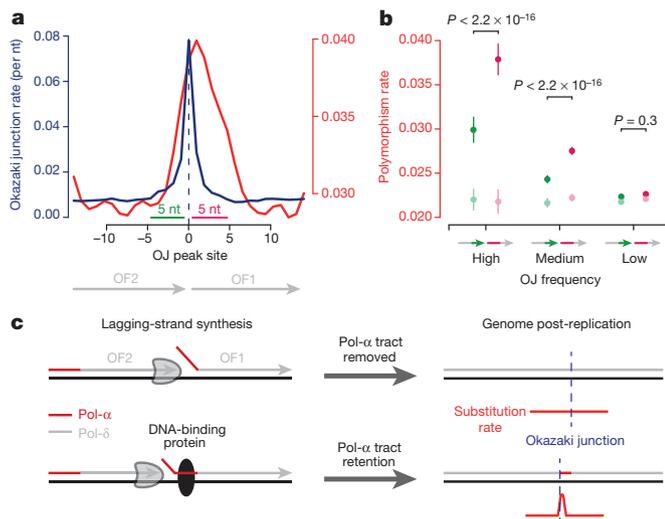


Figure 2 | Frequent nucleotide substitutions at OF 5' ends. **a**, Mutation rates are increased downstream of OJs. Substitution polymorphisms (red) and OJ rate (blue) in regions surrounding high frequency OJs (top 0.1%). $n = 5,660$ sequences orientated for dominant direction of OF synthesis. **b**, Mutation rates correlate with OJ peak size. Mutations are significantly enriched downstream of the junction (pink), compared to genome shuffle controls (light green/pink). Sites grouped by OJ frequency. Points denote mean and error bars denote s.d. from 100 bootstrap samples or genome shuffles (controls); statistics by paired two-sided t -test. **c**, Hypothesis: DNA synthesized by non-proofreading Pol- α is preferentially trapped in regions rapidly bound by proteins post-replication. These act as partial barriers to Pol- δ displacement of Pol- α -synthesized DNA, resulting in locally increased mutations.

This would be desirable, as unlike other replicative DNA polymerases, Pol- α lacks 3'-to-5' proofreading exonuclease activity, limiting its intrinsic fidelity²³. On the other hand, studies on the mutagenesis pattern of reduced fidelity polymerase mutants in yeast demonstrate that Pol- α -synthesized DNA does contribute to the genome^{21,24–26}. How comprehensive the removal or retention of such DNA is *in vivo* is unknown, but notably the retention of error-prone Pol- α -synthesized DNA at the 5' end of OFs would provide a straightforward explanation for the increased mutation rates we observed. Given that protein barriers have been shown to influence OF processing¹⁷, we therefore propose that Pol- α -synthesized DNA is preferentially retained at sites where proteins bind shortly after initial OF DNA synthesis (Fig. 2c). Our model would predict (1) that Pol- α tracts are retained at a considerable level within the mature genome post-replication, and (2) that mutational signatures arising from such Pol- α -synthesized DNA will be increased at many DNA-binding protein sites in eukaryotes.

EmRiboSeq

To address where error-prone Pol- α DNA is retained *in vivo*, we used the incorporation of ribonucleotides into genomic DNA to track the activity of specific DNA polymerases. Ribonucleotides are covalently incorporated into genomic DNA by replicative polymerases^{27,28}, although they are normally efficiently removed by ribonucleotide excision repair, a process initiated by the type 2 RNase H enzyme (RNase H2)²⁹. In RNase-H2-deficient budding yeast, such ribonucleotides are generally well tolerated: Δ *rnh201* yeast has proliferation rates identical to wild type under normal growth conditions²⁷, and therefore in this genetic background ribonucleotides can be used as a 'label' to track polymerase activity. Furthermore, the contribution of specific polymerases can be studied using polymerases with catalytic site point mutations (such as Pol- α (Leu868Met), Pol- δ (Leu612Met) and Pol- ϵ (Met644Gly)) that incorporate ribonucleotides at higher rates than their wild-type counterparts (refs 21, 26, 27, 30 and J. S. Williams, A. R. Clausen & T. A.

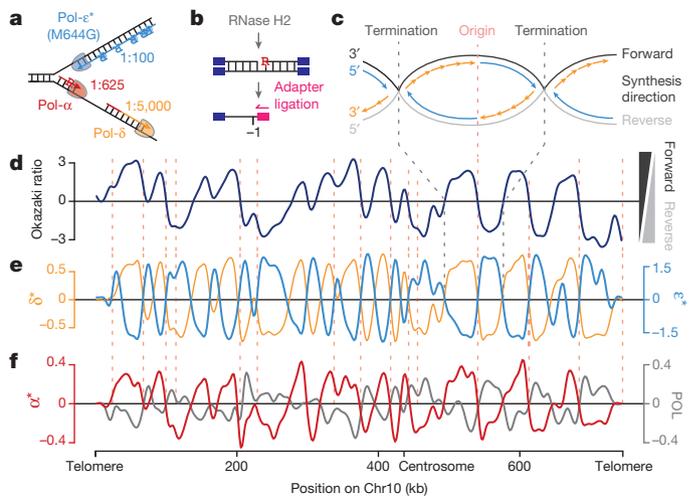


Figure 3 | Mapping DNA synthesis *in vivo* using emRiboSeq. **a**, Replicative polymerases can be tracked using point mutants with increased ribonucleotide incorporation. Schematic of replication fork with Pol- ϵ (asterisk denotes Met644Gly mutant) and ribonucleotide incorporation rates for each polymerase. Embedded ribonucleotides (R) highlighted. **b**, Schematic of emRiboSeq methodology. **c**, Schematic of replication fork with Pol- ϵ^* and Pol- δ . **d**, Mapping of leading/lagging-strand synthesis and replication origins using emRiboSeq. Ratio of OF reads¹⁷ between forward and reverse strands of chromosome 10 (Chr10); **d** corresponds to the ratio of their respective ribonucleotide content (**e**) for Pol- δ^* (orange), whereas Pol- ϵ^* (cyan) shows negative correlation. Intersections with x axis correspond to replication origins and termination regions (**c–e**). Experimentally validated origins (dotted pink lines). **f**, Pol- α^* DNA is detected genome-wide by emRiboSeq as a component of the lagging strand. Strand ratios are shown as best-fit splines, y axes denote \log_2 of ratios (**d–f**).

Kunkel, personal communication; Fig. 3a). Yeast strains expressing these mutant polymerases have previously been used to demonstrate that Pol- ϵ and Pol- δ are the major leading- and lagging-strand polymerases, respectively, by measuring strand-specific alkaline sensitivity of particular genomic loci^{30–32}.

To track directly the genome-wide contribution of polymerases, we developed a next-generation sequencing approach, which we term emRiboSeq (for embedded ribonucleotide sequencing), that determines the strand-specific, genome-wide distribution of embedded ribonucleotides. This is achieved by treatment of genomic DNA with recombinant RNase H2 to generate nicks 5' of embedded ribonucleotides, followed by ligation of a sequencing adaptor to the 3' -hydroxyl group of the deoxynucleotide immediately upstream of the ribonucleotide (Fig. 3b and Extended Data Fig. 2a). Subsequent ion-semiconductor sequencing permits strand-specific mapping of ribonucleotide incorporation sites.

Control experiments using endonucleases of known sequence specificity demonstrated 99.9% strand specificity and 99.9% site specificity for the technique (Extended Data Fig. 2b–d). Using RNase-H2-deficient Pol- ϵ (Met644Gly) and Pol- δ (Leu612Met) yeast strains, we then mapped the relative contributions of these respective polymerases genome-wide (Fig. 3c–e and Extended Data Figs 3 and 4). We found that ribonucleotide incorporation in the Pol- δ (Leu612Met) strain was substantially enriched on the DNA strand that is preferentially synthesized by lagging-strand synthesis¹⁷, in keeping with its function as the major lagging-strand polymerase^{30,33,34}, while ribonucleotide incorporation in the Pol- ϵ (Met644Gly) strain exhibited an entirely reciprocal pattern consistent with its function as the leading-strand polymerase^{31,35} (Fig. 3e). Furthermore, points at which neither enzyme showed strand preference (intersection of both Pol- ϵ and Pol- δ plots with the x axis) corresponded precisely with annotated origins of replication. Other intersection points were also evident that correspond to replication termination regions, as well as putative, non-annotated origins. The latter overlapped with early replicating regions³⁶ (Extended Data Fig. 3b, c). Therefore, we concluded that emRiboSeq can be used to determine the distribution of polymerase activity genome-wide, and has utility for the identification of replication origin and termination sites.

Pol- α -synthesized DNA ~1.5% of genome

Having demonstrated the validity of our technique through detailed mapping of the major replicative polymerases, we next examined the contribution of Pol- α -synthesized DNA to the budding yeast genome. Significantly, the Pol- α (Leu868Met) Δ *rnh201* strain had a strand ratio distribution identical to that seen for Pol- δ (Leu612Met) Δ *rnh201*, consistent with the expected role for Pol- α in lagging-strand replication (Fig. 3f). Furthermore, the Pol- α (Leu868Met) pattern of strand incorporation was reciprocal to that of a wild-type polymerase strain (POL), which displayed leading-strand bias, in keeping with a strong propensity for ribonucleotide incorporation by leading-strand polymerase Pol- ϵ compared to Pol- δ (ref. 37). Increased ribonucleotide retention on the lagging strand was also present in DNA from stationary phase Pol- α (Leu868Met) Δ *rnh201* yeast (Extended Data Fig. 3d), demonstrating that Pol- α -derived DNA is retained in the mature genome post-replication and that this signal was not due to the transient presence of Pol- α DNA during S-phase.

