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REPORT

TINF2 is a major susceptibility gene in Danish patients with multiple primary melanoma

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Summary

TINF2 encodes the TINF2 protein, which is a subunit in the shelterin complex critical for telomere regulation. Three recent studies have associated six truncating germline variants in *TINF2* that have previously been associated with a cancer predisposition syndrome (CPS) caused by elongation of the telomeres. This has added *TINF2* to the *long telomere syndrome* genes, together with other telomere maintenance genes such as *ACD*, *POT1*, *TERF2IP*, and *TERT*.

We report a clinical study of 102 Danish patients with multiple primary melanoma (MPM) in which a germline truncating variant in *TINF2* (p.(Arg265Ter)) was identified in four unrelated participants. The telomere lengths of three variant carriers were >90% percentile. In a routine diagnostic setting, the variant was identified in two more families, including an additional MPM patient and monozygotic twins with thyroid cancer and other cancer types. A total of 10 individuals from six independent families were confirmed carriers, all with cancer history, predominantly melanoma. Our findings suggest a major role of *TINF2* in Danish patients with MPM.

In addition to melanoma, other cancers in the six families include thyroid, renal, breast, and sarcoma, supporting a CPS in which melanoma, thyroid cancer, and sarcoma predominate. Further studies are needed to establish the full spectrum of associated cancer types and characterize lifetime cancer risk in carriers.

High-risk melanoma susceptibility genes include *CDKN2A* (MIM: 600160), *CDK4* (MIM: 123829), and *BAP1* (MIM: 603089), all genes involved in cell cycle regulation; however, *BAP1* is also involved in regulation of DNA damage response, cell senescence, and apoptosis, as well as the more recently discovered *TERT* (MIM: 187270), *POT1* (MIM: 606478), *ACD* (MIM: 609377), and *TERF2IP* (MIM: 605061) genes involved in maintaining telomeres. The most frequently mutated high-risk gene is *CDKN2A*, where pathogenic variants are observed in 11% of families with $\geq$3 melanoma cases in a previous Danish study,[1] while variants in other high-risk genes are rare.[2–8]

Moderate-risk genes include *MC1R* (MIM: 155555) and *MITF* (MIM: 156845). R-alleles of *MC1R,* strongly associated with fair skin, blue eyes, and red hair, are common in the Danish population (minor-allele frequency ~0.2) and confer a per allele risk of ~2 for melanoma.[9] The *MITF* p.(Glu318Lys) variant has been associated with a 2- to 3-fold increased risk of melanoma and renal cell carcinoma (RCC).[10,11] Numerous low-risk melanoma genes have been identified including pigmentation genes such as *ASIP* (MIM: 600201), *OCA2* (MIM: 611409), *IRF4* (MIM: 601900), *TYRP1* (MIM: 115501), and *TYR* (MIM: 606933).[12]

The recent discovery of *TERT*, *POT1*, *ACD*, and *TERF2IP* as high-risk melanoma genes suggests that disruption of telomere maintenance may be a key mechanism of melanoma predisposition.

The telomeres are regions at the end of the chromosomes consisting of repetitive TTAGGG hexamers synthesized and added to the telomeres by telomerase.[13] Low abundance and low activity of telomerase ensure a tight regulation of telomere synthesis. An important regulator of telomerase activity is the shelterin complex,[14] consisting of several protein subunits: TERF1 (telomeric repeat binding factor 1), TERF2 (telomeric repeat binding factor 2), TINF2 (TERF1-interacting nuclear factor 2), TERF2IP (telomeric repeat binding factor 2 interacting protein), ACD (adrenocortical dysplasia protein homolog), and POT1 (protection of telomeres 1). This regulation ensures that telomere synthesis mainly occurs in early embryonal development in somatic cells with subsequent repression of TERT (telomerase reverse transcriptase) in adult cells.[15] Thus, after elongation during embryogenesis, telomeres gradually shorten during each cycle of DNA replication.[13] Some adult tissues, including stem cells, continue to express telomerase, however still have telomere shortening.[16]

This default telomere shortening in cells constitutes a tumor suppressor mechanism, as cells with too short telomeres reach senescence or go into apoptosis (the Hayflick limit) by activation of a DNA damage response.[17] Initially, long telomeres thus increase cancer risk by delaying the Hayflick limit, permitting excessive proliferation that might drive tumorigenesis.[18]

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In addition to telomere length regulation, the shelterin complex maintains several complex functions in telomere maintenance, such as telomere protection, in which the complex allows the cell to distinguish telomere ends from DNA breaks.[13]

Consequently, it has been unclear whether the cancer predisposition associated with *TERT*, *POT1*, *ACD*, and *TERF2IP* is mediated by long telomeres. For example, reviewed in Schmutz et al. 2020[18] and Gong et al. 2021,[19] *POT1* cancer-predisposing variants have, in addition to creating long telomeres,[4,6] been shown to result in genomic instability,[20–23] which has therefore been suggested as the main mechanism driving cancer development.[18] However, evidence has arisen of an increased cancer risk conferred by overelongation of telomeres in early development, thus linking *TERT*, *POT1*, *ACD*, and *TERF2IP* to *long telomere syndrome*. This evidence, consisting of *in vitro* studies as well as epidemiologic studies including GWAS and familial cases, has been reviewed elsewhere.[14,18]

Three recent studies[18,24,25] have linked six germline truncating variants of *TINF2,* located on chromosome 14q12 (MIM: 604319), with high-penetrance cancer predisposition.

He et al.[24] describe a *TINF2* frameshift variant (c.591del, p.(Trp198GlyfsTer12)) leading to a premature stop codon in a family of multiple cases or melanoma; however a wide melanoma, with complete segregation of the variant in affected individuals. Schmutz et al.[18] have identified a frameshift variant (c.557del, p.(Ser186PhefsTer24)) with introduction of a premature stop codon and a variant (c.604G>C, p.(Glu202Gln)), which disrupt the splice donor site of exon 5 resulting in frameshifts and predicted truncations (p.(Leu170ValfsTer12) and p.(Glu202GlyfsTer14)) in a total of four cancer-prone families. These families all have cases or melanoma; however a wide/or melanoma; however a wide range of other cancers and benign tumors are present as well, including a mantle cell lymphoma, two brain tumors (e.g., a cerebellar subependymoma and a diffuse astrocytoma), a tenosynovial giant cell tumor, and several cases of the common cancers: breast, colorectal, lung, and prostate cancer.

Ballinger et al.[25] describe three germline truncating *TINF2* variants (p.(Val67TrpfsTer3), p.(Arg256Ter) and p.Arg265Ter)), one variant in each of three patients with history of undifferentiated pleomorphic sarcoma (one patient) or liposarcoma (two patients) in a large population-based, case control study of 1,644 sarcoma patients. Telomere length analysis of variant carriers in the five families described by He et al.[24] and Schmutz et al.[18] show long telomeres in peripheral blood lymphocytes compared to healthy controls and in the family of He et al.[24] compared to wild-type relatives as well. Schmutz et al.[18] further show telomere overelongation in clonal cell lines with heterozygous knockin of their two variants. Thus, these results argue that truncating *TINF2* variants can lead to long telomeres.

Similarly, Ballinger et al.[25] find that relative telomere length in leukocytes of sarcoma patients is longer in carriers of variants in the shelterin complex genes; however this is not specified for the *TINF2* variant carriers.

Here, we describe a truncating germline *TINF2* variant (NM_001099274.3(TINF2): c.793C>T, p.(Arg265Ter)), identified in six Danish independent families with a history of melanoma. The variant is found to be associated with long telomeres.

102 patients with multiple primary melanoma (MPM), defined as greater than or equal to three cutaneous melanomas (including *in situ* melanomas), participated in a clinical study in the years 2021–2022 at the Department of Clinical Genetics at Rigshospitalet, Denmark (see supplemental methods and Figure S1). Participation involved whole exome sequencing (WES) of lymphocyte DNA to examine potential cancer-predisposing variants, drawing of a three-generation pedigree, and a detailed questionnaire regarding skin type, sun exposure, sun bed use, and tobacco use. Forty (39%) patients had previously received genetic counseling due to their history of melanoma, and 34 (33%) had previous genetic tests including as a minimum *CDKN2A* and *CDK4*.

WES data from all patients were analyzed by an *in silico* gene panel consisting of 390 cancer-related genes (Table S2) and showed no high-risk melanoma gene single nucleotide variants (SNVs) or structural variants (SVs). Two patients (2%) carried the *MITF* moderate-risk variant p.(Glu318Lys), and 74 (73%) patients carried R-alleles of *MC1R*; 32 had two R-alleles, 42 one R-allele, resulting in an allele frequency (AF) 2.6 times the background population (AF = 0.52, OR = 2.60).[1] Nine patients (8.8%) carried a heterozygous pathogenic or likely pathogenic germline SNV in a cancer-related gene other than *MC1R* and *MITF*: *ATM* (MIM: 607585), *BRCA1* (MIM: 113705), *CHEK2* (MIM: 604373), *FANCM* (MIM: 609644), *NTHL1* (MIM: 602656), *PALB2* (MIM: 610355), and *TP53* (MIM: 191170) (Table 1). 14 patients (13.7%) had one or two variants of unknown significance (VUSs) identified, with a total of 19 VUSs (Table S1).

