**LETTERS**

**POT1** loss-of-function variants predispose to familial melanoma

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Deleterious germline variants in **CDKN2A** account for around 40% of familial melanoma cases1, and rare variants in **CDK4**, **BRCA2**, **BAP1** and the promoter of **TERT** have also been linked to the disease2-5. Here we set out to identify new high-penetrance susceptibility genes by sequencing 184 melanoma cases from 105 pedigrees recruited in the UK, The Netherlands and Australia that were negative for variants in known predisposition genes. We identified families where melanoma cosegregates with loss-of-function variants in the protection of telomeres 1 gene (**POT1**), with a proportion of family members presenting with an early age of onset and multiple primary tumors. We show that these variants either affect **POT1** mRNA splicing or alter key residues in the highly conserved oligonucleotide/oligosaccharide-binding (OB) domains of **POT1**, disrupting protein-telomere binding and leading to increased telomere length. These findings suggest that **POT1** variants predispose to melanoma formation via a direct effect on telomeres.

Cutaneous malignant melanoma accounts for around 75% of skin cancer deaths, with around 10% of cases having one first-degree relative and 1% of cases having two or more first-degree relatives who have had a diagnosis of this disease6. As only around half of familial melanoma cases can be attributed to variants in known predisposition genes, principally **CDKN2A**, a substantial proportion of genetic risk for melanoma remains elusive. To identify new mediators of germline genetic risk, we sequenced 184 melanoma cases from 105 pedigrees. The cases sequenced came from pedigrees with between 2 and 11 cases of melanoma (169 cases) or were single cases that presented with either multiple primary melanoma (MPM), multiple primary cancers (one of which was melanoma) and/or an early age of onset (before the fourth decade of life; 15 cases) (Online Methods and Supplementary Tables 1 and 2). Sequencing of two-case pedigrees was preferentially performed for those families enriched with cases of MPM. All cases were previously found to be negative for pathogenic variants in **CDKN2A** and **CDK4**.

After performing exome (168 samples) or whole-genome (16 samples) sequencing, we called and filtered variants, keeping only those predicted to affect protein structure or function. Notably, we found no known pathogenic variants in **BAP1** or **BRCA2**, and we confirmed that all samples had wild-type **CDKN2A** and **CDK4**. We further filtered the calls, taking forward only non-polymorphic variants (Online Methods). When we sequenced more than one member of a pedigree, we retained only cosegregating variants, whereas all variants were considered from pedigrees in which only one affected family member was sequenced. As a result, a total of 23,051 variants remained for downstream analysis. Focusing on the 28 pedigrees for which sequence data were available for 3 or more family members, we found 320 genes carrying cosegregating protein-changing variants (Supplementary Table 3). Of particular interest were five genes that showed previously unreported variants in more than one of these pedigrees (**POT1**, **MPDZ**, **ACD**, **SMG1** and **NEK10**). Analysis of the missense and disruptive variants (nonsense, splice acceptor or donor, and frameshift) in these genes led us to identify a five-case pedigree (UF20) carrying a splice-acceptor variant between **POT1** and **SMG1**.

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Received 29 May 2013; accepted 7 March 2014; published online 30 March 2014; doi:10.1038/ng.2947
Figure 1 Rare variants in POT1 found in familial melanoma pedigrees. (a) We identified four pedigrees carrying deleterious variants in POT1. Shown are a five-case pedigree (UF20) and a six-case pedigree (AF1) carrying the disruptive p.Tyr89Cys OB domain variant and a splice-acceptor variant, respectively. Note that pedigrees have been adjusted to protect the identities of the families without loss of scientific integrity. POT1 genotypes for all samples available for testing are shown in blue, and other types of cancer are indicated. CMM, cutaneous malignant melanoma; CLL, chronic lymphocytic leukemia; WT, wild type. Diamonds represent individuals of undisclosed sex. The cases that were sequenced have a red outline. All melanomas were confirmed by histological analysis, with the exception of two cases (marked by asterisks). The number of primary melanomas in each subject is indicated; age of onset in years is shown in parentheses. Half-filled symbols represent other cancers. (b) Highly conserved residues of POT1 are altered in familial melanoma. Shown are the positions of the variants identified on the POT1 protein (top) and on an amino acid alignment (missense variants; bottom).

