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Nonsense Mutations in the Shelterin Complex Genes *ACD* and *TERF2IP* in Familial Melanoma

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Abstract

Background: The shelterin complex protects chromosomal ends by regulating how the telomerase complex interacts with telomeres. Following the recent finding in familial melanoma of inactivating germline mutations in *POT1*, encoding a member of the shelterin complex, we searched for mutations in the other five components of the shelterin complex in melanoma families.

Methods: Next-generation sequencing techniques were used to screen 510 melanoma families (with unknown genetic etiology) and control cohorts for mutations in shelterin complex encoding genes: *ACD*, *TERF2IP*, *TERF1*, *TERF2*, and *TINF2*. Maximum likelihood and LOD [logarithm (base 10) of odds] analyses were used. Mutation clustering was assessed with χ^2 and Fisher's exact tests. *P* values under .05 were considered statistically significant (one-tailed with Yates' correction).

Results: Six families had mutations in *ACD* and four families carried *TERF2IP* variants, which included nonsense mutations in both genes (p.Q320X and p.R364X, respectively) and point mutations that cosegregated with melanoma. Of five distinct mutations in *ACD*, four clustered in the POT1 binding domain, including p.Q320X. This clustering of novel mutations in the POT1 binding domain of ACD was statistically higher (*P* = .005) in melanoma probands compared with population control individuals (n = 6785), as were all novel and rare variants in both *ACD* (*P* = .040) and *TERF2IP* (*P* = .022). Families carrying *ACD* and *TERF2IP* mutations were also enriched with other cancer types, suggesting that these variants also predispose to a broader spectrum of cancers than just melanoma. Novel mutations were also observed in *TERF1*, *TERF2*, and *TINF2*, but these were not convincingly associated with melanoma.

Conclusions: Our findings add to the growing support for telomere dysregulation as a key process associated with melanoma susceptibility.

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Germline mutations in the high penetrance melanoma susceptibility genes *CDKN2A* and *CDK4* account for cutaneous malignant melanoma (CMM) development in about 40% of multicase families (1). Additionally, rare mutations in *BAP1* have been associated with uveal and cutaneous melanoma predisposition (2). More recently, germline mutations in the promoter of *TERT* (telomerase reverse transcriptase) (3), as well as inactivating mutations in the shelterin component *POT1* (protection of telomeres 1) (4,5), implicate telomere dysregulation as a novel pathway underlying familial melanoma.

Shelterin is a telomere-specific protein complex that protects the ends of chromosomes by mediating the interaction of telomerase with telomeres. It is made up of six family members, encoded by the genes *POT1*, *ACD* (adrenocortical dysplasia protein homolog; also known as *TPP1*, *TINT1*, *PIP1*, and *PTOP*), *TERF1* (telomeric repeat binding factor 1, also known as *TRF1*), *TERF2* (telomeric repeat binding factor 2, also known as *TRF2*), *TERF2IP* (telomeric repeat binding factor 2 interacting protein, also known as *RAP1* and *DRIP5*), and *TINF2* (TERF1-interacting nuclear factor 2, also known as *TIN2*) (Supplementary Figure 1, available online). The shelterin components are collectively necessary for all telomere functions, which include the protection of telomeres from degradation, aberrant recombination, from being inappropriately processed by the DNA-repair pathway, and also the facilitation of chromosome capping to mediate telomerase activity (6). Thus, protein-altering variants located within this complex have recently been shown to have a great impact on diseases related to cellular lifespan, particularly cancer (4,5,7).

Here, following on from recent reports of germline *POT1* mutations in familial melanoma (4,5), we establish a key role for other components of the shelterin complex in susceptibility to CMM.

Methods

We screened for germline *ACD*, *TERF2IP*, *TERF1*, *TERF2*, and *TINF2* variants in exome, whole-genome, or targeted pull-down sequence data from 601 individuals belonging to 510 families with CMM.

Samples Used for Whole-Genome, Exome, or Targeted Sequence Analysis

All case patients gave written informed consent for participation. Each was wild-type for *CDKN2A*, *BAP1*, *POT1*, *BRCA2*, *CDK4*, and the *TERT* promoter. Samples were ascertained through the Queensland Familial Melanoma Project (QFMP) (8,9), the Sydney Genetic Epidemiology of Melanoma study (10), the UK Familial Melanoma Study, the Leiden University Medical Centre, the Danish Project of Hereditary Malignant Melanoma, and the Oncogenetic Clinic at Skåne University Hospital.