In four study participants we identified the *TINF2* nonsense p.(Arg265Ter) variant. No other cancer-predisposing SNVs or SVs were identified in these individuals, except for one participant (proband family F4) carrying two *MC1R* R-alleles (p.(Arg151Cys) and p.(Arg160Trp)). Another participant (proband family F1) had an *MC1R* r-allele (p.(Val60Leu) identified. AF values of R-alleles and r-alleles were thus lower in the four study participants carrying the *TINF2* variant (i.e., 0.25 and 0.13, respectively) compared to the remaining MPM study participants (i.e., 0.54 and 0.18, respectively). Additionally, three individuals with cancer (two of whom are monozygotic [MZ] twins) had the same *TINF2* variant identified via routine diagnostic testing while the clinical study was running. One patient with MPM, who at the time of the study inclusion did not fulfill the inclusion criteria, had the variant identified by WES, while the MZ twins had a clinical cancer gene panel (42 genes, view Table S3) performed, detecting no pathogenic variants, followed by whole-genome sequencing (WGS) of one twin,

Table 1. Pathogenic and likely pathogenic variants in cancer-related genes identified in study participants

Gene	Variant identified[a]	Associated cancers
ATM	NM_000051.3:c.538C>T, NP_000042.3:p.(Gln180Ter)	homozygous/compound heterozygous form: ataxia-telangiectasia; heterozygous form: breast cancer (Girard et al.[26]), melanoma (Dalmasso et al.[27]), possibly pancreatic cancer and colorectal cancer (West et al.[28])
	NM_000051.3:c.1564_1565delGA, NP_000042.3:p.(Glu522IlefsTer43)	
BRCA1	NM_007294.3:c.5089T>C, NP_009225.1:p.(Cys1697Arg)[b]	breast cancer and ovarian cancer (Kuchenbaecker et al.[29]) and lower risk of pancreatic cancer, prostate cancer, and stomach cancer (Cavanagh et al.[30])
	NM_007294.3:c.1556delA, NP_009225.1:p.(Lys519ArgfsTer13)[b]	
CHEK2	NM_007194.4:c.1100del, NP_009125.1:p.(Thr367MetfsTer15)	breast cancer and prostate cancer, possibly renal cancer, colorectal cancer, and thyroid cancer (West el al.,[28] Cybulski et al; 33[31]) and melanoma (Bui et al.[32])
FANCM	NM_020937.4:c.1972C>T, NP_065988.1:p.(Arg658Ter)	in homozygous form: breast cancer (Catucci et al.[33]); in heterozygous form: breast cancer (Neidhardt et al.[34] Figlioli et al.[35]) and likely ovarian cancer (Dicks et al.[36])
MC1R	NM_002386.3:c.252C>A, NP_002377.4:p.(Asp84Glu)	melanoma (Raimondi et al.[9])
	NM_002386.3:c.451C>T, NP_002377.4:p.(Arg151Cys)	
	NM_002386.3:c.478C>T, NP_002377.4:p.(Arg160Trp)	
	NM_002386.3:c.880G>C, NP_002377.4:p.(Asp294His)	
	NM_002386.3:c.425G>A, NP_002377.4:p.(Arg142His)	
	NM_002386.3:c.487C>T, NP_002377.4:p.(Arg163Ter)	
	NM_002386.3:c.86dupA, NP_002377.4:p.(Asn29LysfsTer14)	
	NM_002386.3:c.537dupC, NP_002377.4:p.(Ile180HisfsTer59)	
MITF	NM_000248.3:c.952G>A, NP_000239.1:p.(Glu318Lys)	melanoma, renal cell carcinoma (Bertolotto et al.[11] Yokoyama et al.[10])

(*Continued on next page*)

Table 1. ***Continued***

Gene	Variant identified[a]	Associated cancers
NTHL1	NM_002528.6:c.268C>T, NP_002519.1:p.(Gln90Ter)	colorectal cancer and breast cancer in homozygous or compound heterozygous individuals (Beck et al.[37])
PALB2	NM_024675.3:c.760_761delTC, NP_078951.2:p.(Ser254ArgfsTer2)	breast cancer, pancreatic cancer, ovarian cancer (Yang et al.[38])
TINF2	NM_001099274.1:c.793C>T, NP_001092744.1:p.(Arg265Ter)	melanoma and thyroid cancer (Schmutz et al.,[18] He et al.[24]), dyskeratosis congenita related cancer (Alter et al.[39])
TP53	NM_000546.5:c.542G>A, NP_000537.3:p.(Arg181His)[c]	Adrenocortical carcinoma, breast cancer, brain tumors, and sarcomas (Frebourg et al.[40]), however, also risk of other cancers including melanoma (Sandru et al.[41])

[a]All variants were identified heterozygous in one study participant, respectively, except *MITF* p.Glu318Lys (two heterozygous carriers) and *MC1R* R-alleles (view Table S3 for details).
[b]Known prior to inclusion.
[c]Classified as likely pathogenic.

Table 2. Clinical characteristics of variant carriers in the six families and first-degree relatives with history of cancer

Family	Individual	Malignancies and histo-subtypes if available (age at diagnosis)	Tumor unknown benign/ malignant (age at diagnosis)	Variant status
F1	proband	4 CMs: all SSMM (34 y, 35 y, 41 y, 44 y)	melanocytic skin tumor (50 y)	p.(Arg265Ter)
	mother	breast cancer (not verified)		
F2	proband	2 CMs: both SSMM (25 y), 2 CMs *in situ*: both SSMMIS (25 y, 26 y)		p.(Arg265Ter)
	father	Prostate cancer (60 y), chromophobe RCC grade 3 (60 y), leiomyosarcoma grade 3 (67 y)		p.(Arg265Ter)
	paternal grandfather[a]	multiple myeloma (75 y), lung adenocarcinoma (77 y)		
F3	proband	3 CMs: all SSMM (46, 47, 48 y), 1 CM *in situ*: SSMMIS (46 y)		p.(Arg265Ter)
	sister	CM: SSMM (67 y)		p.(Arg265Ter)
	mother[a]	breast cancer: IDC (58 y), carcinoma cells on breast skin (61 y) perhaps metastasis		
F4	proband	4 CMs: 1 nodular CM, 3 SSMMs (36 y, 38 y, 47 y, 48 y), 1 CM *in situ*: SSMMIS (42 y)		p.(Arg265Ter)
	mother	colorectal cancer (not verified)		
	father[a]	myxoid sarcoma grade 2 (74 y)		
F5	proband	3 CM *in situ*: 1 not classified, 1 likely SSMMIS, 1 lentigo maligna (22 y, 28 y, 41 y) 1 CM: SSMM (51 y)		p.(Arg265Ter)
	brother	CM: SSMM (28 y)		
F6	one MZ twin	thyroid cancer: both papillary and follicular adenocarcinoma (29 y), clear cell RCC (60 y), CM *in situ*: SSMMIS (61 y)		p.(Arg265Ter)
	other MZ twin	thyroid cancer: papillary adenocarcinoma (58 y)		p.(Arg265Ter)
	mother[a]	adenocarcinoma of unknown primary (likely lung, breast, or genitalia) identified in the femoral bone (56 y)		obligate p.(Arg265Ter) carrier (not tested)[b]

Abbreviations: CM, cutaneous melanoma, IDC, invasive ductal carcinoma, RCC, renal cell carcinoma, SSMM, superficially spreading malignant melanoma, SSMMIS, superficially spreading malignant melanoma *in situ*.
[a]Deceased.
[b]A relative of the mother (not shown) was confirmed as variant carrier of p.(Arg265Ter); thus, the mother is an obligate variant carrier.

identifying the *TINF2* variant. No other cancer-predisposing SNVs or SVs were identified in the three patients (however, *MC1R* variants were not evaluated in WGS of the twin).

TINF2 variant carriers from the study cohort all had four to five invasive or *in situ* cutanous melanomas (CMs) (see Table 2). One of the additional three patients clinically identified had history of three *in situ* CMs and one invasive CM, and the last two are the MZ twins, both of whom have had thyroid cancer, and one twin had RCC and CM *in situ* as well. One *TINF2* variant carrier from the study cohort (proband Family F2) had two invasive CMs evaluated for the *BRAF* variants V600E/V600D and V600K/V600R/V600M, showing one CM with the variant V600E or V600D and one CM without the variants.

The five *TINF2* variant carriers with MPM all reported having many nevi. Further, pathological reports show numerous excised nevi clinically suspected of CM in all five individuals; one individual had at least 40 excised non-malignant nevi. Four carriers reported having had skin tumors removed solely on their request, where pathology results showed melanoma, which may suggest an atypical presentation of melanomas.

The fifth *TINF2* patient had an unusual clinical presentation. At 28 years, she was diagnosed with an intraspinal extramedullary meningioma in the region C4-C5. After

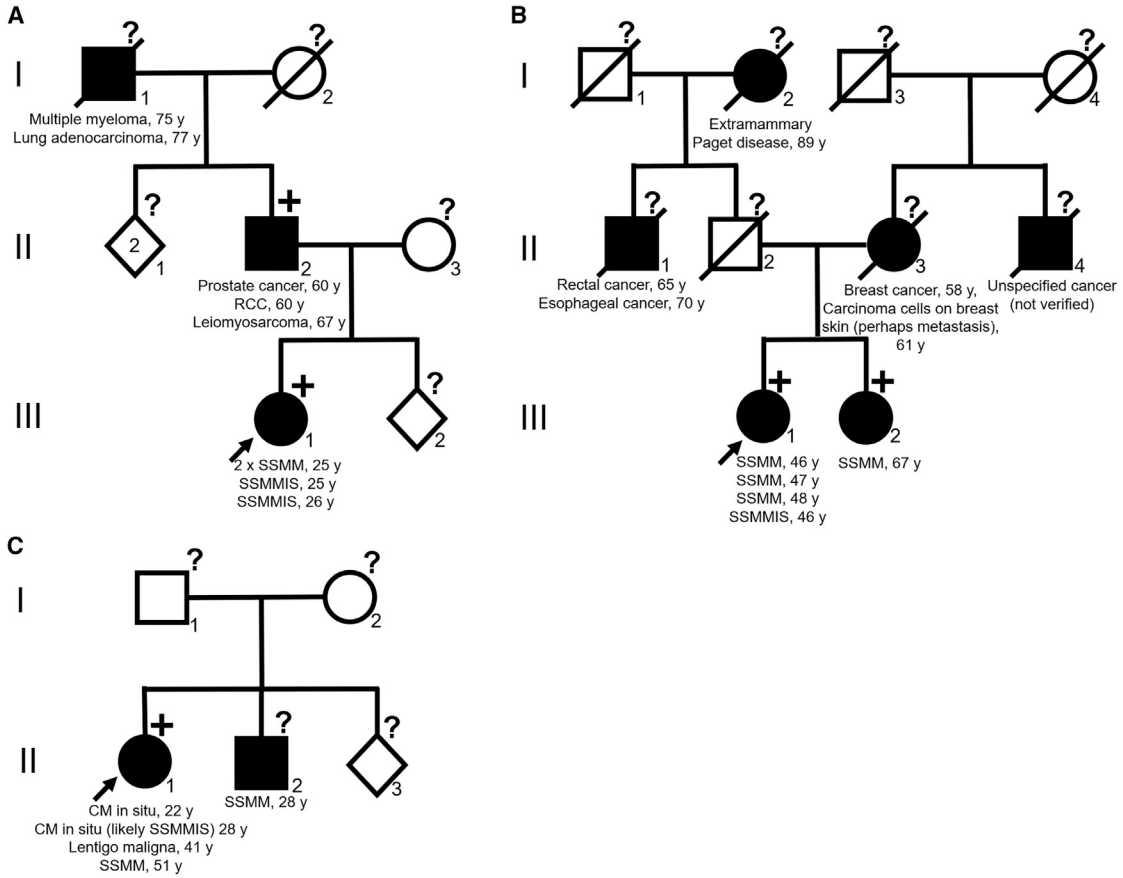


Figure 1. Pedigree of families F2, F3, and F5

Probands are highlighted by arrow. Black symbols indicate family members with history of cancer. Square symbols indicate males, circles females, and diamonds sex unspecified. A number within a square/circle/diamond refers to number of individuals represented by the symbol if more than one individual. A line through a symbol indicates that the individual is deceased. +, TINF2 p.(Arg265Ter) variant carriers. **?**, not tested for TINF2 p.(Arg265Ter) variant. A Family F2. B Family F3. C Family F5.

surgical resection, she developed loss of sensation in one lower extremity as well as partial paresis on the contralateral lower extremity. On the lower extremity with sensation loss, she developed a Spilus-like giant nevus. Within the acquired giant nevus, she has developed four of her five invasive or *in situ* melanomas.