To gather further evidence for an association between POT1 variants and familial melanoma, we compared the representation of POT1 variants in our familial melanoma cases with variants in controls. Notably, the presence of POT1 variants in 4 of 105 families with melanoma represented a statistically significant enrichment of variants (P = 0.016, excluding a discovery pedigree) compared with a control data set of 520 exomes from individuals sequenced as part of the UK10K project (see URLs) in which we found only 1 missense variant located outside the OB domains of POT1 (Online Methods, Supplementary Fig. 4 and Supplementary Table 6). Furthermore, none of the 4 POT1 variants identified in our melanoma pedigrees were found by genotyping 2,402 additional population-matched controls (Online Methods and Supplementary Table 6). Interestingly, genotyping of these positions across a matched cohort of 1,739 population-based sporadic melanoma cases identified 1 individual who carried the POT1 variant encoding p.Arg273Leu who presented with early-onset MPM similar to the phenotype presented by the pedigree UF20. Other malignancies in the tested first- or second-degree relatives of variant carriers included melanoma (pedigrees UF20 and UF31), endometrial cancer (pedigree UF20) and brain tumors (pedigrees UF20 and UF23). Intriguingly, the pedigree with the splice-acceptor variant (AF1) had a member with a history of melanoma and CLL, in keeping with a role for POT1 in CLL development. Collectively, these data suggest a possible role for germline POT1 variants in susceptibility to a range of cancers in addition to melanoma.

To test whether the identified missense variants disrupted telomere binding as was observed for somatic mutations found in CLL, we examined the structure of POT1 protein bound to a telomere-like polynucleotide (dTUAdAgGdGdGdTdTdAdG) (Protein Data Bank (PDB) 3KJP)12,13. According to this model, all 3 altered residues (Tyr89, Gln94 and Arg273) were among 24 residues located in close proximity (<3.5 Å) to the telomeric polynucleotide16 (Fig. 2a). Arg273 interacts with the oxygen at position 2 of telomeric deoxythymidine 7, whereas Gln94 and Tyr89 both interact with the G deoxynucleotide at position 4. Therefore, as described for the somatic mutations in CLL, the POT1 variants we identified are expected to weaken or abolish the interaction of POT1 with telomeres. Analysis of the nucleotides coding for these 24 OB domain residues identified 1 nonsynonymous change in 6,498 control exomes14 compared with 3 in 105 families (P = 1.54 × 10−5) (Online Methods and Supplementary Tables 6 and 7). To further test whether the OB domain variants we identified disrupted POT1 function, we assessed the ability of in vitro-translated POT1 Tyr89Cys, Gln94Glu and Arg273Leu proteins to bind to (TTAGGG)3 sequences. Electrophoretic mobility shift assays showed a complete abolition of POT1-DNA complex formation with mutant POT1 (Fig. 2b and Supplementary Fig. 5). Notably, the POT1 p.Tyr36Asn and p.Tyr223Cys alterations recently described in CLL10, which seem to be functionally analogous to the variants we describe, showed that all nine carriers of POT1 variants from the familial cohort had developed melanoma, presenting with one primary (four cases) to eight melanomas at 25 to 80 years of age (Fig. 1 and Supplementary Fig. 3). One variant carrier from these familial cases also developed breast cancer at 65 years, and another developed small cell lung cancer at 50 years (pedigree UF20). Other malignancies in the tested first- or second-degree relatives of variant carriers included melanoma (pedigrees UF20 and UF31), endometrial cancer (pedigree UF20) and brain tumors (pedigrees UF20 and UF23).
here, promote uncapping of telomeres, telomere length extension and chromosomal aberrations and thereby promote tumorigenesis.

Given the role of POT1 in telomere length maintenance, we next asked whether melanoma cases from pedigrees with mutated POT1 had telomere lengths that differed from those of non-carrier melanoma cases. Using exome sequence data from 41 cases, including 3 members of pedigree UF20, we estimated the telomere length of each subject by counting TTAGGG repeats. This analysis showed that all three members of pedigree UF20 had telomeres that were significantly longer than those in melanoma cases with wild-type POT1 (P < 0.0002; Fig. 2c and Supplementary Fig. 6). This result was confirmed by telomere-length PCR, which also showed longer telomeres for subjects carrying the p.Gln94Glu and p.Arg273Leu variants compared to melanoma cases without POT1 variants (P = 3.62 × 10−5; Fig. 2d and Supplementary Fig. 7). Thus, missense variants in the OB domains of POT1 not only abolish telomere binding but are also associated with increased telomere length, a key factor influencing melanoma risk. Notably, OB domain variants that disrupt the interaction of POT1 with telomeric single-stranded DNA are thought to function as dominant-negative alleles, yet, as we show here, they are compatible with life, suggesting that additional somatic events are required to promote tumorigenesis.

