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## ORIGINAL RESEARCH

# NEK11 as a candidate high-penetrance melanoma susceptibility gene

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**ABSTRACT**

**Background** A proportion of patients diagnosed with cutaneous melanoma reports a positive family history. Inherited variants in *CDKN2A* and several other genes have been shown to predispose to melanoma; however, the genetic basis of familial melanoma remains unknown in most cases. The objective of this study was to provide insight into the genetic basis of familial melanoma.

**Methods** In order to identify novel melanoma susceptibility genes, whole exome sequencing (WES) analysis was applied in a Dutch family with melanoma. The causality of a candidate variant was characterised by performing cosegregation analysis in five affected family members using patient-derived tissues and digital droplet PCR analysis to accurately quantify mutant allele frequency. Functional in-vitro studies were performed to assess the pathogenicity of the candidate variant.

**Results** Application of WES identified a rare, nonsense variant in the *NEK11* gene (c.1120C>T, p.Arg374Ter), cosegregating in all five affected members of a Dutch family. *NEK11* (NIMA-related Kinase 11) is involved in the DNA damage response, enforcing the G2/M cell cycle checkpoint. In a melanoma from a variant carrier, somatic loss of the wildtype allele of this putative tumour suppressor gene was demonstrated. Functional analyses showed that the *NEK11* p.Arg374Ter mutation results in strongly reduced expression of the truncated protein caused by proteasomal degradation.

**Conclusion** The *NEK11* p.Arg374Ter variant identified in this family leads to loss-of-function through protein instability. Collectively, these findings support *NEK11* as a melanoma susceptibility gene.

**INTRODUCTION**

Cutaneous melanoma is an aggressive type of skin cancer resulting from malignant transformation of melanocytes. Incidences of melanoma continue to rise steadily, with more than 230 000 cases diagnosed each year worldwide, accompanied by 55 000 deaths.<sup>1</sup> Ten per cent of cases are found in people with familial predisposition, that is, families with at least two first degree relatives with melanoma.<sup>2</sup>

Several high-penetrance melanoma susceptibility genes have been identified and account for approximately 40% of melanoma families.<sup>3–4</sup> The majority of these families are affected by germline mutations in *CDKN2A*<sup>5</sup>, a key cell-cycle checkpoint regulator and first reported high-penetrance melanoma susceptibility gene.<sup>6–8</sup> Following *CDKN2A*, germline mutations in other genes have been linked

to familial predisposition to melanoma; these include *CDK4*<sup>9</sup> and *BAP1*.<sup>10–11</sup> Biallelic inactivation has been reported in tumour tissues with germline variants in *BAP1* including mesothelioma, uveal melanoma and cutaneous melanoma<sup>12–13</sup> suggesting that genetic analysis is an informative approach for discovering melanoma-predisposition genes. Considering the discovery of germline *MITF* variants<sup>14–15</sup> suggesting that genetic analysis in patient's tissues and functional validation adds to the current value of mutation screening. Application of whole exome sequencing (WES) analysis has been successful in identifying rare variants including *TERT* promoter,<sup>16</sup> *POT1*,<sup>17</sup> *TERF2IP* and *ACD*,<sup>18</sup> *GOLM1*,<sup>19</sup> *EBF3*<sup>20</sup> and *POLE*<sup>21</sup> as candidate high-penetrance melanoma susceptibility genes. Still, the genetic basis of over half of melanoma families remains unknown, impairing genetic testing and counselling in families with predisposition to melanoma.<sup>22–23</sup> Here, *NEK11* gene was identified by WES in a Dutch family with melanoma and characterised as a potential novel high-penetrance melanoma-susceptibility gene.

**METHODS****Whole exome sequencing (WES)****Study population and ethics approval**

WES was carried out in blood-derived DNA samples of two members of a Dutch familial melanoma family. Study approval was obtained by the ethics committee of Leiden University Medical Center (LUMC, P00.117).