Ethics approval was granted by the Committee of Biomedical Research Ethics of the Capital Region of Denmark, the UK Multicentre Research Ethics Committee, and the Human Research Ethics Committees of: the QIMR Berghofer Medical Research Institute, the Lund University, the University of Sydney, and the Leiden University Medical Centre.

Next-Generation Sequencing

Whole-genome or exome sequencing was performed on 113 CMM families from Australia, UK, the Netherlands, Denmark, and Sweden. Between one and five case patients were sequenced from each family, totaling 204 individuals. Supplementary Table 1 (available online) details samples by center. Supplementary Table 2 (available online) summarizes ages of CMM onset and other cancers in individuals who were

sequenced. Sequencing was performed on the Illumina Hiseq 2000 platform with Agilent (CA, USA) SureSelect Human All Exon V4+UTRs enrichment kits. Paired-end reads of 75 to 100 bp were generated, with mean coverage of 60 to 96X. The Burrows-Wheeler Aligner was used to map reads to the UCSC hg19 reference sequence (11). UK and Leiden samples were filtered for duplicate reads using Picard (12), recalibrated, and aligned using Genome Analysis Tool Kit (13). Single nucleotide polymorphisms (SNPs) and indels were annotated using bcftools and SAMtools mpileup with disabled BAQ computation (14). Data were filtered using: quality score over 40, alternate reads over two, and alternate reads of 20% or more of total reads. Variants in dbSNP135 or the 1000 Genomes Project (April 2012) were removed.

Targeted sequencing of the shelterin genes was carried out in 397 QFMP probands (Supplementary Table 3) using two Ampliseq panels. The first panel (105 amplicons; 10202 bp) included the coding regions and had 100% coverage, except *TERF1* (96%). The second panel (68 amplicons; 7723 bp) was comprised of untranslated regions (UTRs), promoters, and alternative exons. Libraries were barcoded and run on an Ion personal genome machine (PGM) using 318 chips, with minimum 30X coverage. Sequence reads were processed using the Ion Torrent Suite (Life Technologies, CA, USA) with alignment and variants called as above. Variants were filtered to exclude those listed in dbSNP, the 1000 Genomes Project, Kaviar, and synonymous changes. Variants had to have a quality score of 30 or more and 10 or more alternate reads.

Sanger Sequencing

Sanger sequencing was used to confirm variants found by next-generation sequencing. Primers are listed in Supplementary Table 4.

Agena iPLEX

Seven variants (*ACD*: p.N249S, p.A200T, p.Q320X, p.I322F; *TERF2IP*: p.M5I, p.D10H, p.R364X) were genotyped in an Australian case-control sample. Case patients (n = 1669) were derived from the QFMP (8,15), and the control individuals (n = 1590) were parents of twins ascertained in the Brisbane Twin Naevus Study (BTNS) (16). The control individuals self-reported their melanoma history and had not developed CMM at the time of sample collection. The Agena iPLEX gold system (Agena Bioscience, CA) was used to genotype the variants; primers were designed using Assay Design Suite (Supplementary Table 4, available online).

Analysis of Novel Variants in Shelterin Genes in Control Exomes

Publicly available exome data from 1965 Danish individuals (17) or a subset of exomes from the UK10K sequencing project (http://www.uk10k.org, accessed on February 13, 2014) or the European-American samples lodged in the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) database (http://evs.gs.washington.edu/EVS/; accessed on February 17, 2014) were also used as control data.

Statistical Analyses

Maximum Likelihood Analysis
The age of the founder mutation was estimated using a statistical model described by Neuhausen and colleagues (18). Assuming 1 cM equals 1 Mb and a de novo mutation rate of

1.2 x 10^{-8} per generation ([19]), a joint likelihood of the genotype data was calculated taking into account ancestral haplotype, number of generations, G, since the ancestor, and allele frequencies of the SNPs among the European population. An estimate of G was calculated that maximized the likelihood. A confidence interval was calculated by finding the range of values of G that yielded a likelihood of at least one tenth of the maximum likelihood.