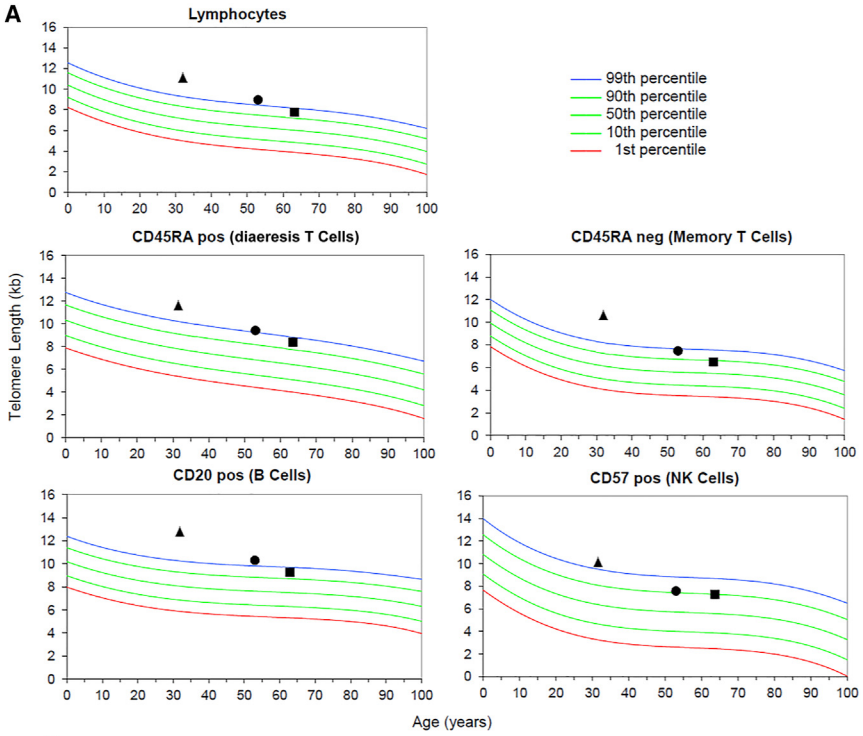
Clinical characteristics of all variant carriers and first-degree relatives with history of cancer are presented in Table 2, and pedigrees of the families F2, F3, and F5 are in Figure 1. Worth highlighting is two first-degree relatives of probands (Families F2 and F3) shown to carry the variant, one with history of melanoma and one with history of three verified primary cancers: prostate cancer, RCC, and leiomyosarcoma. In addition, one proband has a first-degree relative with history of CM not tested for the variant.

The *TINF2* variant (p.(Arg265Ter)) truncates the TINF2 protein by introducing a premature stop codon. The variant has just recently been described by Ballinger et al.[25] in a patient with undifferentiated pleomorphic sarcoma with a pedigree meeting the classic Li-Fraumeni syndrome (LFS) criteria.[25]

The variant has of April 2nd, 2023, been registered in global background population databases (gnomAD datasets) four times (twice in "other" population and twice in Latino/admixed American, with the highest AF of 0.00036 (non-cancer) in the population group "other") in addition to registration of one of our patients. Moreover, the variant is reported twice in ClinVar in addition to registration of one of our patients, once as a VUS regarding dyskeratosis congenita and once as likely pathogenic; however the condition was not provided. We screened for the variant in a Danish database of exomes of 2,000 patients or controls from a diabetes study[42] and in-house data from around 750 patients evaluated for non-cancer genetic diseases and did not identify any individual carrying the *TINF2* variant.

As described, the two previous studies by He et al.[24] and Schmutz et al.[18] have associated three different variants of *TINF2* with a long-telomere CPS. We therefore hypothesized that our variant may confer a similar effect on telomere length. To examine whether this was the case, flow-FISH (fluorescence *in situ* hybridization) was performed to measure telomere length in three of the probands with MPM. All three individuals had unusually long telomeres compared to age-matched controls, i.e., median telomere length in lymphocyte DNA (Figure 2).

A



B

	Proband family 1 (●)			Proband family 2 (▲)			Proband family 3 (■)		
	MTL (kb)	MTLN (kb)	INT	MTL (kb)	MTLN (kb)	INT	MTL (kb)	MTLN (kb)	INT
Lymphocytes	9.0	6.3	VH	11.2	7.1	VH	7.8	6.0	H
CD45RA+ (diaeresis T)	9.4	6.8	VH	11.6	7.8	VH	8.4	6.4	H
CD45RA- (Memory T)	7.5	5.6	H	10.5	6.1	VH	6.5	5.5	N
CD20+ (B Cells)	10.3	7.6	VH	12.7	8.1	VH	9.2	7.5	H
CD57+ (NK Cells)	7.6	5.7	H	10.1	6.4	VH	7.3	5.6	N

Figure 2. Telomere length results of three TINF2 p.(Arg265Ter) variant carriers
Analysis performed by flow-FISH (with permission from RepeatDx Europe). Results show median telomere length percentiles in lymphocytes compared to age-matched controls, shown in graphical form (A) and as a table (B). Square: proband from family 1. Triangle: proband from family 2. Circle: proband from family 3. Abbreviations are as follows: MTL, patient medial telomere length. MTLN, normal MTL at age (50^{th} percentile). INT, telomere length interpretation. VH, very high ($\geq 99^{th}$ percentile). H, high ($\geq 90^{th}$ and $<99^{th}$ percentile). N, normal ($\geq 10^{th}$ percentile and $<90^{th}$ percentile). L, low = ($\geq 1^{st}$ percentile and $<10^{th}$ percentile). VL, very low ($<1^{st}$ percentile). As presented, family one and two had a median telomere length of lymphocytes overall above the 99^{th} percentile, whereas family three had between the 90^{th} and 99^{th} percentile.

To estimate the age of the variant, we modeled the genotypes around the variant for the five MPM carriers using hidden Markov models (see supplemental methods). The most likely model suggested a 417-kb region around the variant for which the five individuals share a haplotype and that they had a common ancestor 277 generations ago (95% confidence interval 245–313). As this suggests that the variant is very old, we cannot say whether the variant is a Danish founder, as the variant may have originated from elsewhere and entered the Danish gene pool through immigration.

In normal circumstances, the TINF2 protein interacts with TERF1 and TERF2, both of which bind double-stranded DNA, and ACD, which in turns binds POT1, a protein that binds single-stranded DNA.[43] TINF2 and ACD thus mediate shelterin complex assembly and are crucial for its stability.[44]

Both He et al.[24] and Schmutz et al.[18] performed co-immunoprecipitation analysis to evaluate if interaction of TINF2 with TERF1 was lost due to TINF2 truncation. Results showed complete loss of TERF1 binding to the truncated TINF2 proteins. Schmutz et al.[18] further found very little or no interaction with TERF2 of the p.(Leu170fs) protein and loss of ACD interaction in the p.(Leu170fs) and p.(Ser186fs) proteins.

It has previously been found that the localization of TINF2 to telomeres is primarily dependent on its interaction with TERF1.[45] In agreement with this, telomeric chromatin immunoprecipitation assays performed by Schmutz et al.[18] indicated that the truncated proteins did not locate to telomeres.[18]

A prevailing model for telomere length homeostasis suggests that TIN F2, POT1, and TERF1 inhibit telomerase activity in *cis* by accumulating on the synthesized TTAGGG hexamers.[18] It has been shown that TINF2 as well as TERF1 and POT1 are negative regulators of telomere length,[18,46] and that telomerase hyper-elongates telomeres when TINF2 is inhibited.[46,47] The telomere length homeostasis model thus may explain how loss of TERF1 interaction leads to overelongation of telomeres.

Schmutz et al.[18] further performed analysis of telomere protection and genomic instability to investigate whether the cancer risk conferred by the *TINF2* variants could be caused by other mechanisms than telomere overelongation. Telomere protection was investigated by a telomere dysfunction-induced foci assay, showing telomere protection was maintained in heterozygous clones. Analysis of metaphase spreads similarly did not detect telomere dysfunction or genomic instability in heterozygous cell lines. Schmutz et al.[18] thus concluded it unlikely that telomere deprotection or genomic rearrangements contribute to cancer predisposition conferred by the variants. Further, Schmutz et al.[18] examined six of their reported tumors, of which no loss of heterozygosity was detected, and in four of the six tumors (i.e., a melanoma, astrocytoma, colorectal cancer, and breast cancer), second hits in *TINF2* were excluded.