**Sequencing analysis and bioinformatics**

Sequencing was performed on HiSeq2000 platform with TruSeq Exome Enrichment kit. Paired-end reads of 110 bp were generated with mean coverage of 40×. The Burrows-Wheeler aligner was used for mapping sequencing reads to the reference UCSC human genome. Single nucleotide variants (SNVs) were detected using samtools/bcftools. Indels were detected with Pindel and annotated to dbSNP144 using ANNOVAR. Variants altering the coding sequence were selected excluding those that were present at a frequency of 0.0005% or higher in the Kaviar (Known VARIants) control population database, including 162 million variants from human genomes of datasets such as ExAc and 1000Gs.<sup>24</sup>



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### Selection, validation and interpretation of variants

Variants identified were assessed to identify pathogenic or potentially pathogenic variants in ClinVar. Variants were then filtered using in-silico prediction algorithms to show if an alteration affects protein function. Details about criteria for interpretation of variants has been reported previously.<sup>25</sup> These included exonic, frameshift, non-synonymous SNVs, splicing and stop-gain SNVs. We then focused on segregating mutations between all family members and functional significance. Cosegregation of *NEK11* p.Arg374Ter mutation was confirmed using Sanger sequencing of germline DNA from family members 1, 4, 5, 7 and 12 (online supplementary table S1) (Macrogen, Amsterdam, The Netherlands).

### Genotyping and LOH analysis

DNA was extracted from primary melanoma FFPE tissue of a *NEK11* p.Arg374Ter mutation carrier (family member 5) using the QIAamp DNA formalin-fixed paraffin-embedded (FFPE) Tissue kit (QIAGEN, Venlo, The Netherlands) according to manufacturer's instructions. DNA extraction of normal and tumour tissue was obtained through microdissection/punch biopsy by the pathology department of LUMC, The Netherlands. Genomic primer sets were used to amplify the region of interest containing the mutation (*NEK11* c.1120C>T) and a common SNP (rs4974475, chr3:130882827, minor allele frequency (MAF) 17%), located at 2 kb upstream from the *NEK11* mutation site to verify Loss of Heterozygosity (LOH) (online supplementary table S1). PCR products were cleaned-up using a PCR clean-up protocol (Bio-Rad, Hercules, California, USA) and sequenced by Sanger sequencing analysis-long run (Macrogen, Amsterdam, The Netherlands). Chromatograms were then analysed by Chromas Technelysium DNA Sequencing Software (Technelysium Pty Ltd, South Brisbane, Australia).

### Droplet digital PCR (ddPCR)

Mutation detection assays specific for ddPCR (Bio-Rad, Hercules, California, USA) describe the incorporation of both wild-type and mutated targets in a single ddPCR mix. In this case, the assay was designed for the detection of *NEK11* p.Arg374Ter mutation. Detailed protocol of mutation detection ddPCR assay has been described previously<sup>26</sup> and ddPCR sequence information is provided in online supplementary table S1. QuantaSoft software (Bio-Rad, Hercules, California, USA) was used to analyse the data by calculating the concentration of the amplified ddPCR product (copies/ $\mu$ l).<sup>26</sup> The wild-type (WT) allele frequency was calculated by dividing the WT allele counts over the total allele counts and the mutant (MT) allele frequency was calculated by dividing MT allele counts over the total allele counts.

### Cell culturing and maintenance

U2OS (human osteosarcoma tumour cell line) and FM6 (human cutaneous malignant melanoma cell line) cells were maintained in DMEM medium supplemented with penicillin (100 I.U./mL)/streptomycin (100  $\mu$ g/mL) and 10% Fetal bovine serum (FBS) and glutamax 100 $\times$  (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All cells were grown in a humidified incubator at 37°C and 5% CO<sub>2</sub> and routinely subcultured when reaching 95% confluency.

### Plasmid construction and introduction of *NEK11* p.Arg374Ter mutation

*NEK11*-FL (full-length isoform<sup>27</sup>) was expressed from a plasmid construct containing WT *NEK11* cDNA, fused with N-terminal

FLAG-epitope tag (Kind gift from professor Andrew Fry, University of Leicester, UK) for expression in U2OS and FM6 cells.<sup>27</sup> Site-directed mutagenesis was applied to introduce *NEK11* p.Arg374Ter mutation in flag-tagged *NEK11* expression vectors. Primer-sets were designed specifically targeting the mutation site of *NEK11* exonic sequence (online supplementary table S1). Thermal cycling was performed to introduce the mutation consisting of 1 min denaturation at 95°C, followed by 10 cycles of 1 min steps at 95°C, 63°C and 68°C. The PCR product was then digested with *DpnI* enzyme and transformed into Top10 bacteria to produce inducible vectors for functional experiments. Sequences of both *NEK11* WT and MT expression vectors were confirmed by Sanger Sequencing analysis long-run (Macrogen, Amsterdam, The Netherlands) (online supplementary table S1).