To provide biochemical validation, we performed alkaline gel electrophoresis on genomic DNA extracted from Pol- α (Leu868Met), Pol- δ (Leu612Met) and Pol- ϵ (Met644Gly) Δ *rnh201* yeast. Increased fragmentation was detected in all three strains (Extended Data Fig. 4a–c) and increased ribonucleotide incorporation was also detected in genomic DNA from stationary phase Pol- α (Leu868Met) yeast (Fig. 4a–c), consistent with Pol- α tract retention in mature genomic DNA. To quantify the contribution of Pol- α DNA to the genome, we used densitometry measurements from the alkaline gels to calculate ribonucleotide incorporation rates²⁸. We detected 1,500 embedded ribonucleotides per genome in Δ *rnh201* genomic DNA, which increased to 2,400 sites per genome for Pol- α (Leu868Met) (Fig. 4c). Observed ribonucleotide

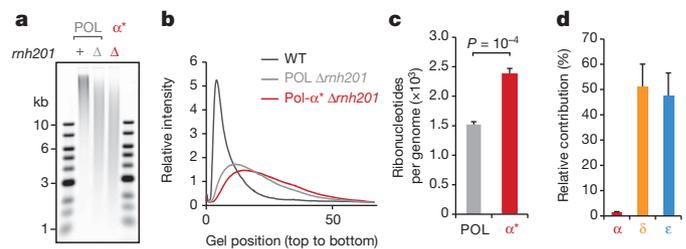


Figure 4 | Pol- α DNA synthesis contributes ~1.5% of the mature genome. **a, b**, Increased ribonucleotide incorporation in Pol- α^* stationary phase yeast is detected by alkaline gel electrophoresis. kb, kilobases; WT, wild type. **c**, Quantification confirms significantly increased rates in the Pol- α^* genome ($n = 6$ independent experiments; error bars denote s.e.m.; statistics by paired two-sided t -test). **d**, Estimate of relative contribution of polymerases to the genome ($n = 4$ independent experiments; error bars denote s.e.m.).

incorporation rates correspond to the product of the incorporation frequency of each polymerase and the amount of DNA it contributes to the genome. Using the *in vitro* ribonucleotide incorporation rates of wild-type and mutant polymerases and the number of embedded ribonucleotides embedded *in vivo* (Extended Data Figs 3a and 4a–c), we estimated the relative contributions of each of the replicative polymerases to the genome (Fig. 4d), calculating the contribution of Pol- α to be $1.5 \pm 0.3\%$ (mean \pm s.d.).

RNase H enzymes may contribute to the removal of OF RNA primers^{16,38} and consequently Δ *rnh201* strains could have altered levels of Pol- α -synthesized DNA to that seen in wild-type strains. This confounding factor was excluded using an *RNH201* separation-of-function mutant³⁹, which established that retention of Pol- α DNA was independent of a role for RNase H2 in RNA primer removal (Extended Data Fig. 5).

In conclusion, Pol- α -synthesized DNA makes a small but significant contribution to the genome, relative to the major replicative polymerases, confirming the first prediction of our model.

Mutational cost of TF binding in humans

As OF processing is a conserved process in eukaryotes, we next considered whether an OF-related mutational signature was also present in humans. Substitution rates are also increased at nucleosome cores in humans⁷ with an identical distribution to yeast. Furthermore, the TF NFYA has an unexplained ‘shoulder’ of increased substitution proximal to its binding sites⁴⁰, reminiscent of the Reb1 pattern (Fig. 1b). We therefore investigated whether similar mutational patterns are present at other experimentally defined human TF and chromatin protein binding sites. Increased inter-species nucleotide substitution rates were detected flanking essential binding site residues, for many, but not all TFs, as well as CTCF binding sites (Fig. 5a, b and Extended Data Fig. 6). Substitution rates were measured using genomic evolutionary rate profiling (GERP) scores, which quantify nucleotide substitution rates relative to a genome-wide expectation of neutral evolution⁴¹, such that a negative GERP score indicates increased nucleotide substitution rates. Furthermore, increases in mutation rate correlated with the degree of enrichment reported in chromatin immunoprecipitation with lambda exonuclease digestion (ChIP-exo) data sets for these proteins, likely reflecting the strength of binding or frequency of occupancy at specific sites, which would be expected to influence Pol- δ processivity and consequent mutation levels.

Finally, to extend our analysis beyond common TF binding sites, we investigated whether the same mutational signature could be found for a broad range of regions at which regulatory proteins bind, regions we identified by the presence of DNase I footprints. Our preceding analysis of TFs suggested that nucleotide substitutions would be increased immediately adjacent to the protein binding region defined by such footprints. In yeast we found that DNase I footprint edges served as a good proxy for increased OJ rate with significantly elevated substitution rates

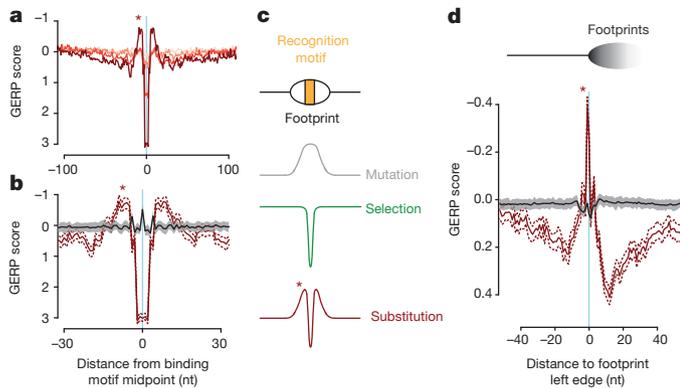


Figure 5 | OF mutational signatures are conserved in humans. **a**, Nucleotide substitutions (plotted as GERP scores) are increased immediately adjacent to TF NFYA binding sites ($n = 5,110$). Pink to brown: lower to higher quartiles of ChIP-exo peak height (reflecting strength of binding/occupancy). Stronger binding correlates with substitution rate in the 'shoulder' region (asterisk). **b**, Increased substitution rates are not a consequence of local sequence composition effects. Strongest binding sites (brown) compared to trinucleotide preserving shuffle (black). **c**, Model showing nucleotide substitution profiles are the sum of mutation rate and selective pressure. **d**, Interspecies substitution rates are also increased adjacent to DNase I footprint edges (asterisk) ($n = 33,350$). Sequences aligned to left footprint edges as indicated in schematic. Right footprint edge is indistinct owing to heterogeneity in footprint length. Substitution rates are no longer increased after trinucleotide preserving shuffle from local flanking sequences (black). Brown dashes and grey shading denote 95% confidence intervals (**b**, **d**).

(Extended Data Fig. 7). Similarly, in humans, aligning regions containing DNase I footprints on the basis of boundary junctions (left-hand edge of footprint), detected substantially increased nucleotide substitution rates close to the junction, relative to the baseline rate in the immediate region (Fig. 5d). These increased substitution rates were related to position rather than sequence content, as this signal was lost when a trinucleotide preserving genome shuffle was applied, both for individual TFs (Fig. 5b and Extended Data Fig. 6a–d) and DNase I footprints (Fig. 5d). Therefore, this mutational signature is not due to the retention of mutagenic sequences (for example, CpG dinucleotides) at such sites⁴², and is a widespread phenomenon in the genome at protein binding sites in both yeast and humans.

Discussion

Here we establish a mutational signature at protein binding sites that we suggest could result from the activity of the replicative polymerase Pol- α . We use a novel technique, emRiboSeq, to demonstrate that error-prone DNA synthesized by Pol- α is retained in the mature lagging strand. EmRiboSeq tracks genome-wide *in vivo* polymerase activity using ribonucleotides as a 'non-invasive' label, and will have significant future use for the *in vivo* study of DNA polymerases in replication and repair. Further optimization of emRiboSeq should permit high resolution examination of the role of polymerases at specific sites, such as Pol- α tract retention at protein binding sites. It will also be a useful method for defining replication origin and termination sites, and furthermore will facilitate the investigation of physiological roles of genome-embedded ribonucleotides^{30,43,44}.

A direct relationship between OF junctions and mutation frequency is indicated by the significant correlations between substitution rate and OF junction sites at diverse protein binding sites, although future experimental validation will be needed to establish causality formally. We find that substitution rates are specifically increased downstream of such junction sites, suggesting a replicative origin for such mutations. As Pol- α DNA tracts occur genome-wide, and Pol- δ processing of OFs is impaired by DNA-bound proteins¹⁷, we propose that retention of Pol- α DNA is increased at these functionally important sites, and is responsible for the increased mutation rate (Fig. 2c). Replication fidelity

processes, including efficient mismatch repair at the 5' end of OFs^{25,45}, will mitigate Pol- α replication errors. Additionally, Pol- α DNA will be incorporated at relatively low frequency (Extended Data Fig. 8), with most DNA at such sites still synthesized by Pol- δ and Pol- ϵ . However, over evolutionary timescales, it seems that these processes are insufficient to compensate fully for the lack of Pol- α proofreading activity. An alternative possibility is that protein binding may impair access of replication-related repair factors, such as Exo1 to correct errors in Pol- α -synthesized DNA⁴⁵. However, it does not appear that the mismatch repair machinery is generally obstructed at such sites, as mismatch repair efficiency at nucleosomes is reported to be uniform with respect to dyad position²⁴.

Nucleosome formation has a key role in ensuring genome stability⁴⁶, and consequently there is an imperative for the rapid repackaging of the genome post-replication. However, we now show that this comes at the cost of increased mutation at specific sites, detectable on an evolutionary timescale. OF-associated mutagenesis could also have importance for human genetics, as it increases mutation rates at TF and regulatory protein binding sites. Such increased mutagenesis has been substantially obscured by strong purifying selection at these sites necessary to maintain functionality. Notably, increased mutation suggests that they will be evolutionary hotspots, and may help to explain the rapid evolutionary turnover of TF sites⁴⁷ and the difficulty in non-coding functional site prediction by interspecies sequence conservation comparisons. Furthermore, as hyper-mutable loci, TF binding sites may be frequently mutated in inherited disease and neoplasia.

In summary, we demonstrate that DNA synthesized by Pol- α contributes to the eukaryotic genome, probably increasing mutations at specific regulatory sites of relevance to both human genetics and the shaping of the genome during evolution.