These results point strongly toward *TINF2* as a haploinsufficient tumor suppressor, in which truncating variants

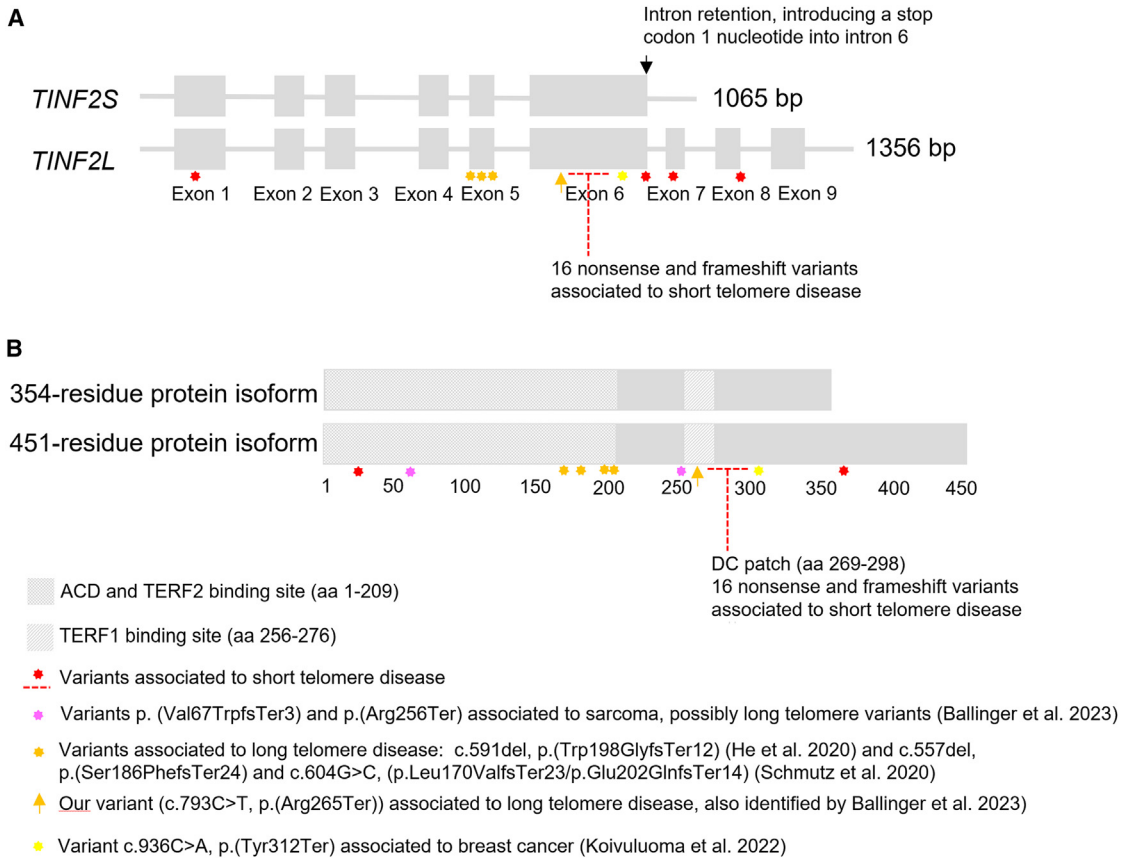


Figure 3. Pathogenic and likely pathogenic nonsense variants, frameshift variants, and splice variants in TINF2 identified in the HGMD and ClinVar databases

(A) The TINF2 gene shown as TINF2S and TINF2L.

(B) The TINF2 protein including the short and long transcript isoforms. Three frameshift variants, and now our nonsense variant (NM_001099274.3(TINF2): c.793C>T, p.(Arg265Ter)), have been associated with long telomere syndrome, all located upstream of the DC patch in exons 5 and 6. A frameshift variant and a nonsense variant identified by Ballinger et al.[25] may be associated with long telomere syndrome as well. One variant (p.(Tyr312Ter) has been associated with moderate breast cancer risk. Location of 20 nonsense, frameshift, and splice variants associated with short telomere disease is shown in the figure (missense variants not included). Details of the variants are shown in Table S5 including literature references.

can cause a cancer-prone state mediated by telomere elongation.

To our knowledge, no studies have measured telomere length in the tumors of carriers of long-telomere-associated *TINF2* variants. Further, only a few non-synonymous somatic variants in *TINF2* have been identified in CM, to our knowledge. In the Australian Melanoma Genome Project, three non-synonymous variants were identified (two missense variants, p.(Leu150Arg) and p.(Pro220Ser), and one splice site variant) in a total of 303 CMs included (i.e., 1%).[48] Thus, this suggests that *TINF2* rarely harbors somatic mutations in CM. The *TINF2* variant (c.793C>T, p.(Arg265Ter)) identified in our study is located within the TERF1 binding region (Figure 3). We thus expected the truncated transcript to contain the TERF2/ACD binding domain and to lack the C-terminal end of the TERF1 binding region.

Interestingly, two *TINF2* transcript isoforms have been described,[49] namely *TINF2L* encoding a 451-residue protein and *TINF2S* encoding a 354-residue protein. The smaller protein isoform is caused by inclusion of intron 6, intron 7, and intron 8, which introduced a premature stop codon one nucleotide into intron 6. This means that in this transcript isoform, the p.(Arg265Ter) variant is located in the last exon and therefore results in a truncated protein, whereas the p.(Arg265Ter) variant is suspected to undergo nonsense-mediated decay in the longer isoform. The expression pattern of the two isoforms in stem cells is currently unknown. However, together with the observed unusually long telomeres in variant carriers, we strongly suspect that the cancer-prone state observed in our families is conferred by telomere elongation due to loss of TERF1 interaction, as indicated in the studies by He et al.[24] and Schmutz et al.[18]

Interestingly, our variant is close to the "DC patch" (*dyskeratosis congenita patch*) located downstream of the TERF1 binding region. Loss of this patch is expected in our truncated protein, and as well in the truncated proteins identified previously.[18,24] The DC patch is the gene region in which *TINF2* variants related to short telomere disease are located.[50] Short telomere disease has a wide range of clinical presentations. The most common is pulmonary fibrosis in adulthood; however, bone marrow failure and immunodeficiency, most often in children, and other

organ failures occur as well.[14] Approximately 10%–15% of the patients are affected by cancer, mainly head and neck carcinomas and hematologic cancers.[39] The TINF2 protein uses the DC patch to stimulate the telomerase to telomere maintenance.[18] Several particularly severe cases of short telomere disease have been associated with *TINF2* variants.[50] A truncating *TINF2* variant (c.805 C>T, p.(Gln 269Ter)) causing severe short telomere disease, located in the DC patch just four amino acids downstream of our variant, has been shown to have impaired but not have completely abolished binding to TERF1.[50] Other truncating variants as well as missense variants associated to DC in *TINF2* have—to our knowledge—not been shown to affect binding of TERF1. Whether slight interaction between TINF2 and TERF1 is adequate to avoid the long telomere phenotype remains undetermined.

Another interesting finding is a truncating *TINF2* variant (c.936C>A, p.(Tyr312Ter)), previously identified in a patient with Ewing sarcoma,[51] which in a recent study by Koivulouma et al. 2022[52] has been associated with moderate risk of breast cancer and in a previous case report was found in a patient with normal telomere length.[53]

Overall, these data show the differential effects on telomere length and phenotypes of truncating *TINF2* variants located close to each other, as we observe both long and short telomere disease, and as well telomere disease not likely related to telomere length. An overview of pathogenic and likely pathogenic nonsense, frameshift, and splice *TINF2* variants, including the abovementioned variants, are shown in Figure 3 together with gene location and related disease manifestation.

The cancer-prone families we observe show evidence of a long telomere syndrome. We identified the *TINF2* p.(Arg265 Ter) variant in 10 individuals from the six families, all heterozygous carriers. All variant carriers had history of cancer, predominantly CM/CM *in situ* (seven individuals), however also thyroid cancer (two individuals), RCC (two individuals), and one case each of a grade 3 leiomyosarcoma, prostate cancer, and an unspecified cancer. Eight carriers had history of multiple malignancies. These findings thus suggest co-segregation of the variant in individuals affected with cancer.

In the families, we identified one case of CM, one case of myxoid sarcoma grade 2 in untested first-degree relatives, and no cases of thyroid cancer. Various cancer types were observed, including multiple cases of renal cancer, breast cancer, prostate cancer, colorectal cancer, and lung cancer. Furthermore, single cases of multiple myeloma, leukemia (not verified), and several other cancers were observed (Table 2). Notably, three family members had multiple malignancies.

The many different cancer types are supportive of a multi-cancer spectrum not limited to melanoma, thyroid cancer, and sarcoma. However, some of the observed cancer types are common (e.g., prostate cancer, colorectal cancer, and lung cancer) and are likely to occur independent of a CPS. Breast cancer, also a frequent cancer, was observed in all four families described by Schmutz et al.[18]; however, there is potential ascertainment bias in families referred to genetic counseling, as the referral typically is because of accumulation of cancer in the family. The families identified in our study cohort (F1–F4) were included only because of MPM in one individual, and in these families, we still observed family members with cancer, supporting a multi-cancer predisposition. In general, similar to our families, Schmutz et al.[18] observed a wide range of cancer types in addition to melanoma and thyroid cancer. On the contrary, the family investigated by He et al.[24] had a striking history of melanoma and thyroid cancer, with eight cases of papillary thyroid cancer and four cases of CM, thus representing a much narrower cancer spectrum and a different clinical phenotype. Detailed cancer history in the three families with a pathogenic *TINF2* variant described by Ballinger et al.[25] is not provided; however, one family met the classic LFS criteria, another the Chompret criteria for LFS, and the third family did not meet the criteria for a cancer syndrome. None of the three families met the GenoMEL familial melanoma criteria.[25]

The hypothesis of a multi-cancer spectrum is, however, also in agreement with other telomere maintenance genes. For example, *POT1* variants have, in addition to familial melanoma, been associated with cases of gliomas, chronic lymphocytic leukemia (CLL), and angiosarcomas[14,54] in families not necessarily having melanomas,[54] suggesting a wider cancer predisposition of the *POT1* gene.[14] Thus, it could suggest a wide cancer spectrum in long telomere syndrome in general. McNally et al.,[14] reviewing existing evidence of long telomere syndrome, suggest a large role of CLL as well. However, CLL has not yet been associated with *TINF2*-mediated long telomere syndrome.

In conclusion, we believe that the data presented (e.g., familial cancer cases, telomere length measurements, evidence of previous long telomere variants, etc.) establish that heterozygosity of the *TINF2* p.(Arg265Ter) variant predisposes to long telomere syndrome.