### Lentivirus production

*NEK11* WT and MT cDNAs were recloned into a lentiviral backbone containing the neomycin resistance gene. Lentiviral stocks were produced by transfections into HEK-293T cells as described previously<sup>28</sup> but calcium phosphate was replaced with polyethylenimine (PEI) in the transfection mix. Virus was quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix, New York, New York, USA).

### Transient transfections

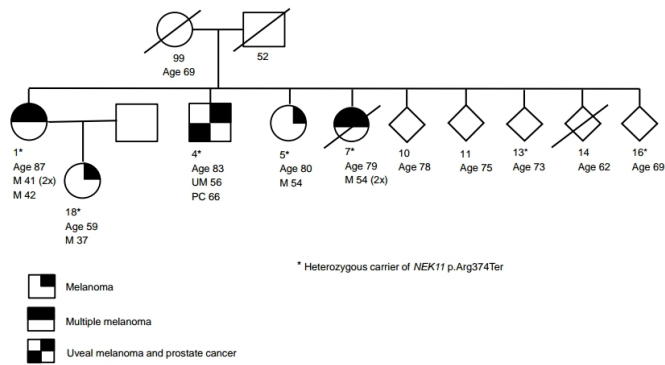
U2OS cells were harvested and seeded in appropriate growth medium in 6-well plates (0.5 $\times$ 10<sup>5</sup> cells/mL) and 60 mm dishes (1.8 $\times$ 10<sup>5</sup> cells /mL). The DNA mix was prepared as follows: 0.8  $\mu$ g *pLV-NEO-NEK11-WT*, *pLV-NEO-NEK11-MT* and *pLV-NEO-empty* lentiviral vectors (see lentivirus production section), 0.1  $\mu$ g *Tomato-Red*, 300 ng of *GFP* expression vector and 0.2  $\mu$ g *pSuper*. The PEI mix was prepared as follows: 3:1 PEI (3 parts of PEI to 1 part of DNA concentration) diluted in Gibco Opti-MEM Reduced Serum medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA). PEI mix was added slowly to DNA mix followed by a short vortex. The mixture was kept at RT for 20 min and then added dropwise to U2OS cells. Growth medium was replaced 16 hours after transfection and U2OS cells were further incubated for another 24 hours.

### Lentiviral transductions

Fresh culture media were prepared with viral supernatants supplemented with 8  $\mu$ g/mL polybrene (Sigma-Aldrich, St. Louis, Missouri, USA). FM6 cells were seeded in 6-well plates at a density of 2 $\times$ 10<sup>5</sup> cells/well. FM6 cells were incubated with virus-containing medium overnight, after which the cells were refed with fresh medium containing G418 (Sigma-Aldrich, St. Louis, Missouri, USA) to produce stable cell lines expressing *NEK11* WT and MT by using neomycin as selection marker.

### RNA isolation, cDNA synthesis and gene expression analysis

Lymphocytic RNA of a *NEK11* p.Arg374Ter carrier (family member 18) and a non-relative spouse was isolated using the RNeasy micro kit from QIAGEN (Venlo, The Netherlands). RNA was isolated from FM6 cells using the SV total RNA isolation kit (Promega, Fitchburg, Wisconsin, USA). First strand cDNA synthesis was carried out using the iScript c-DNA synthesis kit (Bio-Rad, Hercules, California, USA) and Sanger Sequencing analysis (LGTC, LUMC, The Netherlands) was used to detect presence of *NEK11* WT and MT alleles (Primer sequences shown in online supplementary table S1). *NEK11* gene expression was confirmed using SYBR green based quantitative PCR on CFX384 Touch Real-Time PCR Detection System (Bio-Rad,



**Figure 1** Cosegregation of *NEK11* p.Arg374Ter in a Dutch melanoma family. Whole-exome sequencing was carried out for family members 1 and 4. Cosegregation of *NEK11* p.Arg374Ter was confirmed by analysing germline DNA from all family members. Current age and age at death of deceased individuals (those reported) are indicated. Age at diagnosis of each tumour type is noted in affected family members. M, melanoma; PC, prostate cancer; UM, uveal melanoma.