Note added in proof: Three studies, published concurrently with this paper, have independently developed similar methods to determine the genome-wide distribution of embedded ribonucleotides^{48,49,50}, demonstrating the utility of ribonucleotides as markers of replication enzymology in budding yeast.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 14 October 2014; accepted 5 January 2015.

Published online 26 January 2015.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank N. Hastie and P. Burgers for discussions, I. Adams, J. Caceres, T. Aitman and P. Heyn for comments on the manuscript, and A. Gallacher for technical assistance. We are indebted to J. Williams, A. Clausen and T. Kunkel for sharing yeast strains and unpublished data, and to S. Cerritelli and R. Crouch for *RNH201* vectors. Funding: MRC Centenary Award to M.A.M.R.; MRC and Lister Institute of Preventive Medicine to A.P.J.; MRC and Medical Research Foundation to M.S.T.

Author Contributions M.A.M.R. and J.D. performed experiments. H.K., S.M.d.P. and M.S.T. performed computational analysis. M.S.T., M.A.M.R. and A.P.J. designed the study, developed emRiboSeq and wrote the manuscript.

Author Information Next-generation sequencing data submitted to the Gene Expression Omnibus (GEO) under accession number GSE64521; R and PERL code submitted to GitHub repository taylorLab/LaggingStrand (<https://github.com/taylorLab/LaggingStrand>). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.P.J. (andrew.jackson@igmm.ed.ac.uk) or M.S.T. (martin.taylor@igmm.ed.ac.uk).

METHODS

Yeast reference genome and annotation. All analyses were performed on the sacCer3 (V64) *S. cerevisiae* reference genome assembly. Data sets originally obtained with coordinates on other assemblies were projected into the sacCer3 assembly using liftOver (v261)⁵¹ with the corresponding chain files obtained from <http://www.yeastgenome.org>. All regions of the sacCer3 genome were used for read alignment but analyses including strand ratios and all rate estimates excluded the following multi-copy regions: the mitochondrial genome, rDNA locus chrXII:459153–461153 and any 100-nucleotide segment with mappability score of <0.9 (gem-mappability⁵² with k -mer = 100). In total, this masked 951,532 nucleotides (7.8%) of the reference genome. Gene structure annotations were the *Saccharomyces* Genome Database (SGD) consensus annotations extracted from the University of California, Santa Cruz (UCSC) genome browser in November 2013. Annotated origins of replication were obtained from ref. 53. DNase I hypersensitive sites and footprints were obtained from ref. 54, and nucleosome position, occupancy and positional fuzziness (positional heterogeneity) measures were from ref. 55. Yeast replication timing data was obtained from ref. 36, where we have plotted the percentage of heavy–light (replicated) DNA (pooled samples data set). Higher percentage indicates earlier average replication time.

Yeast polymorphisms and between species substitution rates. Yeast polymorphism data was obtained from the *Saccharomyces* Genome Resequencing project⁵⁶. A polymorphic difference between any of the 37 sequenced *S. cerevisiae* strains was called as a polymorphic site. Sites with $n > 2$ alleles were only counted once as a polymorphic site. Only nucleotide point substitutions were considered, insertions and deletions were excluded. The polymorphism rate reported is the number of polymorphic sites divided by the number of sacCer3 sites with sequence coverage in at least two additionally sequenced strains.

Yeast between-species substitution rates were calculated from MultiZ stacked pairwise alignments obtained from the UCSC genome browser (Supplementary Table 1). Alignments for five *sensu stricto* yeast species (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii* and *S. bayanus*) were extracted from the original seven species alignment. The reference assembly names and phylogenetic relationship are represented by the tree ((sacCer3, sacPar), sacMik), sacKud, sacBay). Substitution rates were calculated over whole chromosomes using baseml from the paml⁵⁷ package (version 4.6) under the HKY85 substitution model with ncatG = 5 categorical gamma. Per-nucleotide relative rate estimates (branch length multipliers) were obtained over the sacCer3 genome.

Human conservation measures. GERP scores⁴¹ were used as a measure of between species nucleotide diversity across 46 vertebrate species. Single-nucleotide resolution bigWig files were obtained from UCSC genome browser (hg19). For consistency of presentation with plots of polymorphism rate and yeast between-species nucleotide substitution rate, the y axes in plots showing GERP scores have been inverted so that greater constraint is low and greater diversity is high.

OF sequence processing. OF sequence data was obtained from ref. 17 (GEO accession GSM835651). Analysis primarily focused on the larger ‘replicate’ library but results were confirmed in the ‘sample’ library (GEO accession GSM835650). The OF strand ratio was calculated as the sum of per nucleotide read coverage on the forward strand divided by the same measure for reverse strand reads. OF strand ratios were calculated in windows of 2,001 nucleotides. A pseudo count of 1 read-covered nucleotide was added to both strands in each window to avoid divisions by zero. Results shown are for de-duplicated read data (identical start and end coordinates were considered duplicates). De-duplication minimises potential biases in PCR amplification, qualitatively similar results were obtained with non-de-duplicated data and support identical conclusions.

Rather than using separate Okazaki 5' and 3' end counts that did not always correlate well, probably due to amplification biases, sequencing and size selection biases; we produced a normalized OJ rate measure. This is the average of (1) the fraction of upstream OFs that terminate with a 3' end at a focal nucleotide, and (2) the fraction of downstream OFs whose 5' end is at the focal nucleotide. The upstream and downstream coverage measures were based on mean Okazaki read coverage for the nucleotides located between 5 and 12 nucleotides upstream (downstream) of the focal 3' (5') end. This OJ rate was calculated at single nucleotide resolution over both strands of the sacCer3 genome.

EmRiboSeq alignment and processing. Sequence reads (see Supplementary Table 2 for runs and read numbers) were aligned to the unmasked sacCer3 genome with bowtie2 (version 2.0.0). Subsequent filtering and format conversion were performed using Samtools (version 0.1.18) and BEDTools (version 2.16.2). Only reads with a mapping quality score >30 were kept for analysis. As there had been no pre-sequencing amplification, de-duplication was not performed. Read 5'-end counts were summed per strand at single nucleotide resolution over the yeast reference genome. Note that under the emRiboSeq protocol, the ribonucleotide incorporation site would be one nucleotide upstream and on the opposite strand to the mapped read 5' end. To facilitate comparison between libraries of

differing read depth, read counts were normalized to sequence tags per million mapped into the non-masked portion of the genome.

Defining TF binding sites. Reb1 and Rap1 ChIP-exo data was obtained from ref. 58 (Sequence Read Archive accession SRA044886). Sequence bar codes were clipped and sequences sorted using Perl (version 5.18.2). Reads were aligned using bowtie2 (version 2.0.0). Following the previously published protocol⁵⁸ up to three mismatches across the length of each tag sequence were allowed, and the 3' most 6 base pairs (bp) removed. Peaks were called with MACS (version 2.0.10). Following ref. 58, sites were defined as monomer if no other peaks were present within 100 bp. Where two or more peaks were present within 100 bp the peak with the highest occupancy was labelled as the primary peak. Telomeric sites were excluded using annotations within the sacCer3 sgdOther UCSC table (<http://www.yeastgenome.org>). The presence or absence of a motif was determined using the Motif Occurrence Detection Suite (MOODS)⁵⁹ (version 1.0.1). Consensus binding motifs positional weight matrices were obtained from JASPAR⁶⁰ (<http://jaspar.genereg.net/>). The matching motif significance threshold was set at 0.005. Multiple peaks were aligned ($x = 0$) to the midpoint of the JASPAR defined motif. Human TF binding sites were defined using ChIP-seq data (Supplementary Table 1) as for yeast, except that the peak clustering threshold was reduced to 50 nucleotides.

Computational and statistical analyses. Analysis and all statistical calculations were performed in R (version 3.0.0). Lines of fit used the smooth.spline function with degrees of freedom: Fig. 1a, 18 degrees; Fig. 1b, 34 degrees; Fig. 3d–f, 80 degrees of freedom (strand ratio calculated in 2,001-nucleotide consecutive windows). Sliding window averages used the rollapply function from the Zoo package with centre alignment and null padding. Pearson's correlation was performed with the cor.test function in R, paired Student's t -test with the t.test function, Mann–Whitney tests with the wilcox.test function and lowess (locally weighted scatterplot smoothing) with the lowess function and default parameters.

No statistical methods were used to predetermine sample size.

Rate estimates with compositional correction. Polymorphism and OJ rates were calculated separately for each nucleotide (A, T, C or G) and the average of these for rates used as the reported or plotted measure for a nucleotide site or group of sites. This corrects for mononucleotide compositional biases that are abundant when sampling specific features of a genome. The between-species relative substitution rate calculation incorporates a compositional correction. The rate estimates shown are the number of observations divided by the number of sites with non-missing data.

Trinucleotide preserving shuffles. Every nucleotide of the sacCer3 genome was assigned to one of 64 categories based on the identity of that nucleotide and its flanking nucleotides. A vector of transformations was produced by swapping the genomic coordinate of a nucleotide for one with an identical category chosen at random. Swaps between masked and unmasked sites (see above) were prevented. 100 such vectors were produced. For a set of stacked coordinates (for example, Fig. 1a comprising 27,586 sequences, each of 251 nucleotides), every nucleotide of every sequence was substituted through the transformation vector, for a randomly selected proxy, matched for the same trinucleotide context and their corresponding rate or annotation used. This provides a compositionally well-matched null expectation. With 100 independent transformation vectors we provide empirically derived 95% confidence bounds and standard deviations on those null expectations. For human sites, shuffles were confined to sequences flanking the region of interest (100–300 nucleotides distant from the binding site for TF analysis and 1,000–2,000 nucleotides distant for DNase I footprint analysis). Human genomic coordinates in the ENCODE 'Duke Excluded Regions'⁶¹ and those positions with a uniqueness score of <0.9 (gem-mappability⁵² with k -mer = 100) were excluded from shuffles.