We find it intriguing that the variant frequency in our cohort is 4/102 (∼4%). We believe these results suggest *TINF2* is an important susceptibility gene in Danish MPM patients.

We observe that our families most likely have variant carriers who have not developed cancer, such as one of the two parents in family F5 unaffected by cancer. This argues against the variant being high penetrance for melanoma risk, such as *CDKN2A* variants. We thus expect that observed cancer cases may be influenced by environmental risk factors and/or additional genetic predisposition (e.g., polygenic risk). No interaction of *MC1R* or *MITF* variants with the *TINF2*-associated melanoma risk was detected, and the low AF of *MC1R* R-alleles and r-alleles in study participants carrying the *TINF2* p.(Arg265Ter) variant does not suggest that *MC1R* interaction can explain the melanoma predisposition of the *TINF2* variant carriers. However, additional studies are needed, including potential interaction of low-risk melanoma genes. Although difficult to quantify, we did observe significant environmental risk in several variant carriers; for instance, almost all variant carriers with history

of melanoma have used sunbeds and had sunburns in childhood (as did >70% of the overall study group).

In conclusion, we have established this variant to confer long-telomere-mediated cancer risk similar to previously identified long telomere *TINF2* variants; this most likely is a multi-cancer syndrome dominated by melanoma, thyroid cancer, and sarcoma; however, it may involve breast cancer, renal cancer, or other cancers as well. We have shown that *TINF2* is an important susceptibility gene in Danish patients with MPM. However, as variants in shelterin genes are rare, further research of *TINF2* variants is necessary to characterize this CPS in greater detail and clarify the role of *TINF2* in MPM.

Based on the identified *TINF2* families, we recommend yearly skin examinations by dermatologists as well as self-examination of skin monthly for all variant carriers. Furthermore, we recommend thyroid surveillance is offered to variant carriers in the family with thyroid cancer in terms of ultrasound and scintigraphy of the thyroid gland every second year.

A Danish study of >2,000 melanoma patients showed that three SNPs predicting long telomeres (rs7726159 (*TERT*), rs1317082 (*TERC* [MIM: 602322]), and rs2487999 (*OBFC1* [MIM: 613128]) were associated with increased mortality in the patients.[55] It remains undetermined whether the association holds for germline *TINF2* variants; however, it may add to the importance of surveillance in variant carriers.

Whether more cancer surveillance would be beneficial remains unknown. The *TINF2* gene should be included in future cancer gene panels when testing individuals with multiple melanomas or familial clustering of melanoma for etiological clarification, however, with consideration that variants in *TINF2* causing long telomeres may not explain the entire melanoma predisposition.

Data and code availability

The WES and WGS data are not publicly available due to lack of consent.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.xhgg.2023.100225.

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Author contributions

M.R.J. included and interviewed all participants in the MPM study. K.A.W.W., T.V.O.H., and M.R.J. analyzed WES results. K.A.W.W. and A.M.J. provided genetic counseling of variant carriers and relatives. M.R.J. imaged genetic variants and telomere length results. P.A.J. performed haplotype analysis. All authors contributed to the manuscript and approved the final manuscript and its submission to *American Journal of Human Genetics*.

Declaration of interests

The authors declare no competing interests.

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Web resources

gnomAD, https://gnomad.broadinstitute.org/
OMIM, https://www.omim.org/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
HGMD, https://www.hgmd.cf.ac.uk
GenoMEL, https://genomel.org/)
VarSeq, https://www.goldenhelix.com
RepeatDx, https://repeatdx.com/

References

1. Wadt, K.A.W., Aoude, L.G., Krogh, L., Sunde, L., Bojesen, A., Grønskov, K., Wartacz, N., Ek, J., Tolstrup-Andersen, M., Klarskov-Andersen, M., et al. (2015). Molecular characterization of melanoma cases in Denmark suspected of genetic predisposition. PLoS One *10*, e0122662.
2. Aoude, L.G., Pritchard, A.L., Robles-Espinoza, C.D., Wadt, K., Harland, M., Choi, J., Gartside, M., Quesada, V., Johansson, P., Palmer, J.M., et al. (2015). Nonsense mutations in the shelterin complex genes ACD and TERF2IP in familial melanoma. J. Natl. Cancer Inst. *107*, dju408.
3. Harland, M., Petljak, M., Robles-Espinoza, C.D., Ding, Z., Gruis, N.A., van Doorn, R., Pooley, K.A., Dunning, A.M., Aoude, L.G., Wadt, K.A.W., et al. (2016). Germline TERT promoter mutations are rare in familial melanoma. Fam. Cancer *15*, 139–144.
4. Robles-Espinoza, C.D., Harland, M., Ramsay, A.J., Aoude, L.G., Quesada, V., Ding, Z., Pooley, K.A., Pritchard, A.L., Tiffen, J.C., Petljak, M., et al. (2014). POT1 loss-of-function variants predispose to familial melanoma. Nat. Genet. *46*, 478–481.
5. Potrony, M., Puig-Butille, J.A., Ribera-Sola, M., Iyer, V., Robles-Espinoza, C.D., Aguilera, P., Carrera, C., Malvehy, J., Badenas, C., Landi, M.T., et al. (2019). POT1 germline mutations but not TERT promoter mutations are implicated in melanoma susceptibility in a large cohort of Spanish melanoma families. Br. J. Dermatol. *181*, 105–113.
6. Shi, J., Yang, X.R., Ballew, B., Rotunno, M., Calista, D., Fargnoli, M.C., Ghiorzo, P., Bressac-De Paillerets, B., Nagore, E., Avril, M.F., et al. (2014). Rare missense variants in POT1 predispose to familial cutaneous malignant melanoma. Nat. Genet. *46*, 482–486.
7. Puntervoll, H.E., Yang, X.R., Vetti, H.H., Bachmann, I.M., Avril, M.F., Benfodda, M., Catricalà, C., Dalle, S., Duval-Modeste, A.B., Ghiorzo, P., et al. (2013). Melanoma prone families with CDK4 germline mutation: Phenotypic profile and associations with MC1R variants. J. Med. Genet. *50*, 264–270.
8. Walpole, S., Pritchard, A.L., Cebulla, C.M., Pilarski, R., Stautberg, M., Davidorf, F.H., de la Fouchardière, A., Cabaret, O., Golmard, L., Stoppa-Lyonnet, D., et al. (2018). Comprehensive study of