Hercules, California, USA) (online supplementary table S1). Gene expression results were analysed using Bio-Rad CFX Manager 3.1 Software (Hercules, California, USA) and corrected relative to reference gene expression (*CAPNS1* and *SRPR*) as well as transfection efficiency target *Tomato-Red* (online supplementary table S1).

### Immunofluorescence staining

Transfected U2OS cells on cover slips were washed twice in phosphate-buffered saline (PBS) solution and fixed with 4% paraformaldehyde for 10 min. Cover slips with U2OS cells were then incubated for 10 min in PBS/0.2% Triton-X100 (permeabilisation) and preincubated for 30 min in PBS/0.05% Tween-20 containing 5% normal goat serum (NGS). Subsequently, U2OS cells were incubated with monoclonal anti-flag M2 Catalog Number F1804 (Sigma-Aldrich, St. Louis, Missouri, USA) diluted in PBS/Tween/NGS (1:500) for 60 min at room temperature followed by washing three times in PBS/Tween for a total of 15 min. The secondary antibody, anti-Mouse IgG-Cy2 (#115-225-146, Jackson ImmunoResearch Laboratories, Cambridge, UK) was diluted in PBS/Tween/NGS (1:100) and added at room temperature in the dark. U2OS cells were finally washed for 10 min with PBS/Tween and coverslips were placed on slides for analysis on a Leica DMRA fluorescent microscope (Nijmegen, The Netherlands).

### Drug treatments

MG132 proteasome inhibitor (Sigma-Aldrich, St. Louis, Missouri, USA) was added to U2OS and FM6 cells at a final concentration of 20  $\mu$ M for 5–6 hours before RNA and protein isolation procedures. The cycloheximide (CHX), translation inhibitor, was added at a concentration of 50  $\mu$ g/mL (Sigma-Aldrich, St. Louis, Missouri, USA) to FM6 stable-cell lines expressing *NEK11* WT and *NEK11* MT as a time-course treatment of 0, 1, 2 and 4 hours.

### Protein isolation and Western immunoblotting analysis

U2OS and FM6 cells were washed twice in ice-cold PBS and incubated on ice for 10 min in Giordano buffer (50 mM Tris-HCl pH7.4, 250 mM NaCl, 0.1% Triton X-100 and 5 mM EDTA; supplemented with phosphatase and protease inhibitors). Lysates were collected by scraping and centrifuged at max speed

for 10 min and protein concentration was determined using the Bradford method. Western Blot procedure was followed as described previously.<sup>29</sup> *NEK11* protein was detected by the Anti-Flag antibody (1:1000) (Sigma-Aldrich, St. Louis, Missouri, USA) and the controls were detected by Anti-USP7 (1:1000) (Bethyl Laboratories, Biomol, Montgomery, Texas, USA), Anti-GAPDH (1:1000) (Sigma-Aldrich, St. Louis, Missouri, USA) and Anti-P53 (1:1000) (Santa Cruz Biotechnology, Heidelberg, Germany). After transient expression, protein levels were determined using the Odyssey machine (LI-COR, Lincoln, Nebraska, USA) and analysed using Odyssey software according to manufacturer's instructions. Secondary antibodies used were IRDye 800CW Goat anti-Mouse IgG (H+L), 0.1 mg (1:5000) and IRDye 680LT Goat anti-Rabbit IgG (H+L), 0.1 mg (1:5000) (LI-COR, Lincoln, Nebraska, USA). The bands were analysed with Image lab software (Bio-Rad, Hercules, California, USA) according to manufacturer's instructions. Protein-expression quantification was performed relative to unaffected expression of controls (GAPDH, USP7).