The identification of POT1 mutations in CLL and the probable susceptibility of our POT1-mutated familial melanoma pedigrees to other tumor types suggests that POT1 might have a more general role in tumorigenesis. To investigate this possibility, we examined pan-cancer data from the Catalogue of Somatic Mutations in Cancer (COSMIC) and IntOGen databases (data from The Cancer Genome Atlas).

### Table 1 POT1 variants identified in familial melanoma pedigrees

| Pedigree | Number of cases in pedigree | Number of carriers/tested cases | Genomic change | Coding mutation | Exon | Amino acid change | Variant type | Bioinformatic prediction tools |
|----------|-----------------------------|---------------------------------|---------------|----------------|------|-------------------|--------------|-----------------------------|
| UF20     | 5                           | 4/4                             | g.124503684T>C | c.266A>G       | 8    | p.Tyr89Cys        | Missense     | SIFT Deleterious           |
|          |                             |                                 |               |                |      |                   |              | PolyPhen-2 Probably damaging|
|          |                             |                                 |               |                |      |                   |              | CAROL Deleterious           |
| AF1      | 6                           | 3/3                             | g.124465412C>T | c.1687–1G>A    | –    | Splice acceptor   | Deleterious  | –                           |
|          |                             |                                 |               |                |      | (intron, between |              | –                           |
|          |                             |                                 |               |                |      | exons 17 and 18) |              | –                           |
| UF31     | 2                           | 1/1                             | g.124503670G>C | c.280C>G       | 8    | p.Gln94Glu        | Missense     | SIFT Tolerated              |
|          |                             |                                 |               |                |      |                   |              | PolyPhen-2 Probably damaging|
|          |                             |                                 |               |                |      |                   |              | CAROL Deleterious           |
| UF23     | 2                           | 1/2b                            | g.124493077C>A | c.818G>T       | 10   | p.Arg273Leu       | Missense     | SIFT Deleterious           |
|          |                             |                                 |               |                |      |                   |              | PolyPhen-2 Probably damaging|
|          |                             |                                 |               |                |      |                   |              | CAROL Deleterious           |

a The reference transcript, taken from the Ensembl database (release 70), is POT1-001 (ENST00000357628). b A second case within this pedigree had a different clinical presentation (solitary MPMs and an early age of onset) (Online Methods). c This variant was also detected in a melanoma case from a population-based case-control series that presented with MPNs and an early age of onset (Online Methods).
(TCGA) and the International Cancer Genome Consortium (ICGC)) from 14 cancer types and found that somatic POT1 mutations were more likely to be missense \( (P < 0.03) \), to alter residues in close proximity to DNA \( (P < 0.02) \) and to have a higher functional bias \( (P < 0.03) \) than expected by chance (Online Methods). These results suggest that, although they are rare, somatic POT1 mutations may drive tumorigenesis across multiple histologies.

Here we describe germline variants in the gene encoding the telomere-associated protein POT1 in almost 4% of familial melanoma pedigrees negative for mutations in CDKN2A and CDK4 and in 2 of 34 pedigrees \( (5.8\%) \) with \( \geq 5 \) cases, making POT1 the second most frequently mutated high-penetration melanoma gene reported thus far. This work and a companion study describing germline POT1 variants in Italian, French and US families with melanoma\(^{20,21} \), together with a recent report of a TERT promoter variant\(^{2} \), substantially extend understanding of a newly discovered mechanism predisposing to the development of familial melanoma. As the dysregulation of telomere protection by POT1 has recently been identified as a target for potential therapeutic intervention\(^{21,22} \), it may be possible that the early identification of families with POT1 variants might facilitate better management of their disease in the future.