Sites selected for analysis. Thresholds were applied to define specific subsets of sites to be evaluated. For the presented data (Fig. 1a) nucleosomes with an occupancy of >80%, positional fuzziness⁵⁵ of <30, with at least 30 OF reads over them, and located more than 200 nucleotides from transcription start sites were used. Other combinations (Extended Data Fig. 1) of these parameters gave qualitatively similar results and support the same conclusions. Reb1 (and Rap1) sites were defined as the primary ChIP-exo peak at a site, with sequences aligned ($x = 0$) to the centre of the highest scoring Reb1/Rap1 position weight matrix match within 50 nucleotides of the ChIP-exo peak summit. DNase I footprints from 41 human cell types were previously combined⁶² into consensus footprints (combined.fps.gz). We intersected the combined footprints with those found in each cell type using BEDtools (version 2.17.0) to identify the subset ($n = 33,530$) that were detected in all 41 cell types. The left-edge coordinate as defined in the combined footprint file was used as the focal nucleotide ($x = 0$) for analysis.

Comparison of polymorphism rates. The five nucleotide positions downstream and the five upstream of the focal OJ position (excluding $x = 0$ in both cases) were scored for their polymorphism rate (Fig. 2b). Rate deltas were calculated as upstream minus downstream in 100 bootstrap replicates and a paired two-sided t -test

performed against the same calculation performed on 100 trinucleotide preserving genome shuffles of the same sites. This tests whether the difference in rate between upstream and downstream positions is greater in the observed data than the shuffled data.

DNA purification. Yeast strains were grown at 30 °C in YPDA to mid-log phase (see Supplementary Table 3 for a list of strains) or to saturation for stationary phase. Per 5 $A_{600\text{nm}}$ units, cell pellets were resuspended in 200 μl lysis buffer (2% Triton X-100, 1% SDS, 0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). An equal volume of TE-equilibrated phenol and glass beads (0.40–0.60 mm diameter, Sartorius) were added, and cells lysed by vortexing for 2 min; 200 μl TE buffer was then added, followed by an additional 1 min of vortexing. After centrifugation, the aqueous phase was further extracted with equal volumes of phenol:chloroform:isoamylalcohol (25:24:1) and chloroform. Total nucleic acids were precipitated with 1 ml of 100% ethanol, and dissolved in 0.5 M NaCl. RNA was degraded by treatment with 10 μg RNase A (Roche) for 1 h at room temperature. DNA was finally purified with an equal volume of Ampure XP beads (Beckman Coulter) and eluted in nuclease-free water. For library preparations DNA was isolated from up to 40 $A_{600\text{nm}}$ units.

Alkaline gel electrophoresis. Isolated genomic DNA (0.5 μg) was treated with recombinant RNase H2, purified as previously described⁶³ and ethanol precipitated. DNA pellets were dissolved in alkaline loading dye and separated on 0.7% agarose gels (50 mM NaOH, 1 mM EDTA) as previously described²⁷, and stained with SYBR Gold (Life Technologies). Densitometry measurements and derivation of ribonucleotide incorporation rates as previously described²⁸. Percentage genome contribution for each replicative polymerase (x) was calculated using the following formula: $N_{\Delta\text{polx}} \cdot F_{\text{polx}} / (N_{\text{pola}} \cdot F_{\text{pola}} + N_{\text{pold}} \cdot F_{\text{pold}} + N_{\text{pole}} \cdot F_{\text{pole}})$, with $N_{\Delta\text{polx}}$ the number of ribonucleotides incorporated in one yeast genome for the mutant polymerase, above that detected in the Δrnh201 POL strain, measured on the same alkaline gel, and F_{polx} the frequency of incorporation by that polymerase (see Fig. 3a).

EmRiboSeq library preparation and sequencing. DNA was sonicated using a Bioruptor Plus (Diagenode) to achieve an average fragment length of approximately 400 bp. Fragmented DNA was concentrated by ethanol precipitation and size selected using 1.2 volumes of Ampure XP. DNA was quantified by nanodrop (Thermo Scientific) and up to 5 μg was used for NEBNext End Repair and dA-Tailing (New England Biolabs) following the manufacturer's guidelines. After the end-repair reaction, DNA was purified using 1.2 volumes of Ampure XP. Subsequent steps were performed in the presence of Ampure XP beads, capturing the DNA by adding NaCl and PEG_{8,000} to final concentrations of 1.25 M and 10%, respectively. The trP1 adaptor (see below) was attached using NEBNext Quick Ligation with 120 pmol of adaptor per microgram of DNA for 14–18 h at 16 °C.

Terminal transferase (NEB) was then used to block any free 3' ends with ddATP for 2 h at 37 °C, with 20 U of TdT per microgram of DNA. After Ampure XP purification, beads were removed and DNA nicked using recombinant RNase H2 (10 pmol μg^{-1} of library) or Nb.BtsI (NEB; 10 U μg^{-1}) for 2 h at 37 °C. RNase H2 purification and reaction conditions were as previously described⁶³. Enzymes were inactivated by heating at 80 °C for 20 min, and DNA was purified using 1.8 volumes of Ampure XP. Shrimp alkaline phosphatase (Affymetrix; 5 U) was then used to remove 5' phosphates at 37 °C (1 h per μg of library). After heat inactivation for 15 min at 65 °C and Ampure XP purification, DNA was denatured by heating at 95 °C for 5 min and snap cooling. Subsequently, A adaptor (see below; 120 pmol μg^{-1} of library) was attached using NEBNext Quick Ligation for 14–18 h at 16 °C. Fragments

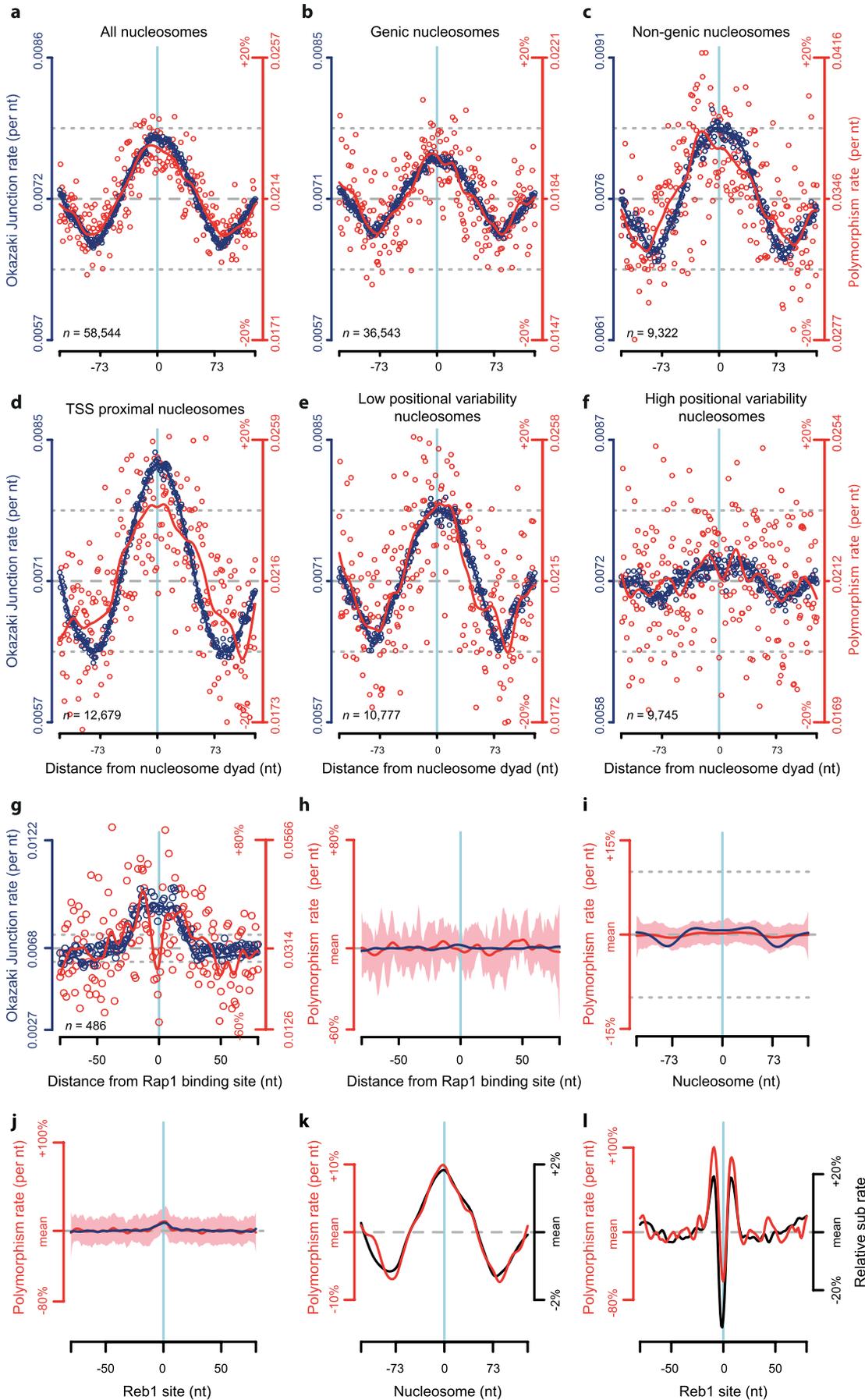
with biotinylated A adaptor were captured on streptavidin-coupled M-280 Dynabeads (Life Technologies) following the manufacturer's guidelines, and non-biotinylated strands were released in 0.15 M NaOH. Single-stranded fragments were concentrated by ethanol precipitation.

Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) was then used for second strand synthesis with primer A to produce a double stranded library. Size selection of fragments between 200 and 300 bp in size was performed using 2% E-Gel EX (Life Technologies). Finally, this library was quality checked and quantified using a 2100 Bioanalyzer (Agilent Technologies) before emulsion PCR, using the Ion Torrent One Touch, and next generation sequencing on the Ion Torrent PGM or Proton platform (Life Technologies).