### Statistical analysis

All data were analysed by calculating the mean and SD and graphs were obtained in GraphPad Prism V.7 (GraphPad software, San Diego, California, USA). Analysis of variance and multiple comparisons were applied to detect statistically significant differences between expression patterns of three independent experiments ( $n=3$ ). Statistical significance was reached when  $p<0.05$ .

## RESULTS

### WES analysis and identification of *NEK11* p.Arg374Ter

A Dutch melanoma family presented four melanoma cases and one uveal melanoma case with prostate cancer. The diagnosis of melanoma in five members in multiple generations strongly suggests an autosomal-dominant mode of inheritance (figure 1) and members were negative for mutations in established melanoma susceptibility genes (*CDKN2A*, *BAP1*, *POT1*, *TERT*, *TERF2IP*, *ACD*, *MITE*, *GOLM1*, *EBF3*). A WES analysis was carried out on DNA from blood cells of two affected members (figure 1). Among a total of 19 rare, cosegregating non-synonymous variants that met our criteria, 17 were missense mutations, probably damaging, predicted deleterious.<sup>30 31</sup> These variants, however, are not plausible candidate melanoma susceptibility genes (table 1) since there is no evidence supporting a strong tumorigenic effect based on published literature. Interestingly, two were nonsense stop-gain SNVs (table 1). One candidate stop-gain variant was p.Arg66Ter in *ZNF192*, a gene possibly regulating transcription. However, no implications in cancer have been mentioned in published literature. In contrast, the other candidate was a truncating variant (p.Arg374Ter) in the never in mitosis-gene A (NIMA)-related kinase 11 (*NEK11*). This family of proteins functions in different aspects of cell cycle regulation, although the in-depth role of NIMA-related kinases remains to be uncovered.<sup>32</sup> *NEK11* has been reported to be somatically mutated in different types of cancer, including lung, breast, prostate and melanoma.<sup>33</sup> The frequency of *NEK11* mutations was  $>5\%$  in melanomas<sup>34 35</sup> suggesting a plausible candidate in melanoma development.

Sanger sequencing confirmed *NEK11* p.Arg374Ter to cosegregate within four cutaneous melanoma cases and one uveal melanoma case in this Dutch melanoma family (figure 1). Family



**Table 1** Summary of WES analysis and identification of segregating novel predicted damaging/deleterious variants in a Dutch melanoma family

Gene	Change	Ch	Ref	Alt	Type*	SIFT	Polyphen	MAF†
<i>GPATCH3</i>	p.Gly131Arg	1	C	T	Missense	Deleterious	probably_damaging	0.00006168
<i>ATPIF1</i>	p.Arg94His	1	G	A	Missense	Deleterious	probably_damaging	0.00004406
<i>KALRN</i>	p.Ser1629Cys	3	C	G	Missense	Deleterious	probably_damaging	0.00002641
<i>NEK11</i>	p.Arg374Ter	3	C	T	Stop-gained			0.00002641
<i>ZNF192</i>	p.Arg66Ter	6	C	T	Stop-gained			0.000008791
<i>GPR111</i>	p.Ser168Arg	6	T	A	Missense	Deleterious	probably_damaging	0.00006486
<i>GPAM</i>	p.Pro403Thr	10	G	T	Missense	Deleterious	probably_damaging	0.000008794
<i>NELL1</i>	p.Val755Met	11	G	A	Missense	Deleterious	probably_damaging	0.0002261
<i>KRT77</i>	p.Asp316Asn	12	C	T	Missense	Deleterious	probably_damaging	0.00001502
<i>OAS2</i>	p.Tyr269Cys	12	A	G	Missense	Deleterious	probably_damaging	–
<i>DNAJC3</i>	p.Arg346Gln	13	G	A	Missense	Deleterious	probably_damaging	0.0001553
<i>TEP1</i>	p.Arg1386Trp	14	G	A	Missense	Deleterious	possibly_damaging	0.001266
<i>PLCB2</i>	p.Arg253Trp	15	G	A	Missense	Deleterious	probably_damaging	0.001074
<i>DNASE1</i>	p.Ala168Val	16	C	T	Missense	Deleterious	possibly_damaging	0.001402
<i>BFAR</i>	p.Phe53Ile	16	T	A	Missense	Deleterious	possibly_damaging	–
<i>ITGB4</i>	p.Arg556Cys	17	C	T	Missense	Deleterious	possibly_damaging	0.0006357
<i>PSG7</i>	p.Trp67Ser	19	C	G	Missense	Deleterious	probably_damaging	0.0001473
<i>GPR50</i>	p.Gly93Ala	X	G	C	Missense	Deleterious	possibly_damaging	0.00001240
<i>GABRQ</i>	p.Arg254Cys	X	C	T	Missense	Deleterious	probably_damaging	0.00007713

\*Variants characterised by ExAC/gnomAD/Genome of The Netherlands/Ensembl databases.