**METHODS**

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

**Accession codes.** Sequence data have been deposited in the European Genome-phenome Archive (EGA), hosted at the European Bioinformatics Institute, under accession EGAS00001000017.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank the UK10K Consortium (funded by the Wellcome Trust; WT091310) for access to control data. D.J.A., C.D.R.-E., Z.D., J.Z.L., J.C.T., M.P. and T.M.K. were supported by Cancer Research UK and the Wellcome Trust (WT098051). C.D.R.-E. was also supported by the Consejo Nacional de Ciencia y Tecnología of Mexico. K.A.P. and A.M.D. were supported by Cancer Research UK (grants C1267/A9540 and C8197/A10123) and by the Isaac Newton Trust. N.K.H. was supported by a fellowship from the National Health and Medical Research Council of Australia (NHMRC). L.G.A. was supported by an Australia and New Zealand Banking Group fellowship from the National Health and Medical Research Council of Australia. C.L.-O., A.J.R. and V.Q. are funded by the Spanish Ministry of Economy and Competitiveness through the Instituto de Salud Carlos III (ISCIII), the Red Temática de Investigación del Cáncer (RTICC) del ISCIII and the Consolider-Ingenio RNAREG Consortium. C.L.-O. is an investigator with the Botín Foundation.

**AUTHOR CONTRIBUTIONS**

C.D.R.-E., M.H., J.A.N.-B., D.T.B., N.K.H. and D.J.A. designed the study and wrote the manuscript. C.D.R.-E., M.H., I.G.A., J.C.T., M.M., J.C., M.P., A.J.R., Z.D., V.Q., A.L.P., J.M.P.J., S.M.S., N.G.M., M.G.G., A.M.D., K.A.P., P.J., J.Z.L., K.M.B., C.L.-O. and T.M.K. performed experiments or analysis. N.A.G., W.G.M., H.S. and N.G.M. provided vital biological resources.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**SUPPLEMENTARY INFORMATION**

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ONLINE METHODS

Case samples and DNA extraction. The families included in this study were recruited to a UK Familial Melanoma Study directed by the Section of Epidemiology and Biostatistics, University of Leeds (Leeds, UK); the Leiden University Medical Center (Leiden, The Netherlands); and the Queensland Familial Melanoma Project (QFMP)26. Informed consent was obtained under the Multicentre Research Ethics Committee (UK); 99/3/045 (UK Familial Melanoma Study cases), Protocol P00.117-gk2/WK/ib (Leiden cases) and from the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute for QFMP cases. Genomic DNA was extracted from peripheral blood using standard methods.

Pedigrees and clinical presentation. The pedigrees in this study are listed by institute and by sequencing center in Supplementary Table 1. All pedigrees, the number of cases of melanoma in each pedigree and the number of cases that were whole-genome sequenced are listed in Supplementary Table 2. To help anonymize the pedigrees, ages were rounded up to the nearest 5-year tier.

Sequence alignment and analysis. DNA libraries were prepared from 5 µg of genomic DNA, and exonic regions were captured with the Agilent SureSelect Target Enrichment System, 50 Mb Human All Exon kit. Whole-genome libraries were prepared using the standard Illumina library preparation protocol. Paired-end reads of between 75 and 100 bp were generated on the HiSeq 2000 platform and mapped to the reference GRCh37/bj9 human genome assembly using the Burrows-Wheeler Aligner (BWA)27. Reads were filtered for duplicates using Picard28 and were recalibrated and realigned around indels using the Genome Analysis Toolkit (GATK) package29 (Familial Melanoma Study and Leiden data). Exome capture and sequencing resulted in an average of 84% of target bases being covered by ≥10× across the autosomes and sex chromosomes. Whole genomes were sequenced to at least 27× mapped coverage. Data for POT1 variant carriers have been released (EGAS00001000017). Variants were then called using SAMtools mpileup30 and filtered for quality.