Oligonucleotides and adaptor design. Custom oligonucleotides were synthesized by Eurogentec. Adaptor primer pairs were annealed by heating at 95 °C for 5 min and cooling gradually. Sequences of the adaptor primer pairs were as follows. Adaptor 1 (trP1): trP1-top, 5'-CCTCTCTATGGGCAGTCCGGTGAT-phosphorothioate-T-3'; trP1-bottom, 5'-phosphate-ATCACCGACTGCCCATAGAGAGGC-dideoxy-3'. Adaptor 2 (A): A-top, 5'-phosphate-CTGAGTCGGA GACACGCAGGGATGAGATGG-dideoxy-3'; A-bottom, 5'-biotin-CCATCTC ATCCCTGCGTGTCTCCGACTCAGNNNNNN-C3 phosphoramidite-3'. The sequence for primer A used in second strand synthesis was 5'-CCATCTCATC CCTGCGTGTCTCCGAC-3'.

Data sources, sequencing data and *S. cerevisiae* strains. Documented in Supplementary Tables 1–3.

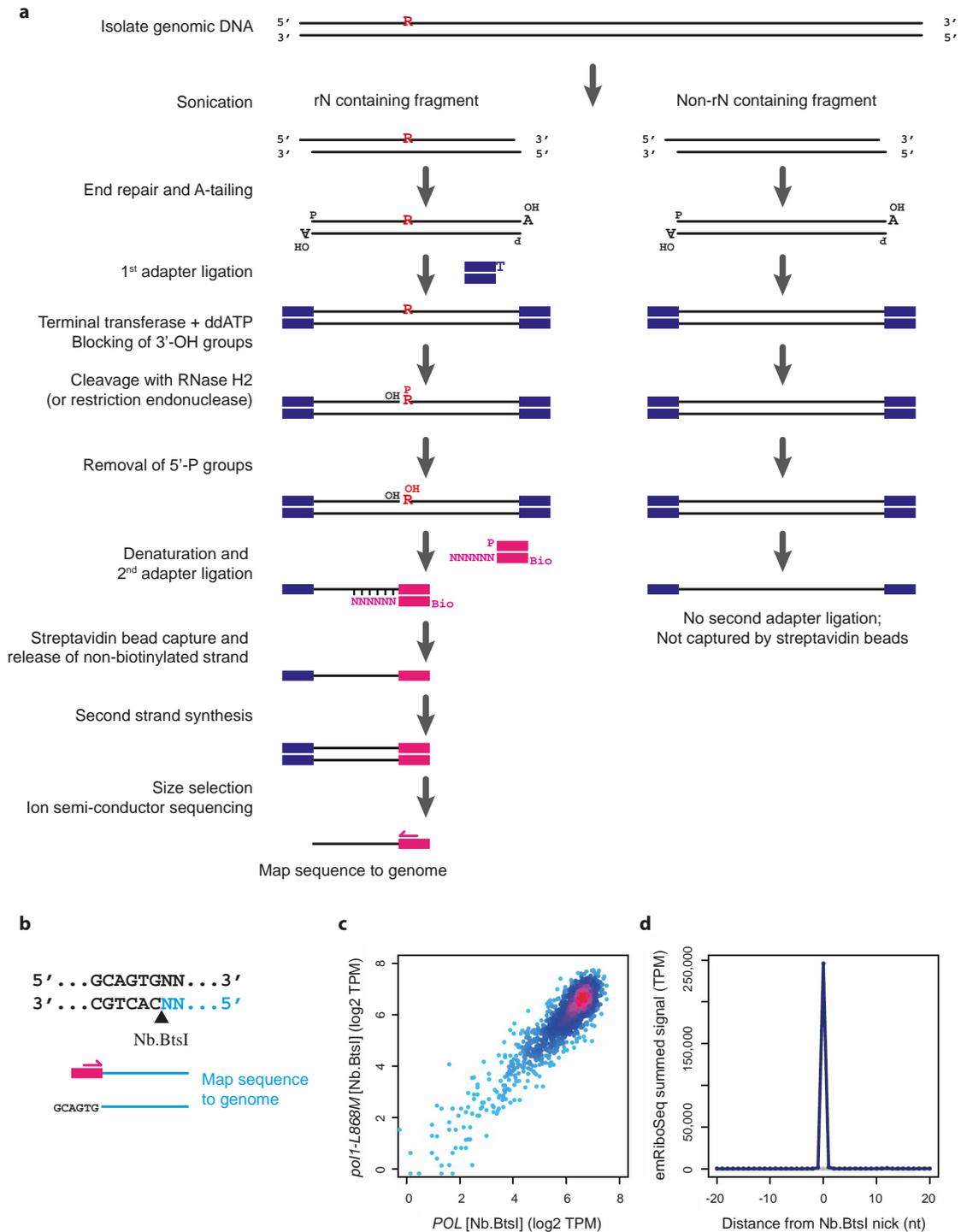
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Extended Data Figure 1 | Increased OJ and polymorphism rates correlate at binding sites of different nucleosome classes and at Rap1 binding sites.

a–f, OJ and polymorphism rates are strongly correlated for different classes of nucleosomes. Data presented as in Fig. 1a, for different sub-classes of *S. cerevisiae* nucleosomes, demonstrating that OJ and polymorphism rates covary in all cases. Transcription start site proximal nucleosomes (**d**) are probably subject to strong and asymmetrically distributed selective constraints, which is likely to explain the modestly reduced correlation for this subset. Such transcription start site proximal nucleosomes were excluded from analyses of other categories presented (**b**, **c**, **e**, **f**), except ‘all nucleosomes’ (**a**). **g**, OJ and polymorphism rates are correlated for the *S. cerevisiae* TF, Rap1. Data presented, as for Reb1 in Fig. 1b, show increased OJ and polymorphism rates

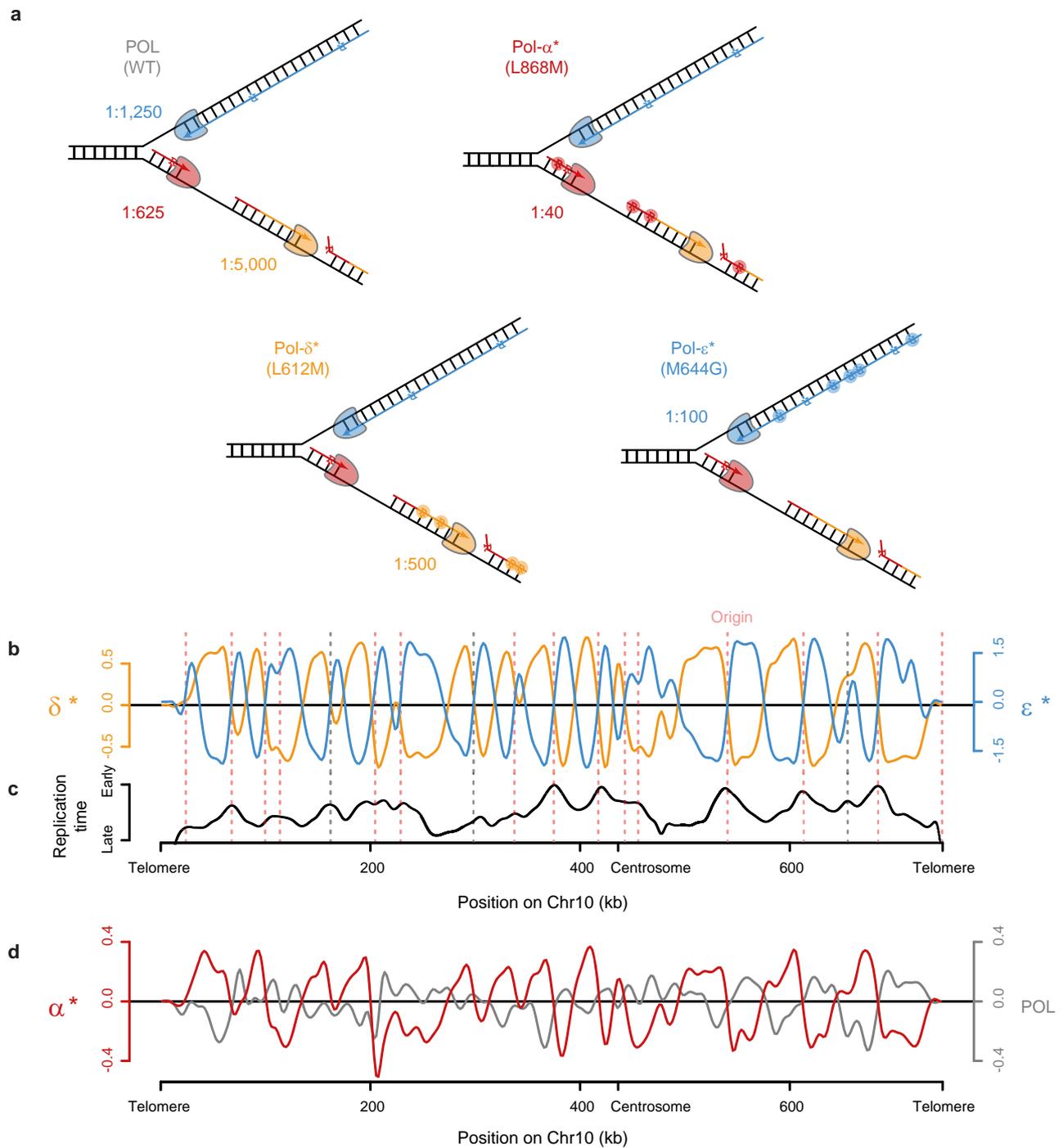
around its binding site, with a dip corresponding to its central recognition sequence. **h–j**, Increased polymorphism and OJ rates at Rap1 (**h**), nucleosome (**i**) and Reb1 (**j**) binding sites are not caused by biases in nucleotide content. Distributions calculated as for **g**, Fig. 1a and b, respectively, using a trinucleotide preserving genome shuffle. Pink shaded areas denote 95% confidence intervals for nucleotide substitution rates (100 shuffles). **k**, **l**, Polymorphism (red) and between-species (black) substitution rates are highly correlated for nucleosome (**k**) and Reb1 (**l**) binding sites. Best fit splines shown only. *y* axes scaled to demonstrate similar shape distribution. Values plotted as percentage relative to the mean rate for all data points (central 11 nucleotides excluded for calculation of mean in **g**, **l**).