†MAF in European (Non-Finnish) population.

WES, whole-exome sequencing.

members 13 and 16 were also found to be *NEK11* p.Arg374Ter carriers although with no clinical presentation of cancer/melanoma. Considering the current age of these individuals and absence of the melanoma phenotype, the possibility for non-penetrance is very likely (figure 1). Somatic loss of the wild-type (WT) allele was detected in primary cutaneous melanoma tissue of a mutation carrier (figure 2A–C) and confirmed by the highly sensitive and quantitative method, droplet digital PCR (ddPCR)<sup>36</sup> whereby, a higher fraction of *NEK11* p.Arg374Ter mutant (MT) than *NEK11* WT allele was detected in melanoma tissue when compared with normal tissue micro dissected from the same biopsy sample (figure 2D). Furthermore, examination of a common SNP (rs4974475, chr3:130882827, MAF 17%), showed loss of this variant in the melanoma tissue of a *NEK11* p.Arg374Ter carrier, suggesting LOH over a longer genetic region (online supplementary figure S1). Collectively, these data suggest a potential loss-of-function (LOF) mutagenic effect of *NEK11* p.Arg374Ter.

### Functional analysis of *NEK11* p.Arg374Ter

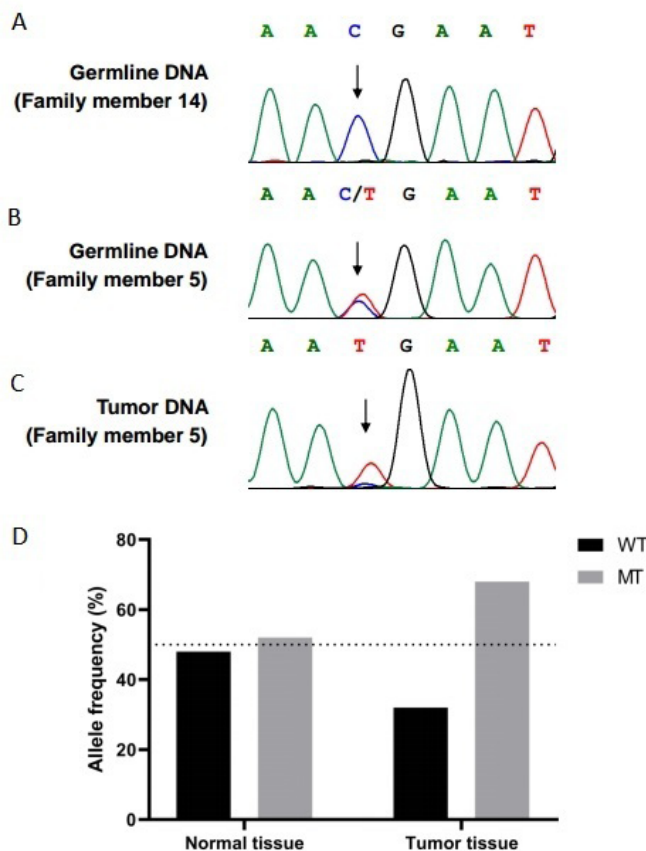
Following genetic characterisation of *NEK11* p.Arg374Ter as a potential LOF mutation, functional analyses were performed to investigate the effects of this truncating mutation on the expression level of *NEK11* mRNA and *NEK11* protein. On transient transfection of *NEK11* expressing plasmids in osteosarcoma tumour cell line U2OS, the expression of *NEK11* MT mRNA was lower than *NEK11* WT mRNA, although the difference was statistically not significant (online supplementary figure S2). U2OS cell-line provided the ideal conditions for functional analysis since it is an easily transfectable, fast-growing cell line and has been previously used to functionally characterise *NEK11*.<sup>27 37 38</sup> *NEK11* MT mRNA expression was detected in lymphocyte mRNA of a mutation carrier by Sanger sequencing and ddPCR analyses (figure 3A–C) suggesting that the premature stop codon does not result in significant transcript degradation by nonsense-mediated mRNA decay (NMD). Combined with the finding that the mutant *NEK11* mRNA was detected

in U2OS cells (online supplementary figure S2), these results indicate no significant effect of the mutation on *NEK11* mRNA expression levels.