LOD Score Analysis

An autosomal dominant model was used to generate the LOD score for families carrying the *ACD* p.N249S variant. Parametric linkage analysis was done using the Genehunter MOD score algorithm. Penetrance was specified as 5%, 95%, and 95% for the three genotype classes, with allele frequency at the disease and marker locus specified as 0.001.

Mutation Clustering

Chi-squared tests were used to determine whether mutations were statistically significantly enriched in melanoma case patients versus control individuals and whether they clustered more often in the POT1 binding domain of ACD. Fisher's Exact test was used where any value was less than five. A P value of less than .05 was considered statistically significant (one-tailed with Yates' correction).

Results

Novel Germline *ACD* and *TERF2IP* Mutations in Melanoma

Six families had mutations in *ACD*, and four families carried *TERF2IP* variants. Segregating nonsense mutations in *ACD* and *TERF2IP* were found in a five-case and a four-case family, respectively. The p.Q320X mutation in *ACD*, in family AUS1, was present in all four case patients available for genotyping ([Figure 1]) and results in a truncated protein, disrupting the POT1 binding domain and eliminating the TINF2 binding domain downstream ([Figure 2A]). Three CMM case patients in family UK1 were carriers of a p.R364X nonsense mutation in *TERF2IP* ([Figure 1]). This results in truncation of the protein 36 amino acids from the C-terminus, disrupting the TERF2 binding domain ([Figure 2A]).