the clinical phenotype of germline BAP1 variant-carrying families worldwide. J. Natl. Cancer Inst. *110*, 1328–1341.
9. Raimondi, S., Sera, F., Gandini, S., Iodice, S., Caini, S., Maisonneuve, P., and Fargnoli, M.C. (2008). MC1R variants, melanoma and red hair color phenotype: A meta-analysis. Int. J. Cancer *122*, 2753–2760.
10. Yokoyama, S., Woods, S.L., Boyle, G.M., Aoude, L.G., MacGregor, S., Zismann, V., Gartside, M., Cust, A.E., Haq, R., Harland, M., et al. (2011). A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. Nature *480*, 99–103.
11. Bertolotto, C., Lesueur, F., Giuliano, S., Strub, T., De Lichy, M., Bille, K., Dessen, P., D'Hayer, B., Mohamdi, H., Remenieras, A., et al. (2011). A SUMOylation-defective MITF germline mutation predisposes to melanoma and renal carcinoma. Nature *480*, 94–98.
12. Landi, M.T., Bishop, D.T., MacGregor, S., Machiela, M.J., Stratigos, A.J., Ghiorzo, P., Brossard, M., Calista, D., Choi, J., Fargnoli, M.C., et al. (2020). Genome-wide association meta-analyses combining multiple risk phenotypes provide insights into the genetic architecture of cutaneous melanoma susceptibility. Nat. Genet. *52*, 494–504.
13. Palm, W., and De Lange, T. (2008). How shelterin protects mammalian telomeres. Annu. Rev. Genet. *42*, 301–334.
14. McNally, E.J., Luncsford, P.J., and Armanios, M. (2019). Long telomeres and cancer risk: The price of cellular immortality. J. Clin. Invest. *129*, 3474–3481.
15. Stanley, S.E., and Armanios, M. (2015). The short and long telomere syndromes: Paired paradigms for molecular medicine. Curr. Opin. Genet. Dev. *33*, 1–9.
16. Armanios, M., and Blackburn, E.H. (2012). The telomere syndromes. Nat. Rev. Genet. *13*, 693–704.
17. D'Adda Di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., and Jackson, S.P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194–198.
18. Schmutz, I., Mensenkamp, A.R., Takai, K.K., Haadsma, M., Spruijt, L., De Voer, R.M., Choo, S.S., Lorbeer, F.K., Van Grinsven, E.J., Hockemeyer, D., et al. (2020). Tinf2 is a haploinsufficient tumor suppressor that limits telomere length. Elife *9*, e61320.
19. Gong, Y., Stock, A.J., and Liu, Y. (2020). The enigma of excessively long telomeres in cancer: lessons learned from rare human POT1 variants. Curr. Opin. Genet. Dev. *60*, 48–55.
20. Ramsay, A.J., Quesada, V., Foronda, M., Conde, L., Martínez-Trillos, A., Villamor, N., Rodríguez, D., Kwarciak, A., Garabaya, C., Gallardo, M., et al. (2013). POT1 mutations cause telomere dysfunction in chronic lymphocytic leukemia. Nat. Genet. *45*, 526–530.
21. Pinzaru, A.M., Hom, R.A., Beal, A., Phillips, A.F., Ni, E., Cardozo, T., Nair, N., Choi, J., Wuttke, D.S., Sfeir, A., and Denchi, E.L. (2016). Telomere Replication Stress Induced by POT1 Inactivation Accelerates Tumorigenesis. Cell Rep. *15*, 2170–2184.
22. Chen, C., Gu, P., Wu, J., Chen, X., Niu, S., Sun, H., Wu, L., Li, N., Peng, J., Shi, S., et al. (2017). Structural insights into POT1-TPP1 interaction and POT1 C-terminal mutations in human cancer. Nat. Commun. *8*, 14929.
23. Gu, P., Wang, Y., Bisht, K.K., Wu, L., Kukova, L., Smith, E.M., Xiao, Y., Bailey, S.M., Lei, M., Nandakumar, J., and Chang, S. (2017). Pot1 OB-fold mutations unleash telomere instability to initiate tumorigenesis. Oncogene *36*, 1939–1951.
24. He, H., Li, W., Comiskey, D.F., Liyanarachchi, S., Nieminen, T.T., Wang, Y., Delap, K.E., Brock, P., and De La Chapelle, A. (2020). A Truncating Germline Mutation of TINF2 in Individuals with Thyroid Cancer or Melanoma Results in Longer Telomeres. Thyroid *30*, 204–213.
25. Ballinger, M.L., Pattnaik, S., Mundra, P.A., Zaheed, M., Rath, E., Priestley, P., Baber, J., Ray-Coquard, I., Isambert, N., Causeret, S., et al. (2023). Heritable defects in telomere and mitotic function selectively predispose to sarcomas. Science *379*, 253–260.
26. Girard, E., Eon-Marchais, S., Olaso, R., Renault, A.L., Damiola, F., Dondon, M.G., Barjhoux, L., Goidin, D., Meyer, V., Le Gal, D., et al. (2019). Familial breast cancer and DNA repair genes: Insights into known and novel susceptibility genes from the GENESIS study, and implications for multigene panel testing. Int. J. Cancer *144*, 1962–1974.
27. Dalmasso, B., Pastorino, L., Nathan, V., Shah, N.N., Palmer, J.M., Howlie, M., Johansson, P.A., Freedman, N.D., Carter, B.D., Beane-Freeman, L., et al. (2021). Germline ATM variants predispose to melanoma: a joint analysis across the GenoMEL and MelaNostrum consortia. Genet. Med. *23*, 2087–2095.
28. West, A.H., Blazer, K.R., Stoll, J., Jones, M., Weipert, C.M., Nielsen, S.M., Kupfer, S.S., Weitzel, J.N., and Olopade, O.I. (2018). Clinical interpretation of pathogenic ATM and CHEK2 variants on multigene panel tests: navigating moderate risk. Fam. Cancer *17*, 495–505.
29. Kuchenbaecker, K.B., Hopper, J.L., Barnes, D.R., Phillips, K.A., Mooij, T.M., Roos-Blom, M.J., Jervis, S., Van Leeuwen, F.E., Milne, R.L., Andrieu, N., et al. (2017). Risks of breast, ovarian, and contralateral breast cancer for BRCA1 and BRCA2 mutation carriers. JAMA *317*, 2402–2416.
30. Cavanagh, H., and Rogers, K.M.A. (2015). The role of BRCA1 and BRCA2 mutations in prostate, pancreatic and stomach cancers. Hered. Cancer Clin. Pract. *13*, 16–17.
31. Cybulski, C., Górski, B., Huzarski, T., Masojć, B., Mierzejewski, M., Dębniak, T., Teodorczyk, U., Byrski, T., Gronwald, J., Matyjasik, J., et al. (2004). CHEK2 is a multiorgan cancer susceptibility gene. Am. J. Hum. Genet. *75*, 1131–1135.
32. Bui, A.N., LeBoeuf, N.R., and Nambudiri, V.E. (2021). Skin cancer risk in CHEK2 mutation carriers. J. Eur. Acad. Dermatol. Venereol. *35*, 353–359.
33. Catucci, I., Osorio, A., Arver, B., Neidhardt, G., Bogliolo, M., Zanardi, F., Riboni, M., Minardi, S., Pujol, R., Azzollini, J., et al. (2018). Individuals with FANCM biallelic mutations do not develop Fanconi anemia, but show risk for breast cancer, chemotherapy toxicity and may display chromosome fragility. Genet. Med. *20*, 452–457.
34. Neidhardt, G., Hauke, J., Ramser, J., Groß, E., Gehrig, A., Müller, C.R., Kahlert, A.K., Hackmann, K., Honisch, E., Niederacher, D., et al. (2017). Association between loss-of-function mutations within the FANCM gene and early-onset familial breast cancer. JAMA Oncol. *3*, 1245–1248.
35. Figlioli, G., Bogliolo, M., Catucci, I., Caleca, L., Lasheras, S.V., Pujol, R., Kiiski, J.I., Muranen, T.A., Barnes, D.R., Dennis, J., et al. (2019). The FANCM:p.Arg658* truncating variant is associated with risk of triple-negative breast cancer. Npj Breast Cancer *5*, 38.
36. Dicks, E., Song, H., Ramus, S.J., Oudenhove, E.V., Tyrer, J.P., Intermaggio, M.P., Kar, S., Harrington, P., Bowtell, D.D., Group, A.S., et al. (2017). Germline whole exome sequencing and large-scale replication identifies FANCM as a likely high grade serous ovarian cancer susceptibility gene. Oncotarget *8*, 50930–50940.
37. Beck, S.H., Jelsig, A.M., Yassin, H.M., Lindberg, L.J., Wadt, K.A.W., and Karstensen, J.G. (2022). Intestinal and extraintestinal neoplasms in patients with NTHL1 tumor syndrome: a systematic review. Fam. Cancer *21*, 453–462.

38. Yang, X., Leslie, G., Doroszuk, A., Schneider, S., Allen, J., Decker, B., Dunning, A.M., Redman, J., Scarth, J., Plaskocinska, I., et al. (2020). Cancer Risks Associated With Germline PALB2 Pathogenic Variants: An International Study of 524 Families. J. Clin. Oncol. *38*, 674–685.
39. Alter, B.P., Giri, N., Savage, S.A., and Rosenberg, P.S. (2009). Cancer in dyskeratosis congenita. Blood *113*, 6549–6557.
40. Frebourg, T., Bajalica Lagercrantz, S., Oliveira, C., Magenheim, R., Evans, D.G., European Reference Network GENTURIS, Ligtenberg, M., Kets, M., Oostenbrink, R., Sijmons, R., et al. (2020). Guidelines for the Li–Fraumeni and heritable TP53-related cancer syndromes. Eur. J. Hum. Genet. *28*, 1379–1386.
41. Sandru, F., Dumitrascu, M.C., Petca, A., Carsote, M., Petca, R.-C., and Ghemigian, A. (2022). Melanoma in patients with Li-Fraumeni syndrome (Review). Exp. Ther. Med. *23*, 75–76.
42. Lohmueller, K.E., Sparsø, T., Li, Q., Andersson, E., Korneliussen, T., Albrechtsen, A., Banasik, K., Grarup, N., Hallgrimsdottir, I., Kiil, K., et al. (2013). Whole-exome sequencing of 2,000 Danish individuals and the role of rare coding variants in type 2 diabetes. Am. J. Hum. Genet. *93*, 1072–1086.
43. De Lange, T. (2018). Shelterin-mediated telomere protection. Annu. Rev. Genet. *52*, 223–247.
44. O'Connor, M.S., Safari, A., Xin, H., Liu, D., and Songyang, Z. (2006). A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly. Proc. Natl. Acad. Sci. USA *103*, 11874–11879.
45. Frescas, D., and de Lange, T. (2014). TRF2-Tethered TIN2 Can Mediate Telomere Protection by TPP1/POT1. Mol. Cell Biol. *34*, 1349–1362.
46. Kim, S.H., Kaminker, P., and Campisi, J. (1999). TIN2, a new regulator of telomere length in human cells. Nat. Genet. *23*, 405–412.
47. Ye, J.Z.S., and De Lange, T. (2004). TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. Nat. Genet. *36*, 618–623.
48. Newell, F., Johansson, P.A., Wilmott, J.S., Nones, K., Lakis, V., Pritchard, A.L., Lo, S.N., Rawson, R.V., Kazakoff, S.H., Colebatch, A.J., et al. (2022). Comparative Genomics Provides Etiologic and Biological Insight into Melanoma Subtypes. Cancer Discov. *12*, 2856–2879.
49. Kaminker, P.G., Kim, S.H., Desprez, P.Y., and Campisi, J. (2009). A novel form of the telomere-associated protein TIN2 localizes to the nuclear matrix. Cell Cycle *8*, 931–939.
50. Sasa, G.S., Ribes-Zamora, A., Nelson, N.D., and Bertuch, A.A. (2012). Three novel truncating TINF2 mutations causing severe dyskeratosis congenita in early childhood. Clin. Genet. *81*, 470–478.
51. Brohl, A.S., Patidar, R., Turner, C.E., Wen, X., Song, Y.K., Wei, J.S., Calzone, K.A., and Khan, J. (2017). Frequent inactivating germline mutations in DNA repair genes in patients with Ewing sarcoma. Genet. Med. *19*, 955–958.
52. Koivuluoma, S., Vorimo, S., Mattila, T.M., Tervasmäki, A., Kumpula, T., Kuismin, O., Winqvist, R., Moilanen, J., Mantere, T., and Pylkäs, K. (2023). Truncating TINF2 p.Tyr312Ter variant and inherited breast cancer susceptibility. Fam. Cancer *22*, 13–17.
53. Hautala, T., Chen, J., Tervonen, L., Partanen, T., Winqvist, S., Lehtonen, J., Saarela, J., Kraatari, M., Kuismin, O., Vuorinen, T., et al. (2020). Herpes simplex virus 2 encephalitis in a patient heterozygous for a TLR3 mutation. Neurol. Genet. *6*, e532–e537.
54. Calvete, O., Martinez, P., Garcia-Pavia, P., Benitez-Buelga, C., Paumard-Hernández, B., Fernandez, V., Dominguez, F., Salas, C., Romero-Laorden, N., Garcia-Donas, J., et al. (2015). A mutation in the POT1 gene is responsible for cardiac angiosarcoma in TP53-negative Li-Fraumeni-like families. Nat. Commun. *6*, 8383.
55. Ismail, H., Helby, J., Hölmich, L.R., H Chakera, A., Bastholt, L., Klyver, H., Sjøgren, P., Schmidt, H., Schöllhammer, L., Nordestgaard, B.G., and Bojesen, S.E. (2021). Genetic predisposition to long telomeres is associated with increased mortality after melanoma: A study of 2101 melanoma patients from hospital clinics and the general population. Pigment Cell Melanoma Res. *34*, 946–954.