Introduction of p.Arg374Ter mutation in the *NEK11* expression vector, resulted in synthesis of a truncated *NEK11* protein lacking the whole C-terminal PEST-like domain as well as part of the coiled-coil motifs (online supplementary figure S3a). The coiled-coil region regulates protein activation suggesting that loss or absence of these motifs would affect protein function.<sup>39</sup> The truncated protein runs at approximately 45 kDa, which reasonably fits with the size of 373 amino acids (online supplementary figure S3a). Immunoblot analysis of protein lysates made from transfected U2OS cells showed that the level of the truncated protein was threefold lower than *NEK11* WT expression ( $p < 0.005$ ; figure 4A, B) when corrected for mRNA expression. Treatment with the proteasome inhibitor MG132 increased *NEK11* protein level, particularly of the truncated product (~2-fold). Still, the difference between *NEK11* WT and MT protein levels in lysates of MG132-treated U2OS cells is significant ( $p < 0.005$ ). Collectively, statistically significant lower protein expression was correlated with the *NEK11* p.Arg374Ter mutation.

Since a distinct subcellular localisation of *NEK11*-FL (645 amino acids) and *NEK11*-S (450 amino acids) has been reported,<sup>27</sup> which might affect the protein expression level, the subcellular localisation of the Flag-tagged *NEK11* MT was investigated in comparison to Flag-tagged *NEK11*-FL. Interestingly, both proteins were mainly localised in the nucleus of U2OS cells (online supplementary figure S3b), in contrast to the earlier publication. However, in that publication GFP-tagged constructs were used. Indeed, using the same GFP-tagged constructs the subcellular localisation of *NEK11*-FL and *NEK11*-S was as reported; the GFP-*NEK11* MT protein localised in the nucleus, similar to GFP-*NEK11*-S (data not shown).

Since transient overexpression yields very high expression levels which might partly mask normal regulation of *NEK11* protein expression, a putative difference in protein expression of the *NEK11* WT and the *NEK11* MT was studied in more