In addition, we found novel (not in dbSNP135 or the 1000 Genomes Project data at the time of analysis) missense mutations in *ACD* and *TERF2IP* that cosegregated in all available invasive CMM case patients in four other families ([Table 1], [Figure 1]). This included a p.N249S mutation in *ACD* in two separate families, which, like the p.Q320X mutation, also occurs in the POT1 binding domain ([Figure 2A]). This mutation segregated in all seven available case patients of AUS2, with eight confirmed and four unconfirmed cases of CMM. Danish family, DK1, which harbored the same mutation ([Figure 1]),
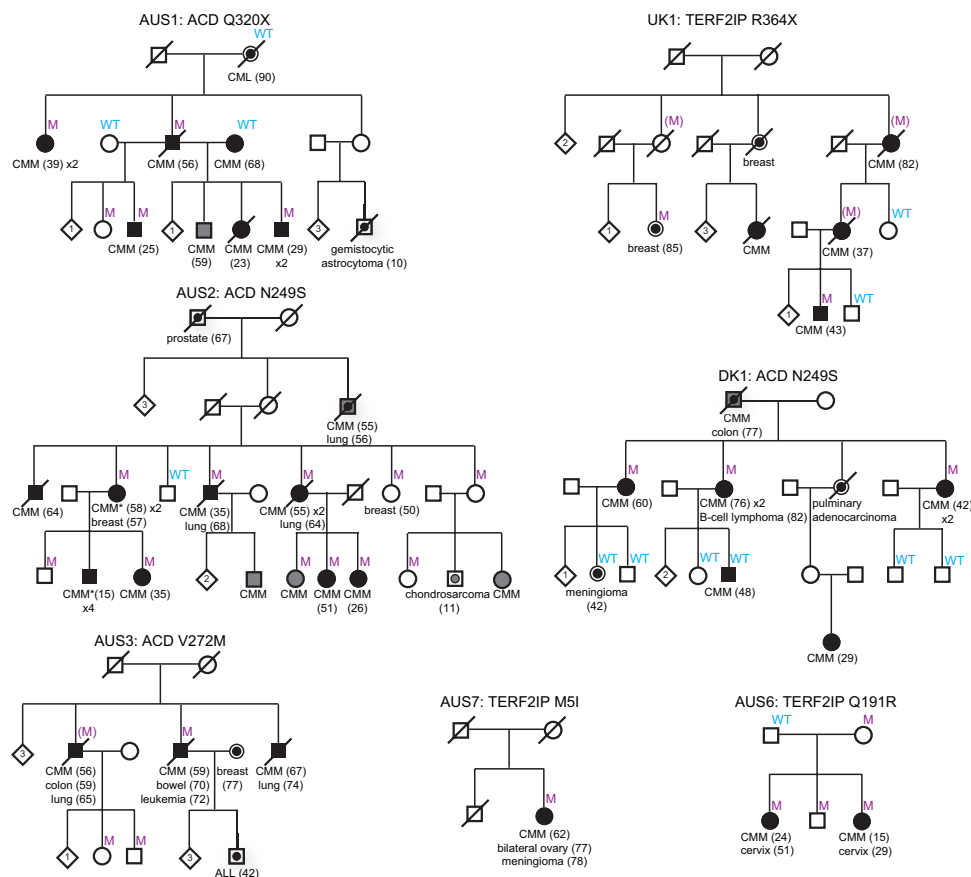
Figure 1. Mutations in *ACD* and *TERF2IP* that segregate with melanoma. The age at first diagnosis of cutaneous malignant melanoma (CMM) is indicated in **brackets**. If the individual has had more than one primary melanoma, the first age at onset is annotated and the total number of CMMs is given. A line through a symbol indicates that the individual is deceased. Individuals carrying a mutation are indicated by "M," while family members that are wild-type for the indicated variant are annotated "WT." "(M)" indicates an individual is an obligate carrier. **Square symbols** indicate males and **circles** females. **Black symbols** represent confirmed CMM case patients and **gray symbols** unconfirmed case patients. Symbols with a **central black circle** represent individuals with a confirmed cancer other than CMM. Those family members with other unconfirmed cancers are indicated by symbols containing a **central gray circle**. Unaffected siblings are indicated by a **diamond** with the number of siblings shown in the center of the symbol. *ACD* = adrenocortical dysplasia protein homolog; *TERF2IP* = telomeric repeat binding factor 2 interacting protein.
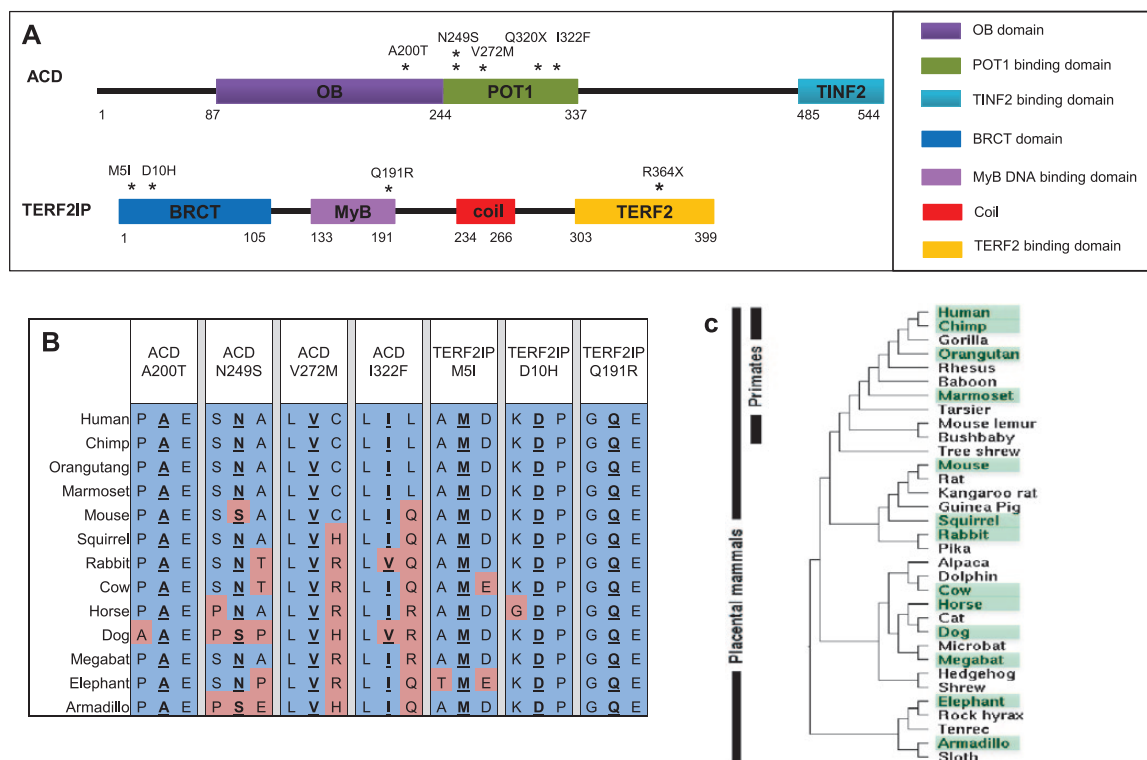
Figure 2. Relative location of germline variants in melanoma case patients with respect to adrenocortical dysplasia protein homolog (ACD) and TERF2IP protein domains (**A**) and conservation of ACD and TERF2IP variants in melanoma families across placental mammals (**B**) and spanning species clades (**C**). *ACD* = adrenocortical dysplasia protein homolog; *TERF2IP* = telomeric repeat binding factor 2 interacting protein.