Extended Data Figure 2 | EmRiboSeq methodology and validation.

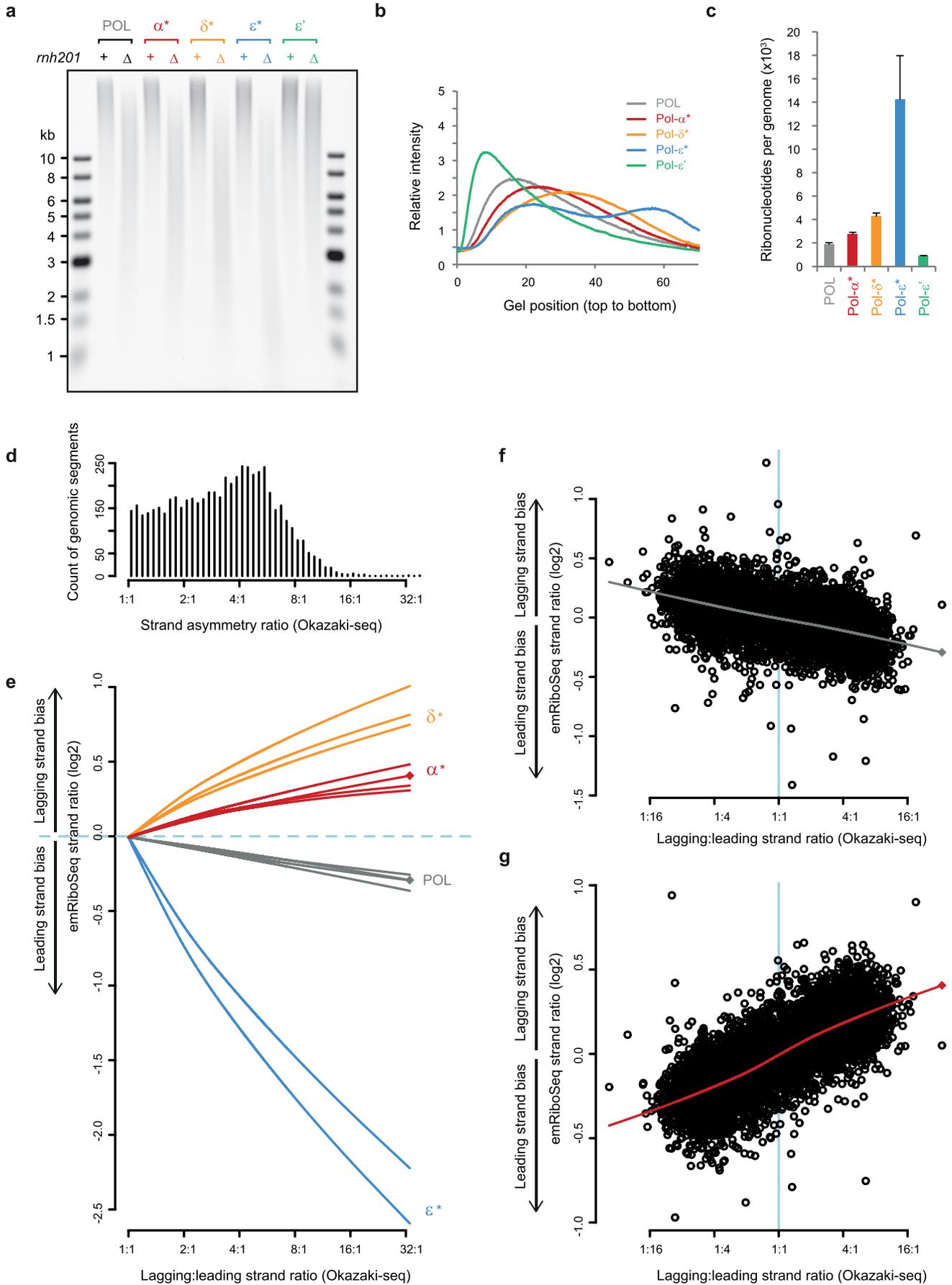
a, Schematic of emRiboSeq library preparation. rN, ribonucleotide.
b–d, Validation of strand-specific detection of enzymatically generated nicks through linker-ligation. Nb.BtsI nicking endonuclease cleaves the bottom strand of its recognition site releasing a 5' fragment (cyan) with a free 3'-OH group after denaturation, to which the sequencing adaptor (pink) is ligated, allowing sequencing and mapping of this site to the genome (**b**). Nb.BtsI libraries have high reproducibility between $\Delta rnh201$ POL and $\Delta rnh201$ Pol- α^* (*pol1-L868M*) strains after normalizing read counts to sequence tags per million

(TPM). Bona fide Nb.BtsI sites were equally represented, at maximal frequency, in both libraries (**c**). Those with lower frequencies represented sites in close proximity to other Nb.BtsI sites, causing their partial loss during size selection. Additionally, Nb.BtsI-like sites were detected as the result of star activity. Libraries were also prepared using BciVI restriction enzyme digestion, that did not show such star activity (data not shown), allowing calculation of the site specificity for the method (>99.9%). Summed signal at Nb.BtsI sites shows >99.9% strand specificity (blue, correct strand; grey, opposite strand) and >99% single nucleotide resolution (**d**).



Extended Data Figure 3 | Mapping replicative polymerase DNA synthesis using emRiboSeq. **a**, Point mutations in replicative polymerases elevate ribonucleotide incorporation rates, permitting their contribution to genome synthesis to be tracked. Schematic of replication fork with polymerases and their ribonucleotide incorporation rates (refs 27, 30 and J. S. Williams, A. R. Clausen & T. A. Kunkel, personal communication) as indicated (POL denotes wild-type polymerases; asterisk denotes point mutants). Embedded ribonucleotides indicated by 'R'; additional incorporation events due to polymerase mutations highlighted by shaded circles. **b, c**, Mapping of leading/

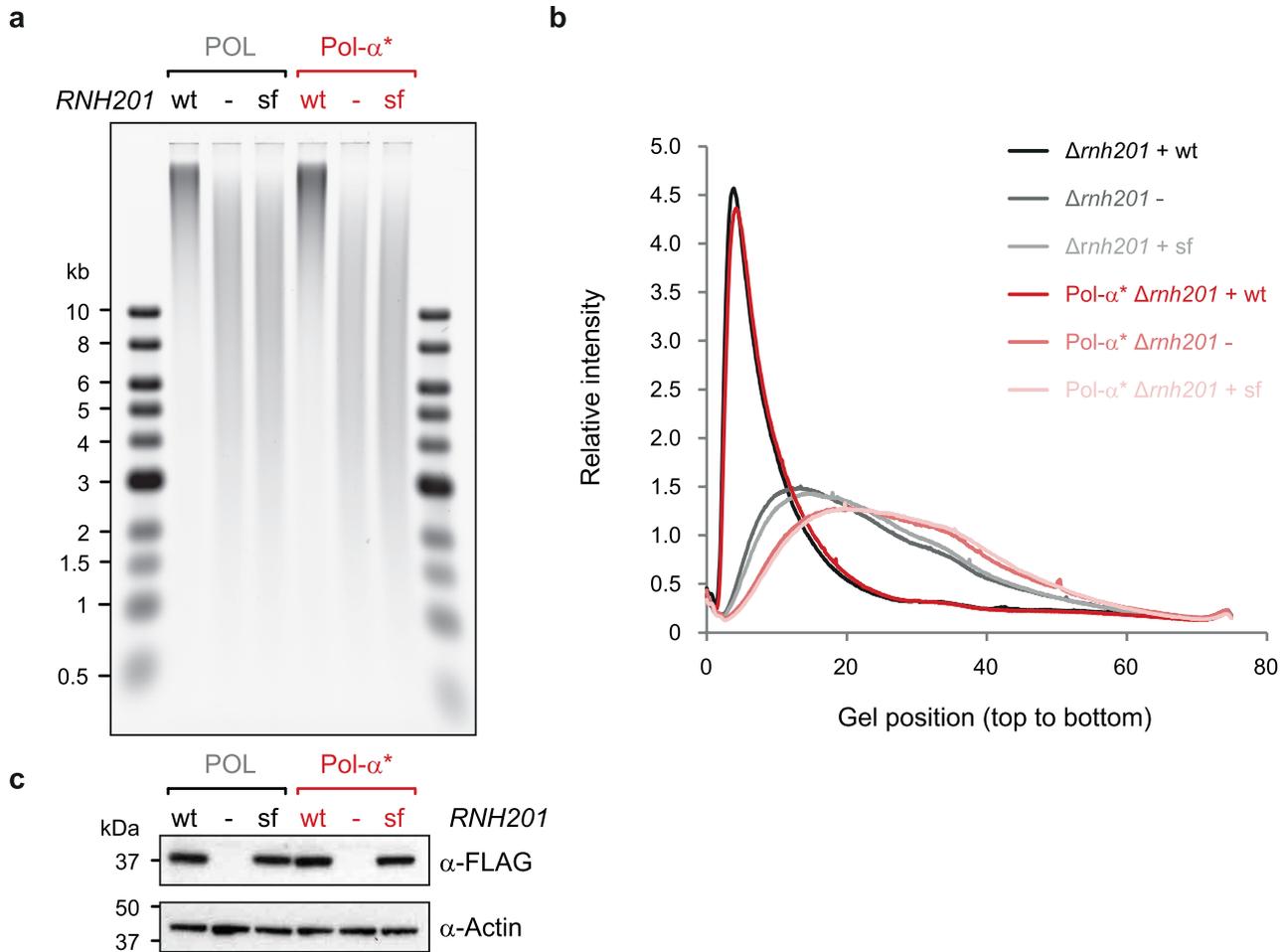
lagging-strand synthesis by Pol- δ^* and Pol- ϵ^* yeast strain using emRiboSeq (as in Fig. 3) highlights both experimentally validated (pink dotted lines) and putative (grey dotted lines) replication origins. These often correspond to regions of early replicating DNA³⁶ (**c**). **d**, Pol- α^* DNA is detected genome-wide by emRiboSeq as a component of the lagging strand in stationary phase yeast, as shown by the opposite pattern for a polymerase wild-type strain. Strand ratios are shown as best-fit splines with 80 degrees of freedom, y axes show \log_2 of the strand ratio calculated in 2,001-nucleotide windows (**b-d**).