HGGA, Volume *4*

Supplemental information

TINF2 is a major susceptibility gene in Danish patients with multiple primary melanoma

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE

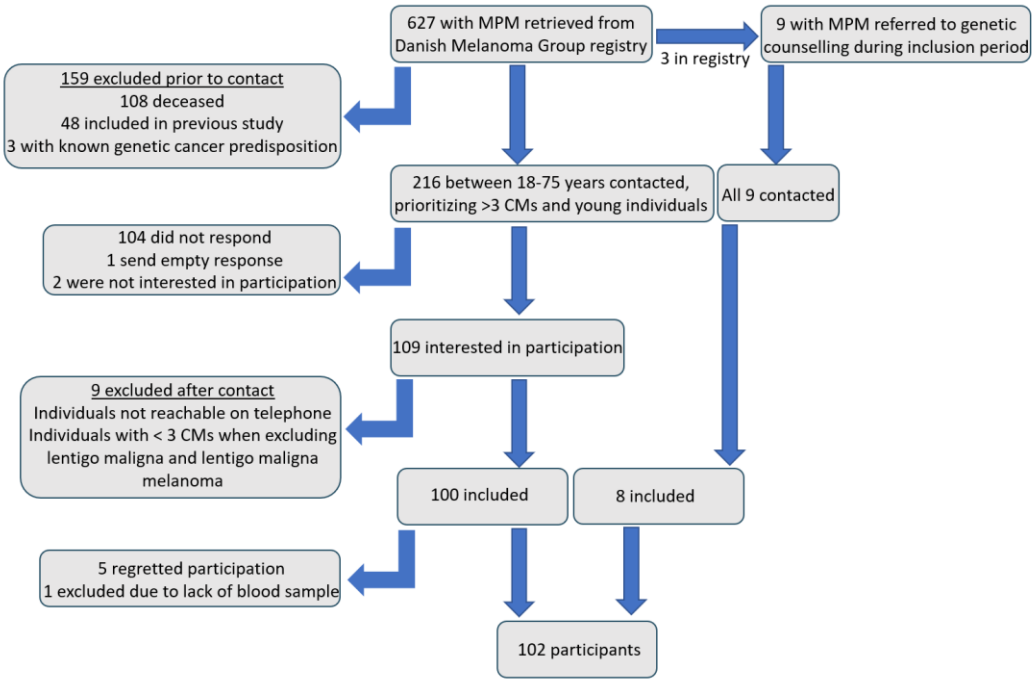

Figure S1. Inclusion flowchart. Flowchart showing the inclusion process, beginning with retrieval of MPM cases from the Danish Melanoma Group registry and patients referred to genetic counselling, ending with 102 included participants.

SUPPLEMENTAL TABLES

Gene	Variant identified[a]
ACD	NM_001082486.1:c.1332C>A , NP_001075955.1:p.(His444Gln)
ATM	NM_000051.4: c.9094G>C, NP_000042.3:p.(Val3032Leu)[b]
BAP1	NM_004656.3:c.2057-4G>T, NP_p.(?)[c]
	NM_004656.3:c.255+7T>C, NP_p.(?)
BRCA2	NM_000059.3:c.5021G>C, NP_000050.2:p.(Ser1674Thr)[d]
	NM_000059.3:c.3890_3892delATA, NP_000050.2:p.(Asn1297del)[b]
MC1R	NM_002386.3:c.836A>G, NP_002377.4:p.(Asn279Ser)
	NM_002386.4:c.637C>T, NP_002377.4:p.(Arg213Trp)[e]
	NM_002386.3:c.667C>T, NP_002377.4:p.(Arg223Trp)
	NM_002386.3:c.295C>A, NP_002377.4:p.(Leu99Ile)[f]
	NM_002386.3:c.292A>G, NP_002377.4:p.(Ile98Val)[f]
MET	NM_001127500.2:c.751C>G, NP_001120972.1:p.(His251Asp)[g]
MITF	NM_000248.3:c.895C>T, NP_000239.1:p.(Leu299Phe)
	NM_000248.3:c.719G>A, NP_000239.1:p.(Arg240His)
MLH1	NM_000249.3:c.2051A>G, NP_000240.1:p.(Tyr684Cys)
MSH2	NM_000251.3:c.698C>G, NP_000242.1:p.(Ser233Cys)[e]
MSH6	NM_000179.2:c.3647-5_3647-2dupAACA, NP_p.(?)
PMS2	NM_000535.6:c.608C>G, NP_000526.2:p.(Thr203Ser)[g]
TP53	NM_000546.5:c.572C>G, NP_000537.3:p.(Pro191Arg)[d]

[a]All variants were identified heterozygous. No VUSs were identified in more than one individual.

[b]Identified in the same individual. [c]Identified in the same individual as one of the pathogenic *MITF* variants (p.Glu318Lys). [d]Identified in the same individual. [e]Identified in the same individual. [f]Identified in the same individual. [g]Identified in the same individual.

Table S1. VUSs in cancer-related genes.

Genes
ACD, APC, AXIN2, BAP1, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CDKN2B, DICER1, EPCAM, FH, FLCN, GREM1, MET, MLH1, MLH3, MSH2, MSH3, MSH6, MUTYH, NTHL1, PALB2, PMS2, POLD1, POLE, POT1, PTEN, RAD51C, RAD51D, RNF43, SDHB, SDHC, SMAD4, STK11, TERF2IP, TERT, TP53 and *VHL.*

Table S3. Clinical cancer gene panel initially performed on MZ twins.

Variant	Variant carriers	Heterozygous carriers
NM_002386.3:c.252C>A, NP_002377.4:p.(Asp84Glu)	9	9
NM_002386.3:c.451C>T, NP_002377.4:p.(Arg151Cys)	39	34
NM_002386.3:c.478C>T, NP_002377.4:p.(Arg160Trp)	39	34
NM_002386.3:c.880G>C, NP_002377.4:p.(Asp294His)	3	3
NM_002386.3:c.425G>A, NP_002377.4:p.(Arg142His)	2	2
NM_002386.3:c.487C>T, NP_002377.4:p.(Arg163Ter)	1	1
NM_002386.3:c.86dupA, NP_002377.4:p.(Asn29LysfsTer14)	2	2
NM_002386.3:c.537dupC, NP_002377.4:p.(Ile180HisfsTer59)	1	1

Table S4. *MC1R* R-alleles identified in MPM study participants.

Variants
c.81C>A, p.(C27Ter)[a]
c.805C>T, p.(Q269Ter)[4]
c.811C>T, p.(Q271Ter)[4]
c.815G>A, p.(W272Ter)[5]
c.826delA, p.(R276GfsTer41)[6]
c.838A>T, p.(K280Ter)[7]
c.839delA, p.(K280RfsTer36)[4]
c.849delC, p.(T284QfsTer33)[6]
c.849_850insC, p.(T284HfsTer8)[7]
c.851_852delCA, p.(T284SfsTer7)[8]
c.851_855delCAGTC, p.(T284NfsTer6)[9]
c.857delTinsGC, p.(M296SfsTer6)[6]
c.866_867delCC, p.(P289LfsTer2)[10]
c.867_868insC, p.(F290LfsTer2)[7]
c.872_875delGGAA, p.(R291IfsTer25)[11]
c.889delA, p.(T297PfsTer20)[12]
c.892delC, p.(Q298RfsTer19)[7]
c.1061+1G>T[13]
c.1090dup, p.(L364PfsTer9)[a]
c.1221+1G>C[14]
[a]*To our knowledge, these variants have not been published. However, they have been registered in ClinVar.*

Table S5. Pathogenic and likely pathogenic nonsense variants, frameshift variants and splice variants identified in *TINF2* associated with DC.

SUPPLEMENTAL METHODS

Approvals

This study was approved by the Capitol Region Committee on Health Research Ethics (H-3-2011-050) and the Danish Data Protection Agency (P-2021-154). The study has been conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was collected from all study participants. All patients described in this article have provided written consent to publication.

Inclusion

The study was performed from February 2021 to December 2022. 627 individuals with ≥ 3 primary cutaneous melanomas were identified using the Danish Melanoma Group registry (a nationwide registry established in 1985). 216 individuals aged 29-75 years were contacted by e-Boks (a Danish personal digital mailbox), prioritizing individuals with a history of > 3 CMs and secondly the youngest individuals at the time of data extraction. Participants ≥ 18 years of age with ≥ 3 primary CMs were offered inclusion. Cases of lentigo maligna or lentigo maligna melanoma were not included in the 3 CM cases due to their strong association to sun exposure. In situ melanomas, such as superficial spreading malignant melanoma in situ (SSMM in situ), were included in the 3 CM cases. Exclusion criteria were individuals with known genetic cancer predisposition syndromes involving melanoma susceptibility (3 individuals), deceased individuals (108 individuals), and individuals participating in a previous study of familial melanoma published by our group (44 individuals)[1].

112 of the 216 responded by e-Boks; two were not interested in participation and one send an empty response. 104 did not respond. The remaining (109) respondents agreed to participate (i.e., 50.5 % of contacted individuals). All 109 were subsequently contacted by telephone, of which 100 were included. The remaining 9 were excluded due to one of several reasons (patients not reachable by telephone and patients with < 3 CMs when excluding lentigo maligna and lentigo maligna melanoma).

Of the 100 left, 5 regretted their participation, several due to health-related issues or lack of time. Further, one participant was excluded due to lack of blood sample. Thus, 94 participants included by e-Boks fulfilled participation. During the study, patients with ≥ 3 primary CMs referred to genetic counseling for suspicion of familial melanoma in the Department of Clinical Genetics in Copenhagen and Roskilde were offered inclusion in the study, of which 8 agreed to participate. 3 of the patients were in the registry and thus not contacted by e-Boks. Thus, a total of 102 participants. One of the participants had one of the three melanomas as "uncertain if primary tumor or metastasis", all other participants had ≥ 3 confirmed primary cases. None of the participants were near relatives, to our knowledge. A flow chart of the inclusion process can be viewed in Figure S1.

Participation

All participants received project information by telephone or in person, as well as written information. All participants performed a questionnaire by telephone or in-person in the Department of Clinical Genetics, Rigshospitalet. The questionnaire included a questionnaire developed by GenoMEL (https://genomel.org/) with questions regarding sun-seeking behavior, sunburns, use of sunscreen and other precautions to sun exposure, as well as sunbed use, and questions regarding tobacco use. Our questionnaire further included questions regarding skin type (skin-, hair- and eye color, skin reaction to sun exposure, freckling) and number of nevi.