presented with five confirmed cases and one unconfirmed case of melanoma. Of the individuals available for analysis, three invasive CMM case patients were found to harbor this mutation; a person diagnosed with a melanoma in situ was not a carrier. A second novel mutation, which cosegregated with all three melanoma case patients in family AUS3, was identified in the POT1 binding domain of ACD (p.V272M). Unfortunately, as the crystal structure of the POT1 binding domain of ACD, containing the p.N249 and the p.V272 residues, has not been resolved, there is insufficient information for in silico modeling of the effects of these variants. Another completely cosegregating mutation we discovered was in the MyB domain of TERF2IP (p.Q191R) in a two-case melanoma family (Table 1, Figure 1).

Additionally, four novel variants were observed in *ACD* or *TERF2IP*, which did not fully segregate with all melanoma case patients in the respective families (Supplementary Figure 2A, available online). These included a p.A200T substitution within the OB domain of ACD, a p.I322F substitution within the POT1 binding domain of ACD, as well as p.M5I and p.D10H substitutions, both of which lie in the BRCT domain of TERF2IP (Figure 1; Supplementary Figure 2A, available online). All of the cosegregating missense mutations in *ACD* and *TERF2IP* occur at highly evolutionarily conserved sites across species (Figure 2, B and C).

Prevalence, Linkage, and Haplotype Analysis of the ACD p.N249S Mutation

The ACD p.N249S variant was the only novel mutation at that time of initial analysis of our exome data that was subsequently observed in the ESP database, albeit exceedingly rarely (3/8600

European-American chromosomes). We therefore additionally screened an Australian population-based case-control panel for this variant and did not see it in any of 1669 case patients or 1590 control individuals; it has also not been seen in the 1000 Genomes Project data, or in the exomes from approximately 1000 Danish diabetes case patients and approximately 1000 metabolically healthy control individuals (17).

Linkage analysis of the two families segregating the ACD p.N249S variant gave a combined LOD score of 1.14, which equates to a *P* value of .011. Analysis of a possible common founder in these families was carried out using whole-genome (hg19 reference genome) SNP arrays. Each of the carriers tested shared an allele for 112 SNPs, stretching from rs12918121 (chr16:67187795) to rs16957597 (chr16:67946356), a region 758561bp long, spanning *ACD* (Supplementary Table 5, available online). The data are thus consistent with all affected individuals sharing the same haplotype. Estimates of when the mutation arose gave a maximum likelihood for 129 generations ago (with a 90% confidence interval of 28 to 362 generations).

ACD and *TERF2IP* Variants in Control individuals

To further assess the association between *ACD* and *TERF2IP* variants and familial melanoma susceptibility, we screened 6785 publicly available control exomes for rare (variant allele frequency [VAF] < 0.001) variants in these genes. Control individuals included the ESP cohort (n = 4300), Danish control individuals (n = 1965) (17), and the UK10K sequencing project (http://www.uk10k.org, accessed February 13, 2014; n = 520). *ACD* and *TERF2IP* variants in these control cohorts are listed in Supplementary Tables 6–8 (available online) and the relative positions with respect to protein domains given in

Table 1. Germline mutations in the ACD and TERF2IP genes identified in melanoma families by exome sequencing