The variant collection was filtered to remove positions found in Phase 1 of the 1000 Genomes Project October 2011 release31 and the dbsNP 135 release (see URLs). Variants were also filtered for positions found in a collection of 805 in-house control exomes. Only variants in exonic regions, as defined in Ensembl release 70, were taken forward for analysis. Positions resulting in protein-altering changes were then identified using the Ensembl Variant Effect Predictor, version 2.8 (Ensembl release 70)32, a combination of VCFTools33 and custom scripts. Variants marked as ‘transcript_ablation’, ‘splice donor variant’, ‘splice acceptor variant’, ‘stop gained’, ‘frameshift variant’, ‘stop lost’, ‘initiator codon variant’, ‘inframere_insertion’, ‘inframere_deletion’, ‘mature miRNA variant’, ‘transcript_amplification’, ‘splice region variant’, ‘incomplete_terminal_codon_variant’, ‘mature miRNA variant’, ‘TFBS_ablation’, ‘TFBS amplification’, ‘TF_binding_site_variant’, ‘feature elongation’ and ‘feature truncation’ were kept for further analyses. We retained only those variants found in all affected cases of a single pedigree (to reduce the impact of systematic mapping errors). An identity-by-descent (IBD) analysis was performed to confirm that cases from different pedigrees within the study were not related.

Genes with cosegregating variants from the 28 pedigrees for which we had sequence data for 3 or more family members are shown in Supplementary Table 3 with their Gene Ontology (GO) terms. Variants in POT1 identified from this analysis were confirmed by capillary sequencing (Supplementary Fig. 1). Several low-penetrance variants in MC1R were also identified in the pedigrees with POT1 variants; all of these are common variants associated with freckling and sun sensitivity (Supplementary Table 8). We also identified a variant at the +6 intronic splice site of introns 17 and 18 of POT1 (g.12446726A>C) in one melanoma pedigree, but our analyses suggested that this variant was unlikely to be deleterious (Supplementary Note), although the temoleres of the subject carrying this variant at +6 appeared longer than the temoleres of controls, suggesting some effect on temolere regulation (Supplementary Fig. 8).

MaxEntScan scoring of splice-site acceptor variant g.124465412C>T. We used the MaxEntScan algorithm34, which yielded scores of ~3.22 for the mutated splice-acceptor site (g.124465412C>T) and 5.53 for the wild-type splice-site sequence. To put these values in context, we retrieved 10,000 splice-acceptor and splice-donor sites from random genes (choosing always the second exon) and obtained a distribution of their scores. The splice-acceptor variant lowered the score of the wild-type sequence from the 9.2 to the 0.57 percentile compared to the score distribution of real splice acceptors (Supplementary Fig. 2) and is thus predicted to be highly deleterious.

RT-PCR sequencing of the POT1 product in two individuals carrying the splice-acceptor variant g.124465412C>T. RNA extracted from the whole blood of two carriers of the splice-acceptor variant was converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). RT-PCR was then performed to confirm that POT1 g.124465412C>T (c.1687–1G>A; ENST00000357628) was indeed disruptive to splicing. M13-tagged forward primer and reverse primer were designed to flank the spliced region (primer sequences available upon request). The product was visualized on a 3% NuSieve Agarose gel, and the sequence was verified using standard Sanger sequencing methods. Sequencing traces for one control and one carrier sample are shown in Supplementary Figure 1c.

Frequency of POT1 variants in a control data set. All exomes from the UK10K sequencing project (REL 14/03/12) cohorts UK10K_NEURO_MUIR, UK10K_NEURO_IOP_COLLIER and UK10K_NEURO_ABERDEEN (see URLs) were selected as controls because these exomes were captured with the same Agilent SureSelect exome probes as those used for the melanoma cases described above and were also sequenced on the Illumina HiSeq 2000 platform (n = 546). One exome was discarded at random from each of three pairs of relatives within this set. UK10K exomes were aligned, filtered for duplicates, and recalibrated and realigned around indels as described above. Variants were then called and filtered for base quality with the same tools and parameters as the melanoma cohort. For 104 of 105 families, we had exome data for at least 1 individual in the pedigree; for 1 melanoma family, we used whole-genome sequence.