Extended Data Figure 4 | Quantification of *in vivo* ribonucleotide

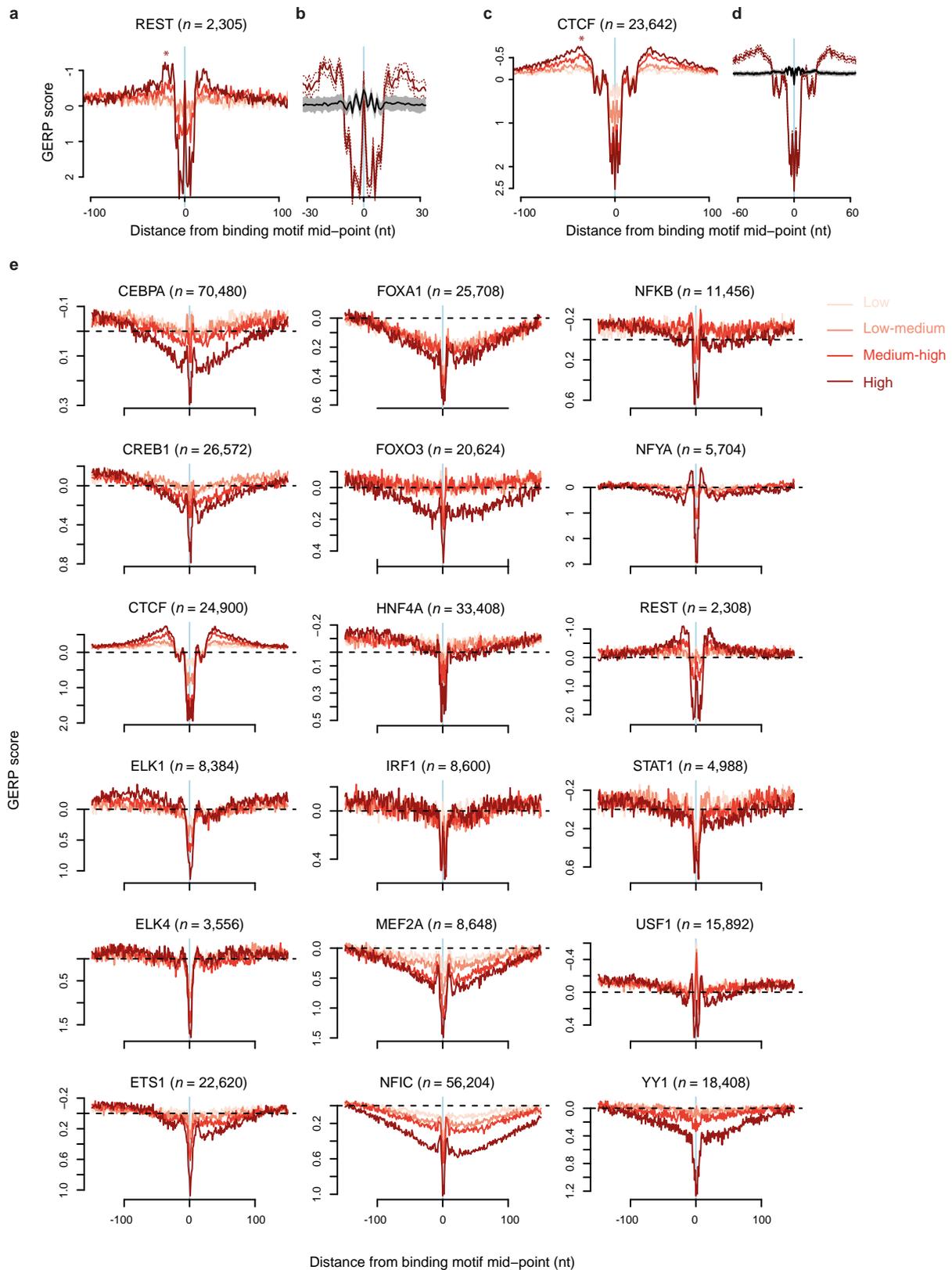
incorporation by replicative polymerases. **a, b,** Representative alkaline gel electrophoresis of genomic DNA from yeast strains with mutant replicative DNA polymerases (**a**), with accompanying densitometry plots (**b**). Embedded ribonucleotides are detected by increased fragmentation of genomic DNA following alkaline treatment in an RNase H2-deficient ($\Delta rnh201$) background. Increased rates are seen with all three mutant polymerases (indicated by asterisk, as defined in Extended Data Fig. 3a), and are reduced in Pol- ϵ' which contains the point mutation Met644Leu, a mutation that increases selectivity for dNTPs over rNTPs²⁷. **c,** Quantification of average ribonucleotide incorporation in polymerase mutants from four independent experiments. DNA isolated from mid-log phase cultures; error bars denote s.e.m. Overall ribonucleotide content is the product of incorporation frequency and the total contribution of each polymerase, resulting in the total ribonucleotide content detected to be highest for Pol- ϵ^* (14,200 per genome), followed by Pol- δ^* (4,300 per genome), Pol- α^* (2,700 per genome), POL (1,900 per genome) and Pol- ϵ' (860 per genome). **d,** Most of the yeast genome exhibits directional asymmetry in replication (median 4:1 strand ratio). Count of genomic

segments calculated for consecutive 2,001-nucleotide windows over the yeast genome based on reanalysis of OF sequencing data¹⁷ denoted as 'Okazaki-seq'. The strand asymmetry ratio was calculated after re-orienting all regions such that the predominant lagging strand was the forward strand. **e-g,** Genome-wide quantification of strand-specific incorporation of wild-type and mutant replicative DNA polymerases determined by emRiboSeq reflects their roles in leading- and lagging-strand replication. A close to linear correlation with Okazaki-seq strand ratios is observed. The strand ratio preference for lagging-strand ribonucleotide incorporation for independent libraries (including stationary phase libraries for POL and Pol- α^* , marked by diamonds) was plotted against the lagging:leading-strand ratio determined using Okazaki-seq data (only ratios $\geq 1:1$ for the latter are shown for clarity). There was high reproducibility between experiments in strand ratio preferences. Lines are lowess smoothed (see Methods) representations of the full data sets (representative examples given in **f** and **g**). **f, g,** Scatter plots illustrating the individual strand ratio data points for 2,001-nucleotide windows, for stationary phase POL (**f**) and Pol- α^* (**g**) yeast. Pearson's correlation = 0.49, $P < 2.2 \times 10^{-16}$ for POL (**f**); correlation = 0.75, $P < 2.2 \times 10^{-16}$ for Pol- α^* (**g**).



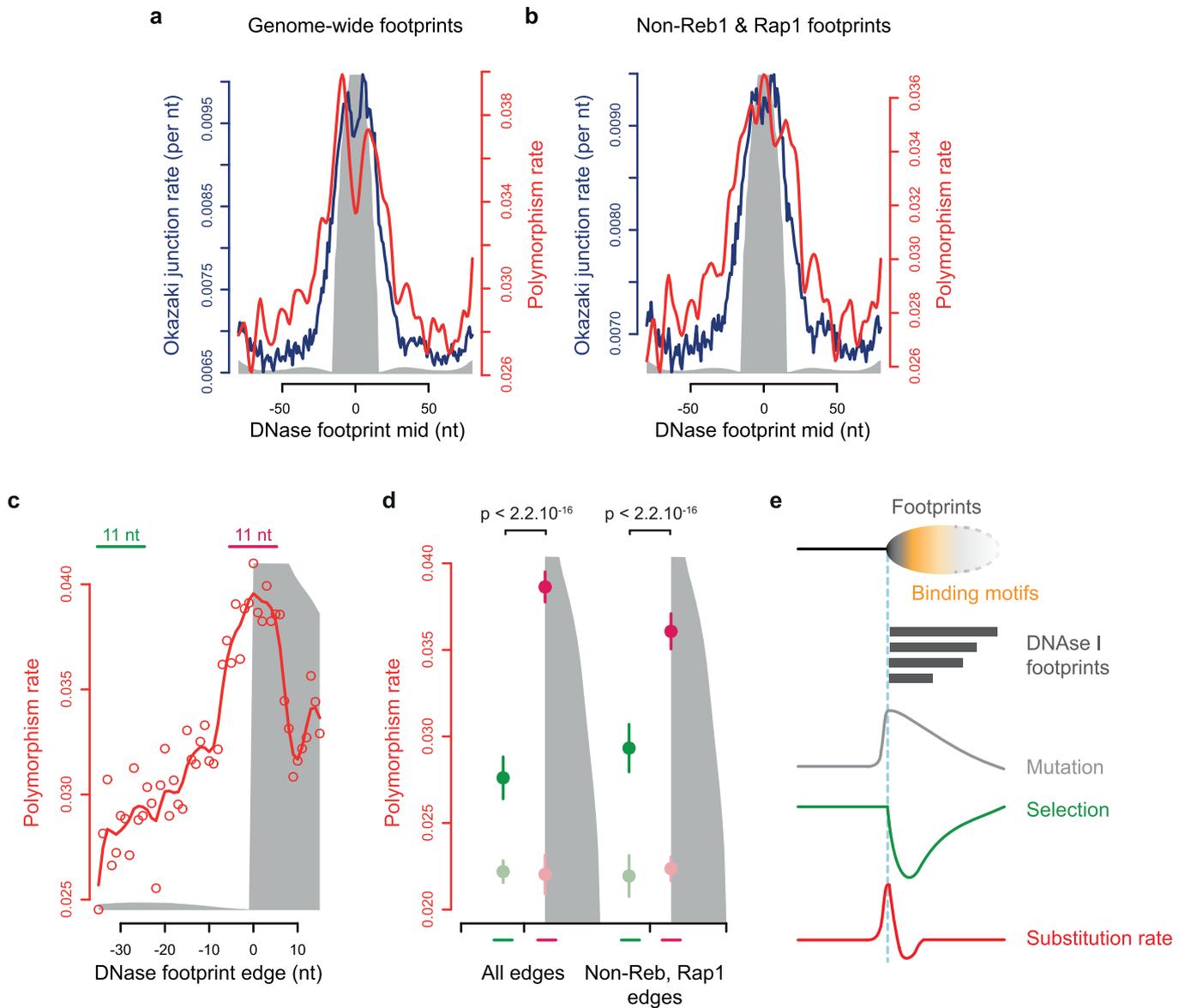
Extended Data Figure 5 | Pol- α -synthesized DNA retention is independent of RNase H2 processing of RNA primers. **a, b,** The ribonucleotide content of genomic DNA is unchanged between $\Delta rhn201$ strains transformed with empty vector (–) or vector expressing Rnh201 separation-of-function mutant (sf), that retains the ability to cleave RNA:DNA hybrids, including RNA primers, but cannot cleave single embedded ribonucleotides³⁹. In contrast, the same vector expressing wild-type Rnh201 (wt) fully rescues alkaline sensitivity of the