Family ID	Chr	Start*	Gene	cDNA change	Protein change	mRNA refSeq	Protein refSeq	Case patients in family	Genotyped case patients who are carriers†	VAF ESP6500‡	Polyphen2 prediction§	SIFT prediction§	GERP
AUS1	16	67692665	ACD	c.958C>T	p.Q320X	NM_001082486	NP_001075955.1	5	4/4	0	(truncating)	(truncating)	2.28
AUS2	16	67693137	ACD	c.746A>G	p.N249S	NM_001082486	NP_001075955.1	5	7/7	3/8597	benign	tolerated	-9.69
DK1	16	67693137	ACD	c.746A>G	p.N249S	NM_001082486	NP_001075955.1	4	3/4	3/8597	benign	tolerated	-9.69
AUS3	16	67692920	ACD	c.814G>A	p.V272M	NM_001082486	NP_001075955.1	3	2/2	0	probably damaging	damaging	3.44
AUS4	16	67693513	ACD	c.598G>A	p.A200T	NM_001082486	NP_001075955.1	6	4/6	0	probably damaging	damaging	3.4
AUS5	16	67692659	ACD	c.964A>T	p.I322F	NM_001082486	NP_001075955.1	5	3/4	0	possibly damaging	damaging	0.87
UK1	16	75690399	TERF2IP	c.1090C>T	p.R364X	NM_018975	NP_061848.2	4	3/3	0	(truncating)	(truncating)	4.88
AUS6	16	75682352	TERF2IP	c.572A>G	p.Q191R	NM_018975	NP_061848.2	2	2/2	0	benign	tolerated	4.62
SE1	16	75681808	TERF2IP	c.28G>C	p.D10H	NM_018975	NP_061848.2	5	1/3	0	probably damaging	damaging	5.75
AUS7	16	75681795	TERF2IP	c.15G>A	p.M5I	NM_018975	NP_061848.2	1	1/1	0	benign	damaging	2.55

* Genome build GRCh37, hg19. GERP = Genomic Evolutionary Rate Profiling score.
† Includes obligate carriers.
‡ European American population.
§ Effect of nonsense mutations not predicted by either Polyphen2 or SIFT are annotated as truncating.

Supplementary Figure 3 (available online). Taking all *ACD* variants with a VAF under 0.001, we found statistically significant enrichment in melanoma case patients vs control individuals (6/510 melanoma probands vs 33/6785 control individuals; $P = .040$). Moreover, if only novel *ACD* variants were considered, the association with melanoma was stronger (6/510 melanoma probands vs 8/2485 control individuals [note ESP control individuals were not included because by definition variants in the ESP were not considered novel]; $P = .013$). The most statistically significant association was found when we only considered variants with VAFs under 0.001 that occurred in the POT1 binding domain; five of 510 CMM probands vs 16 of 6785 control individuals ($P = .005$).

No novel or rare variants were observed in *TERF2IP* in the 520 UK10K samples or the 1965 Danish control individuals. There were eight variants (12 individuals) listed amongst the 4300 European-American ESP control individuals. Thus there was statistically significant enrichment ($P = .022$) of novel plus rare (VAF < 0.001) *TERF2IP* variants in melanoma probands (4/510) vs control individuals (12/6785).

Lastly, to assess the potential contribution of *ACD* and *TERF2IP* to sporadic melanoma, we conducted a case-control analysis for a total of six variants (other than *ACD* p.N249S reported above) in these two genes (*ACD*: p.A200T, p.Q320X, p.I322F; *TERF2IP*: p.M5I, p.D10H, p.R364X) and did not observe any other individual that carried one of these variants amongst 1669 QFMP case patients and 1590 BTNS control individuals.

Germline Variants in Other Shelterin Genes

Novel variants were also observed in *TERF1*, *TERF2*, and *TINF2* (Supplementary Table 9, available online), but these were not convincingly associated with melanoma (see Supplementary Results, available online). Supplementary Table 10 (available online) lists incidences of all cancers in families carrying shelterin mutations.

Discussion

The aim of this study was to screen individuals with strong personal or family histories of CMM who do not carry a mutation in one of the known high-penetrance melanoma risk genes, for germline mutations in *ACD, TERF1, TERF2, TERF2IP,* and *TINF2*. We identified ten mutations in *ACD/TERF2IP*, including two fully segregating nonsense mutations and four novel cosegregating missense mutations.