To ensure that the controls were matched by ancestry to the melanoma cohort, we performed a principal-component analysis (PCA) using 1,092 individuals across 14 populations from the 1000 Genomes Project Phase 1 data set35. A subset of high-quality variant positions (quality score >10, minimum mapping quality >10, strand bias P value >0.0001, end distance P value >0.0001) that were common to the melanoma cohort and the UK10K controls, as well as the 1000 Genomes Project data set, were taken forward for analysis. SNPs with a minor allele frequency of <0.05 or that were in linkage disequilibrium with another SNP (pairwise r2 >0.1) in the 1000 Genomes Project data set or that had a Hardy-Weinberg P value of <1×10−5 in the UK10K controls were excluded. After filtering, 7,196 SNPs remained that were spread across all autosomes. The first ten principal components were estimated using the 1000 Genomes Project individuals and were then projected onto the melanoma cohort samples and UK10K controls using EIGENSTRAT34. Controls lying greater than 2 s.d. from the mean scores for principal component 1 or 2 (PC1 or PC2, respectively), calculated using only European individuals in the 1000 Genomes Project data set, were removed from subsequent analyses (n = 20). This analysis is shown in Supplementary Figure 4. An IBD analysis was performed to ensure that members of the UK10K cohort were not related. This analysis was performed using the PLINK toolset35 and the same set of variants that were used for PCA. For each pair of individuals with an estimated IBD of >0.2, 1 individual was removed at random (n = 3). This filtering left 520 exomes for comparison against the melanoma cohort.

Variants in this collection of 520 UK10K control exomes were then filtered as described above (keeping positions with exonic coordinates ±100 bp and removing all variants in Phase 1 of the 1000 Genomes Project October 2011 release31, the dbsNP 138 release (see URLs) and a collection of 805 control exomes). Because we used an updated version of dbsNP for this step, we also checked that the POT1 variants found in this study passed this filter. Consequences were then predicted and filtered as described above. From this analysis, we identified 1 individual in 520 control exomes that carried a rare, potentially disruptive variant in POT1 (a missense variant located outside of the OB domains). We performed a two-tailed Fisher’s exact test comparing the 3 out of 104 families with melanoma, excluding a discovery pedigree, to
the 1 individual out of 520 controls carrying rare variants in \textit{POT1}, yielding a \(P\) value of 0.016.

**Genotyping in a population-based case-control series (TaqMan).** The variants encoding p.Tyr89Cys, p.Gln94Glu and p.Arg273Leu and the splice-acceptor variant were genotyped in 2,402 control samples belonging to the Leeds Melanoma Case-Control Study. This control set included 499 population-matched control DNA samples, 370 family controls (family members of melanoma cases without a diagnosis of melanoma) and 1,533 DNA samples from the Wellcome Trust Case Control Consortium. All 2,402 samples were wild type for the \textit{POT1} variants. We also genotyped the corresponding positions in 1,739 population-based melanoma cases that were recruited from across Yorkshire, UK, as part of the same study. One case, presenting with MPMs with early onset (48 years old), was found to be a carrier of the variant encoding p.Arg273Leu (Table 1 and Supplementary Tables 5 and 6). This variant was confirmed by PCR sequencing.

**Protein alignment, structural modeling and characterization of \textit{POT1} variants.** The amino acid sequences of \textit{POT1} from evolutionarily diverse species were gathered from NCBI and aligned with Clustal Omega\(^{22}\). Alignments were displayed using Jalview v2.7 (ref. 37). To estimate the number of substitutions per site in this amino acid alignment, we used the ProtPars routine from PHYLIP\(^{38}\). This analysis showed higher conservation for the three altered amino acids (2, 2 and 0 substitutions at positions 89, 94 and 273, respectively) than the average for the OB domains (2.42 substitutions per site) and, in fact, the whole protein (3.49 substitutions per site) across ~450 million years of evolutionary history (since the divergence of the zebrafish and human lineages). If only sequences from eutherian organisms were taken into account, then no substitutions have occurred in any of the three residues, compared to 0.8 substitutions per site in the OB domains and 1.39 substitutions per site in the whole protein. The OB domain regions were defined as amino acids 8–299 in the human sequence, according to Ensembl superfamily domain annotation. The structure of the OB domains of \textit{POT1} (3KJO) was obtained from PDB and was rendered with PyMOL v0.99 (see URLs).