DNA. As complementation with the separation-of-function mutant had no detectable effect on the ribonucleotide content seen in the Pol- α (Leu868Met) $\Delta rhn201$ strain, retention of Pol- α -synthesized DNA appears to be independent of a putative role for RNase H2 in RNA primer removal. Representative result shown for $n = 3$ independent experiments. **c,** Wild-type and mutant Rnh201 are expressed at equal levels, as shown by immunodetection of the C-terminal FLAG tag. Loading control, actin.



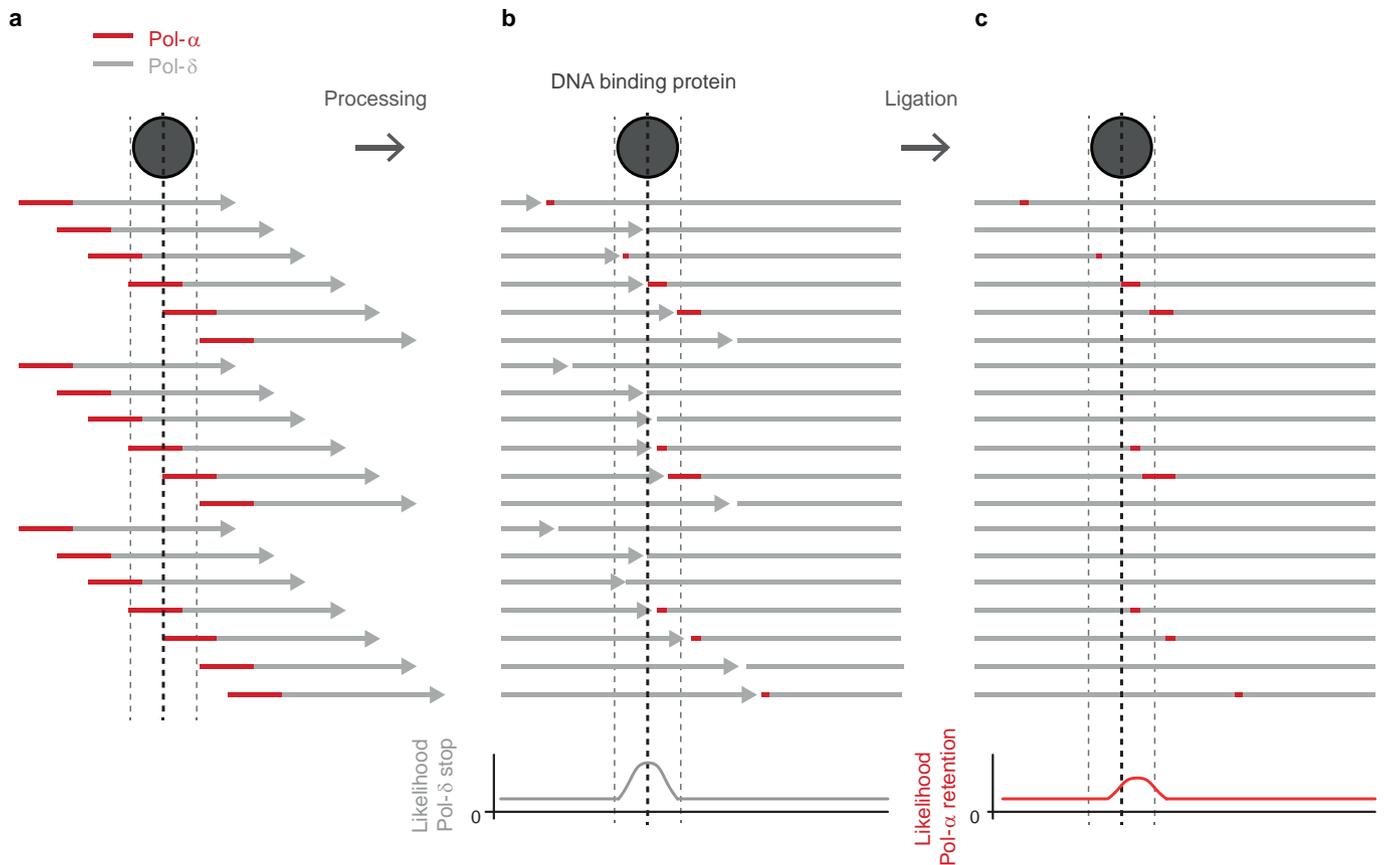
Extended Data Figure 6 | Elevated substitution rates are observed adjacent to many human TF binding sites. **a–d**, Nucleotide substitution rates (plotted as GERP scores) are elevated immediately adjacent to REST (**a**, **b**) and CTCF binding sites (**c**, **d**). Colour intensity shows quartiles of ChIP-seq peak height (pink to brown), reflecting strength of binding/occupancy. Stronger binding correlates with greater increases of proximal substitution rate in the ‘shoulder’ region (asterisk). Increased substitution rates are not a consequence of local sequence composition effects (**b**, **d**). Strongest binding

quartile of sites (brown) is shown compared to a trinucleotide preserving shuffle (black) based on the flanking sequence (100–300 nucleotides from motif midpoint) of the same genomic locations. Brown dashed line and grey shading denote 95% confidence intervals. **e**, Substitution rates plotted as GERP scores for human TF binding sites identified in ChIP-seq data sets (in conjunction with binding site motif). Sites aligned ($x = 0$) on the midpoint of the TF binding site within the ChIP-seq peak (colours as for **a–d**). Dashed black line shows $y = 0$, the genome wide expectation for neutral evolution.



Extended Data Figure 7 | OJ and polymorphism rates are increased at yeast DNase I footprints. **a, b**, DNase I footprint edges correspond, genome-wide, to increased OJ rates and locally elevated polymorphism rates in *S. cerevisiae* (**a**), a pattern that is maintained when footprints associated with Reb1 and Rap1 binding sites are excluded (**b**). Genome-wide DNase I footprints ($n = 6,063$) and excluding those within 50 nucleotides of a Reb1 or Rap1 binding site ($n = 5,136$) were aligned to their midpoint. **c, d**, Aligning DNase I footprints on their left edge rather than midpoint (to compensate for substantial heterogeneity in footprint size) demonstrates a distinct shoulder of elevated polymorphism rate at the aligned edge (**c**), with a significant elevation compared to nearby sequence upstream from the footprint (**d**). DNase I footprints from **a** were aligned to their left edge ($x = 0$) with corresponding polymorphism rates shown (**c**). The increased polymorphism rate cannot be explained by local sequence compositional distortions (**d**). Nucleotide substitution rates in the 11 nucleotides centred on the DNase footprint edge (pink line), and another 11 nucleotides encompassing positions -35 to -25 relative to the footprint edge (green line) were quantified. Darker pink and green filled circles denote the mean of observed substitution rates and lighter

shades denote the mean for the same sites after trinucleotide preserving genomic shuffles. Error bars denote s.d.; statistics by Mann–Whitney test. **e**, Model shows that correlation of increased nucleotide substitution and OJ rates are consistent with increased mutation frequency across heterogeneous DNase I footprints. Polymorphism is reduced at sequence-specific binding sites within the footprints, owing to functional constraint. Therefore, the effect of OF-related mutagenesis in these regions is most sensitively detected in the region immediately adjacent to the binding site (left of vertical dashed blue line, representing footprints aligned to their left edge). This ‘shoulder’ of increased nucleotide substitutions represents sites with increased, OJ-associated mutation is followed by a region of depressed substitution rates, owing to selective effects of the functional binding sites within the footprints (to the right of the dashed blue line). Signals further to the right are not interpretable given the heterogeneity in DNase I footprint sizes. Given strong selection at TF and DNase I footprint sites, this ‘shoulder’ of elevated nucleotide substitutions could represent a measure for the local mutation rate for such regions, analogous to that measured by the fourfold degenerate sites in protein coding sequence.



Extended Data Figure 8 | Model to show Pol- α DNA tract retention downstream of protein binding sites. **a**, OF priming occurs stochastically, with the 5' end of each OF initially synthesized by Pol- α and the remainder of the OF synthesized by Pol- δ . **b**, **c**, OF processing: when Pol- δ encounters the previously synthesized OF, Pol- δ continues to synthesize DNA displacing the 5' end of the downstream OF, which is removed by nucleases to result in mature OFs which are then ligated. The OJs of such mature OFs before ligation were detected previously¹⁷ after depletion of temperature-sensitive

DNA ligase I. They demonstrated that if a protein barrier is encountered (grey circle), Pol- δ progression is impaired, leading to reduced removal of the downstream OF (**b**). Given that $\sim 1.5\%$ of the mature genome is synthesized by Pol- α , a proportion of lagging strands will retain Pol- α -synthesized DNA (red). When Pol- δ progression is impaired by protein binding, this will lead to an increased fraction of fragments containing Pol- α -synthesized DNA downstream of such sites (**c**).