ACD, in a subunit with POT1, mediates the interaction between shelterin and TERT through its oligonucleotide/oligosaccharide-binding folds (OB-folds) (Figure 2A) (20). When the ACD/POT1 subunit is inhibited, the telomerase complex increases telomere length, indicating that this subcomplex is required to inhibit the elongation of chromosome ends (21–25). Additionally, within the shelterin complex, ACD links POT1 to other members (Supplementary Figure 1, available online) and increases the affinity of POT1 for telomeric single stranded DNA (26). We identified a nonsense mutation in ACD, p.Q320X, which disrupts the POT1 binding domain and abolishes the TINF2 binding domain, so would therefore be predicted to result in an unformed shelterin complex. Individuals in this family presented with early onset melanoma at: age 23, 25, 29, 39, and 56 years (Figure 1). The p.N249S mutation in ACD was identified in two families, from Australia and Denmark, which shared a large founder haplotype across the ACD locus. A cosegregating ACD p.V272M mutation was identified in a lower-density

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melanoma family (Figure 1). Two *ACD* mutations were identified that did not fully segregate with all CMM case patients in the family; p.A200T was found in four of seven case patients that could be tested from an eight-case family and p.I322F was found in three of four available case patients from a six-case family (Supplementary Figure 2A, available online). Missense mutations p.A200T, p.V272M, and p.I322F were predicted to be damaging or possibly damaging by the Polyphen2 and SIFT prediction programs (Table 1). Overall, of the five distinct mutations in ACD, four clustered in highly conserved residues in the POT1 binding domain (Figure 2A) and are enriched for occurrence in melanoma case patients compared with control individuals (P = .005), indicating this domain plays an important role in melanoma susceptibility.

TERF2IP associates with the shelterin complex via its C-terminus to a central region of TERF2, forming a stable 1:1 complex. TERF2IP, as part of the shelterin complex, is vital for the repression of homology-directed repair of double strand chromosomal break at the telomere (27). *TERF2IP* p.R364X results in premature truncation of the protein 36 amino acids from the C-terminus, resulting in disruption of the TERF2-binding domain and is therefore predicted to result in a loss of binding to the shelterin complex. Of the three novel missense variants observed in *TERF2IP*, p.Q191R was found in the two case patients in family AUS6, who both developed CMM at an early age (15 and 24 years) and p.D10H was predicted to be damaging/probably damaging by Polyphen2 and SIFT (Table 1); all three missense mutations occurred at highly conserved amino acid residues (Figure 2, B and C).

Many families harboring mutations in *ACD/TERF2IP* included members with multiple primary melanomas (MPMs) and other cancer types (Figure 1; Supplementary Figure 2A, available online). The *TERF2IP* nonsense mutation, in family UK1, was found in an individual without CMM, but who developed breast cancer at age 85. Family AUS2, harboring the ACD p.N249S mutation, included six individuals with MPM and/or early onset melanoma (age 15, 26, and 35 years); four CMM case patients also developed other cancers (three lung and one breast), and a mutation carrier without CMM developed breast cancer at age 50 years. In family DK1, harboring ACD p.N249S, two mutation carriers developed MPM, one of whom also developed B-cell lymphoma at age 82 years. The ACD p.V272M mutation occurred in a family (AUS3) with three case patients of CMM, all of whom developed other cancers; two mutation carriers developed three different primary cancers: CMM, colon, and lung or CMM, bowel, and leukemia. The two TERF2IP p.Q191R carriers in family AUS6 had both CMM and cervical cancer. The p.M5I missense mutation occurred in a sporadic melanoma case, with bilateral ovarian cancer at 77 years, and meningioma at age 78 years. Two ACD p.A200T carriers had CMM and prostate cancer, and two other carriers had MPM. Finally, a carrier of the ACD p.I322F mutation in family AUS5 had MPM. Taken together, these data are strongly suggestive that mutations in *ACD* and *TERF2IP* are associated with early onset CMM and MPM and may predispose to a broader spectrum of cancers than just melanoma. Limitations of this study are that numbers of these other tumor types are too low to determine whether they are robustly associated with germline mutations in *ACD* and *TERF2IP* and that tumor blocks were not available on these cancers, or melanomas, to determine whether loss of heterozygosity is required for tumorigenesis.

In summary, the loss-of-function mutations we report here in *ACD* and *TERF2IP*, along with those previously published in *POT1*, suggest that multiple components of the shelterin complex play a role in melanoma predisposition. Collectively, mutations in *ACD, TERF2IP,* and *POT1* account for about 9% (12/132) of high-density melanoma families (≥ 3 CMM case patients) lacking mutations in *CDKN2A, CDK4, TERT*, and *BAP1*. Given that the shelterin complex directly interacts with the product of *TERT*, a recently reported melanoma predisposition gene, the evidence we document here indicates that dysregulated telomere maintenance is a key pathway controlling melanoma development.

Notes

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