**Analysis of nucleotide variants coding for the 24 key OB domain residues in close proximity (3.5 Å) to telomeric DNA.** Ramsay \textit{et al.}\(^{10}\) defined a list of 24 residues that lie closer than 3.5 Å to telomeric DNA in the crystal structure of \textit{POT1} (PDB 3KJP): residues 31, 33, 36, 39–42, 48, 60, 62, 87, 89, 94, 159, 161, 223, 224, 243, 245, 266, 266, 270, 271 and 273. To assess the statistical significance of finding amino acid substitutions affecting these residues, we searched 6,503 exomes that were part of the National Heart, Lung, and Blood Institute (NHBLI) Grand Opportunity (GO) Exome Sequencing Project (ESP)\(^{14}\) for substitutions at any of the bases that would cause a change in these residues. The genomic positions that encode these 24 residues are shown in Supplementary Table 7. In summary, a minimum of 6,498 exomes had all bases covered at a minimum average coverage of 59x. The variant encoding p.Asn224Asp was found at an overall allele frequency of 1 in 13,005. No other amino acid–changing variants were found. We compared the number of variants found in the 24 key OB domain residues in controls (1 in 6,498) to the number of variants found in all analyzed pedigrees (3 in 105), obtaining a \(P\) value of \(1.54 \times 10^{-5}\) using a two-tailed Fisher’s exact test (Supplementary Table 6).

**In vitro translation and G strand binding assays.** We mutated human \textit{POT1} in a T7 expression vector (Origene) by site-directed mutagenesis to generate cDNAs encoding the \textit{POT1} Tyr89Cys, Gln94Glu and Arg273Leu variants. Mutant and control T7 expression vectors were used in an \textit{in vitro} translation reaction using the TNT coupled reticulocyte lysate kit (Promega) following the manufacturer’s instructions. Briefly, a 50-µl reaction mixture containing 1 µg of plasmid DNA, 2 µl of EasyTag \(^{35}\)S-labeled \(\alpha\)-methionine (1,000 Ci/mmol; PerkinElmer) and 25 µl of rabbit reticulocyte lysate was incubated at 30 °C for 90 min. A 5-µl fraction of each reaction was analyzed by SDS-PAGE; proteins were visualized and relative amounts were quantified using the FLA 7000 phosphorimager system (Fujifilm) (Supplementary Fig. 5). DNA binding assays were performed as described previously with minor modifications\(^{39}\). In 20-µl reaction mixtures, 5 µl of each translation reaction was incubated with 10 nM telomeric oligonucleotide 5’ labeled with \(^{32}\)P (5’-GGTTAGGTTAGGTTAGG-3’) and 1 µg of the nonspecific competitor DNA poly(Deoxyinosinic-deoxyyctidylic) acid in binding buffer (25 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 1 mM EDTA and 5% glycerol). Reactions were incubated for 10 min at room temperature, and protein-DNA complexes were analyzed by electrophoresis on a 6% polyacrylamide Tris-borate-EDTA gel run at 80 V for 3 h. Gels were visualized by exposure to a phosphorimager screen.

**Analysis of telomere length from next-generation sequencing data.** Telomere length was determined essentially as described\(^{15}\). The investigator who performed this analysis was blinded to the \textit{POT1} status of the 41 eligible samples (which were all sequenced at the Sanger Institute, as they were the only ones with enough data available; Supplementary Table 2).

After calculation of relative telomere length, the 38 samples without germline \textit{POT1} variants were adjusted for age at blood draw and sex using a linear model (Supplementary Fig. 6). The corresponding values for \textit{POT1} variant carriers were estimated on the basis of the same adjustment. A Wilcoxon rank–sum test comparing adjusted telomere lengths for non-carrier melanoma cases and the three members of pedigree UF20 (p.Tyr89Cys variant carriers) (\(P = 0.00019\)) supported the finding of increased telomere lengths for variant carriers. In Figure 2c, all values are shown relative to the largest sample measurement.