In the interview, a pedigree covering 1st to 3rd generation family members was constructed for all participants regarding their history of cancer. Parents and siblings were defined as 1st degree relatives, grandparents, parents' siblings and patient's half-siblings as 2nd degree relatives, and great grandparents, grandparents' siblings, cousins and half-siblings of parents, 3rd degree relatives. After obtainment of consent, the participants' melanoma cases as well as other cancers were verified by pathological descriptions and/or medical records. Cancer cases in living family members were seized verified by pathological description (in the Danish Pathology Data Bank)

and/or medical records after obtainment of written consent. Cancer cases in deceased relatives were seized verified as well. For participants that had received genetic counseling prior to participation, the pedigree from previous counseling was retrieved if consent from the patient.

Whole exome sequencing (WES)

DNA library preparation and exome sequencing

DNA library preparation was performed using the Twist human core exome kit with custom region enrichment (Twist Bioscience). Exome sequencing was performed on a NovaSeq 6000 sequencer (Illumina) with a coverage of at least 20X in 98 % of the exome in all samples.

Data analysis pipeline

A clinical pipeline was used to generate BAM/CRAM and VCF files. Annotation and variant filtering was performed using VarSeq (https://www.goldenhelix.com). Initially, we identified families with pathogenic alterations in the known high penetrance melanoma genes (*CDKN2A, CDK4, BAP1, POT1, TERT, ACD, TERF2IP*), and selected known or putative cancer predisposition genes (in-house panel of 390 genes).

Further, structural rearrangements were investigated by searching for copy number variations (CNVs) in exome data.

Exome variant interpretation

All exonic non-synonymous variants were evaluated for their potential effect on protein function using a suite of publicly available tools, including CADD, Alamut (also including SIFT and Polyphen), and PMut. Moreover, selected variants were evaluated for their putative effect on splicing (MaxEntScan). Variants were evaluated and classified according to the ACMG guidelines[2] jointly by three researchers (e.g., M.R.J., T.v.O.H. and K.W.).

Identified pathogenic variants were reported back to the participants, and family members at risk of carrying an identified pathogenic variant were offered genetic counseling as well, either at the Department of Clinical Genetics at Rigshospitalet or their regional Department of Clinical Genetics.

Telomere length analysis

3 of the 4 clinical study participants carrying the identified *TINF2* p.(Arg265Ter) variant had subsequently performed telomere length analysis by RepeatDx Europe (Aachen, Germany), using Flow-FISH analysis on DNA from patient peripheral blood lymphocytes. A description of the technique can be viewed at RepeatDx's homepage (https://repeatdx.com/).

Polymerase chain reaction (PCR) and sequencing

Carrier screening of relatives of the *TINF2* variant carriers agreeing to be tested was performed on purified genomic DNA from whole blood sample. The *TINF2* c.793C>T, p.(Arg265Ter) was amplified using the following primers: 5'-CTGACTCAGTGAACCTGGCTGAGC-3' and 5'-GCTGCTCTTGTGCCCATGGCTAGG-3', followed by sequencing using an ABI3730 DNA analyzer (Applied Biosystems).

***BRAF* analysis**

BRAF analysis was performed in a clinical setting on two invasive melanomas of one individuals with the Idylla *BRAF* method that detects presence of the variants: 1. V600E/V600D and 2. V600K/ V600R/V600M. The analysis does not differentiate between variants within the two groups. The sensitivity of the analysis is 5% tumor cells.

Haplotype analysis

The age of the mutation was estimated using a statistical model[3]. A pedigree was built as a binary tree with the five carriers as leaf nodes, three ancestral nodes and seven edges. Each edge is associated with a number of meioses, *M*, describing the closeness between the two nodes the edge connects. The probability that a recombination has occurred between loci *m* and *n* and not

between locus *m* and the *TINF2* mutation is $\theta_{n,m}$ = exp(-$\lambda x_n M$) – exp(-$\lambda x_m M$), where x_i is the distance from the *TINF2* mutation to locus *i*, and lambda is 10^{-8} assuming 1cM equals 1 Mb. The joint likelihood of the genotypes (g) across the *N* loci is then given by

$$P(g|M) = \sum_{n_1}^{N} \theta_{n_1,n_1+1} \sum_{n_2}^{N} \theta_{n_2,n_2+1} \sum_{n_3}^{N} \theta_{n_3,n_3+1} \sum_{n_4}^{N} \theta_{n_4,n_4+1} \sum_{n_5}^{N} \theta_{n_5,n_5+1} \sum_{n_6}^{N} \theta_{n_6,n_6+1} \sum_{n_7}^{N} \theta_{n_7,n_7+1} P(g|n),$$

where we for simplicity set $x_{N+1}=\infty$ and $P(g|n)$ is given by

$P(g|n) = \prod_{i=1}^{N} P(g_i|n) = \prod_{i=1}^{N} \prod_{j=1}^{G} P(g_{ij}|n)$, where *i* runs over the loci and *j* runs over groups of individuals defined by the recombination pattern, *n*, such that individuals are identical by descent (i.e. sharing a haplotype) if and only if they are in the same group. For singletons $P(g_{ij}|n)$ is provided by the genotype caller as Phred-scaled genotype likelihoods and capped at 0.999 and for larger groups the likelihood is calculated similarly by conditioning on a shared allele within the group. This can viewed as a hidden Markov model in which the state of the model is defined by the grouping, i.e., which individuals are in linkage at that locus and it only depends on the grouping at the previous locus and the transition probabilities, i.e., the probabilities for recombination. The Viterbi method was used to determine the most likely path of the model, which corresponds where the recombinations occurred. The number of meioses, and thereby the age of the mutation, was estimated by maximizing the likelihood for the model and each permutation of the pedigree. The confidence interval was determined using Monte Carlo summation such that $\sum_{age \in CI} L \Big/ \sum L < 0.95$.

SUPPLEMENTAL REFERENCES

1. Wadt, K.A.W., Aoude, L.G., Krogh, L., Sunde, L., Bojesen, A., Grønskov, K., Wartacz, N., Ek, J., Tolstrup-Andersen, M., Klarskov-Andersen, M., et al. (2015). Molecular characterization of melanoma cases in Denmark suspected of genetic predisposition. PLoS One. *10*, 1–16.

2. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., et al. (2015). Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet. Med. *17*, 405–424.

3. Neuhausen, S.L., Mazoyer, S., Friedman, L., Stratton, M., Offit, K., Caligo, A., Tomlinson, G., Cannon-Albright, L., Bishop, T., Kelsell, D., et al. (1996). Haplotype and phenotype analysis of six recurrent BRCA1 mutations in 61 families: Results of an international study. Am. J. Hum. Genet. *58*, 271–280.

4. Sasa, G.S., Ribes-Zamora, A., Nelson, N.D., and Bertuch, A.A. (2012). Three novel truncating TINF2 mutations causing severe dyskeratosis congenita in early childhood. Clin. Genet. *81*, 470–478.

5. Norris, K., Walne, A.J., Ponsford, M.J., Cleal, K., Grimstead, J.W., Ellison, A., Alnajar, J., Dokal, I., Vulliamy, T., and Baird, D.M. (2021). High-throughput STELA provides a rapid test for the diagnosis of telomere biology disorders. Hum. Genet. *140*, 945–955.

6. Vulliamy, T., Beswick, R., Kirwan, M.J., Hossain, U., Walne, A.J., and Dokal, I. (2012). Telomere length measurement can distinguish pathogenic from non-pathogenic variants in the shelterin component, TIN2. Clin. Genet. *81*, 76–81.

7. Walne, A.J., Vulliamy, T., Beswick, R., Kirwan, M., and Dokal, I. (2008). TINF2 mutations result in very short telomeres: Analysis of a large cohort of patients with dyskeratosis congenita and related bone marrow failure syndromes. Blood. *112*, 3594–3600.

8. Gutierrez-Rodrigues, F., Donaires, F.S., Pinto, A., Vicente, A., Dillon, L.W., Clé, D. V., Santana, B.A., Pirooznia, M., Ibanez, M. del P.F., Townsley, D.M., et al. (2019). Pathogenic TERT promoter variants in telomere diseases. Genet. Med. *21*, 1594–1602.

9. Sakwit, A., Rojanaporn, D., Mekjaruskul, P., Suriyajakryuththana, W., Sasanakul, W., and Sirachainan, N. (2019). Novel mutation of the TINF2 gene resulting in severe phenotypic Revesz syndrome. Pediatr. Blood Cancer. *66*, 1–2.

10. Yamaguchi, H., Sakaguchi, H., Yoshida, K., Yabe, M., Yabe, H., Okuno, Y., Muramatsu, H., Takahashi, Y., Yui, S., Shiraishi, Y., et al. (2015). Clinical and genetic features of dyskeratosis congenita, cryptic dyskeratosis congenita, and Hoyeraal-Hreidarsson syndrome in Japan. Int. J. Hematol. *102*, 544–552.

11. Fukuhara, A., Tanino, Y., Ishii, T., Inokoshi, Y., Saito, K., Fukuhara, N., Sato, S., Saito, J., Ishida, T., Yamaguchi, H., et al. (2013). Pulmonary fibrosis in dyskeratosis congenita with TINF2 gene mutation. Eur. Respir. J. *42*, 1752–1759.

12. Muramatsu, H., Okuno, Y., Yoshida, K., Shiraishi, Y., Doisaki, S., Narita, A., Sakaguchi, H., Kawashima, N., Wang, X., Xu, Y., et al. (2017). Clinical utility of next-generation sequencing for inherited bone marrow failure syndromes. Genet. Med. *19*, 796–802.

13. Thaventhiran, J.E.D., Lango Allen, H., Burren, O.S., Rae, W., Greene, D., Staples, E., Zhang, Z., Farmery, J.H.R., Simeoni, I., Rivers, E., et al. (2020). Whole-genome sequencing of a sporadic primary immunodeficiency cohort. Nature. *583*, 90–95.

14. Bluteau, O., Sebert, M., Leblanc, T., De Latour, R.P., Quentin, S., Lainey, E., Hernandez, L., Dalle, J.H., De Fontbrune, F.S., Lengline, E., et al. (2018). A landscape of germ line mutations in a cohort of inherited bone marrow failure patients. Blood. *131*, 717–732.