**Analysis of telomere length (PCR).** We measured telomere length in melanoma cases recruited from the Leeds Melanoma cohort who did not carry a \textit{POT1} variant, seven \textit{POT1} missense variant carriers (pedigrees UF20, UF31 and UF23 and the carrier individual from the Leeds Melanoma cohort) and two non-carrier family controls (UF23, individual III-1 and UF20, individual III-1). The investigator who performed this analysis was blinded to the \textit{POT1} status of all samples. Relative mean telomere length was ascertained by SYBR Green RT-PCR using a version of the published Q-PCR protocols\(^{40,41}\) that was modified as described previously\(^{42}\). In brief, genomic DNA was extracted from whole blood, and telomere length was ascertained by determining the ratio of detected fluorescence from the amplification of telomere repeat units (TEL) relative to fluorescence for a single-copy reference sequence from the \textit{HBB} (β-globin) gene (CON). Telomere and control reactions were performed separately. For each assay, the PCR cycle at which each reaction crossed a predefined fluorescence threshold was determined (\(C_T\) value). The difference in the \(C_T\) values, \(\Delta C_T = C_T\) TEL – \(C_T\) CON, was the measure of telomere length used in the analysis, as in other published data generated using this assay\(^{43,44}\). For the analysis, samples with \(C_T\) CON < 18, \(C_T\) CON > 27 or \(C_T\) CON > 2 s.d. away from the mean were removed and considered to represent failed reactions. This filtering left 252 samples from the Leeds Melanoma cohort for further analyses, with no missense variant carriers or non-carrier family controls removed. All samples had between two and eight technical replicates. Mean \(\Delta C_T\) values for each sample were estimated from all replicates. The estimated mean values of \(\Delta C_T\) obtained from melanoma cases without germline \textit{POT1} variants were adjusted for age at blood draw and sex using a linear model (Supplementary Fig. 7). The corresponding values for \textit{POT1} variant carriers were estimated on the basis of the same adjustment. Adjusted mean \(\Delta C_T\) values are plotted (Fig. 2d), with the histogram showing the non-carrier melanoma cases compared to the missense variant carriers and the non-carrier family controls plotted above. A Wilcoxon rank-sum test comparing the adjusted mean \(\Delta C_T\) values for the 252 non-carrier melanoma cases with those for the 7 missense variant carriers (\(P = 3.62 \times 10^{-5}\)) supported the finding of increased telomere lengths for variant carriers.

**Analysis of \textit{POT1} mutations in cancer databases.** Although mutations in \textit{POT1} have not been found at a high frequency in the cancer studies deposited in COSMIC\(^{38}\) and IntOGen\(^{19}\) (which integrates only whole-exome data from ICGC and TCGA as well as other studies), the mutations that have been reported show a tendency to be missense, alter residues that are predicted to interact with DNA and have a high functional impact bias. To statistically assess the mutational patterns affecting \textit{POT1} in cancer, we compiled a list of all residues closer than 3.5 Å to the telomeric DNA in the crystal structure of \textit{POT1} (PDB 3KJP)\(^{10}\). We then mined COSMIC database.
v66 for confirmed somatic mutations absent from the 100 Genomes Project affecting the ORF of POT1 across 14 cancer types (breast, central nervous system, endometrium, hematopoietic and lymphoid tissue, kidney, large intestine, liver, lung, ovary, parathyroid, prostate, skin, urinary tract and not specified). This analysis yielded 35 somatic mutations, including 4 that were silent. We also compiled the total frequency of each reference/mutated base pair in the same COSMIC database. Finally, we performed a Monte Carlo simulation with 100,000 groups of 35 mutations at random locations in the POT1 ORF. The probability of a given mutation from a reference base (for example, A to G) was forced to equal the frequency for that pair in the whole COSMIC database.

Of the 100,000 simulations performed with this method, only 2,971 contained 4 or fewer silent mutations. Therefore, the COSMIC database contains fewer silent mutations affecting POT1 than expected by chance ($P < 0.03$). To assess the clustering of mutations at sites encoding DNA-binding residues (Supplementary Table 7), we only considered missense mutations, as no selection would be expected for nonsense mutations. In the COSMIC database, we found 27 POT1 missense mutations, 4 of which affected telomere-binding residues. In the Monte Carlo experiment, 9,244 simulations had exactly 27 missense mutations. In only 176 of these simulations were 4 or more residues identified that were classified as disrupting telomere binding. This result suggests that POT1 missense mutations affect DNA-binding residues at a higher than expected rate in the COSMIC database ($P < 0.02$).

To assess the functional impact bias of somatic mutations in POT1, we also looked at mutations in POT1 that are present in the IntOGen database. We chose this database because it integrates only samples that have been whole-exome sequenced and thus can provide a valid, non-biased estimate of the functional impact of mutations in POT1 when they are compared with mutations in the rest of the exome. The frequency of POT1 mutations in this data set is $0.01$ across 9 cancer sites (all of those available in the database, contained in the 14 sites listed above). $P$ values for the three studies for which the gene passed set thresholds defined in the database, calculated with Oncodrive-fm, were combined to yield a $P$ value of 0.021, indicating that this gene is biased toward the accumulation of functional mutations.

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