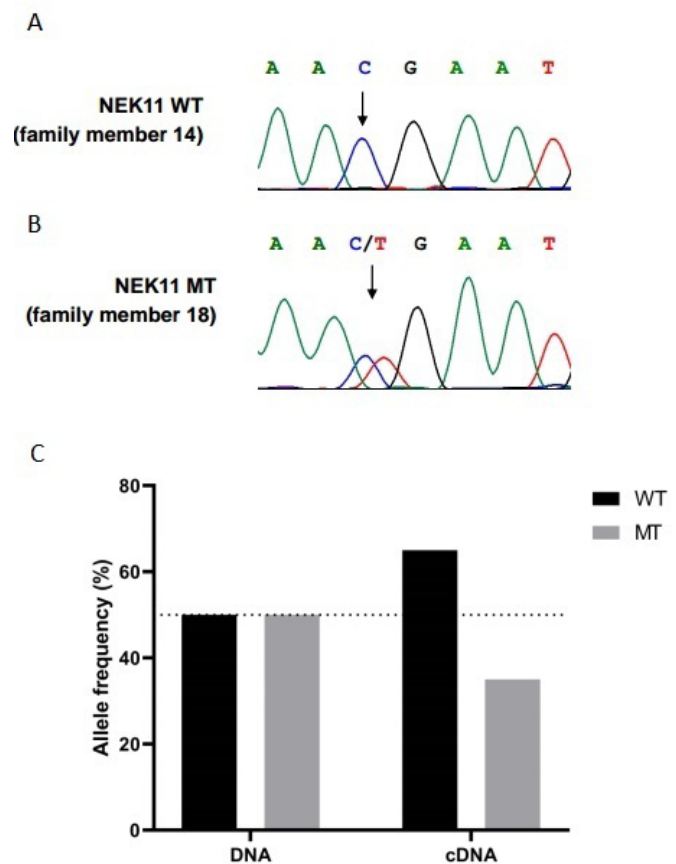


**Figure 2** LOH analysis of a *NEK11* p.Arg374Ter mutation carrier. Chromatogram showing DNA sequence of (A) healthy family member (14), (B) a *NEK11* p.Arg374Ter carrier (family member 5) and (C) tumour of a *NEK11* p.Arg374Ter carrier (family member 5). Arrows indicate the *NEK11* p.Arg374Ter mutation site. (D) ddPCR *NEK11* mutation assay showing the *NEK11* (WT) and *NEK11* p.Arg374Ter (MT) allele frequency detected in normal and tumour tissue from FFPE derived DNA of family member 5. WT, wildtype.

detail in stably-transduced, disease-relevant FM6 cutaneous melanoma cells. Strikingly, *NEK11* MT protein expression was hardly detectable with sixfold difference compared with *NEK11* WT when corrected to mRNA levels ( $p=0.0024$ ) (figure 5A, B). Furthermore, treating FM6 cells with MG132 strongly increased *NEK11* MT protein level (figure 5C), while the effect of MG132 on *NEK11* WT levels was much less pronounced, suggesting that the truncated *NEK11* protein is prone to faster protein degradation. To examine protein half-life of the *NEK11* WT and MT proteins, we decided to treat these FM6 cells with the protein translation inhibitor CHX and harvest at different time-points. The *NEK11* WT protein appeared to be a stably expressed protein with half-life of approximately 4 hours, in contrast to the *NEK11* MT protein showing a half-life of approximately 1 hour in FM6 cells (figure 6A–C). Collectively, we provide evidence that the *NEK11* p.Arg374Ter mutation leads to the synthesis of a truncated protein with a very short half-life, suggesting a LOF mutation, supporting a tumour-suppressive role for *NEK11* in familial melanoma.

## DISCUSSION

Here, a novel nonsense protein truncating variant (PTV) in *NEK11* p.Arg374Ter was identified as a possible familial melanoma predisposition mutation in a Dutch family. The possibility

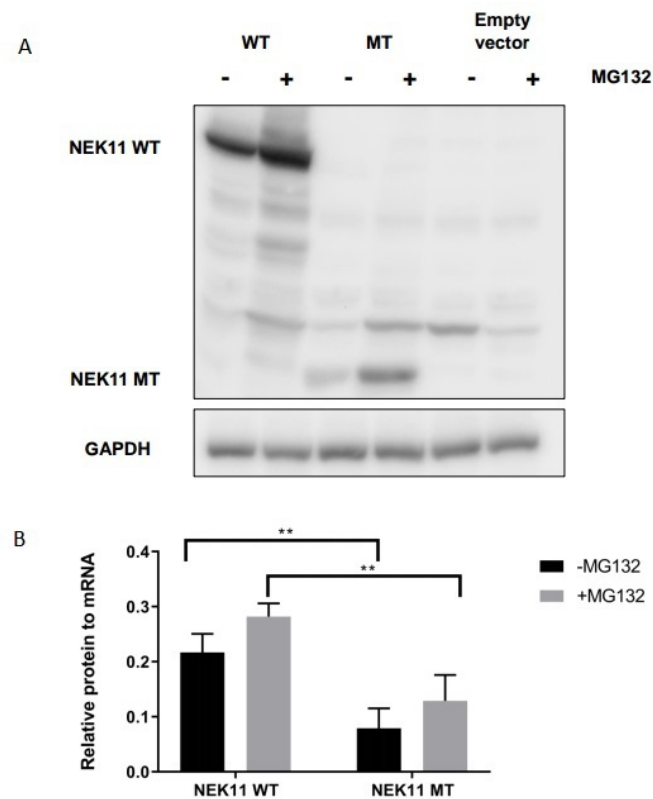


**Figure 3** *NEK11* wildtype and *NEK11* p.Arg374Ter mRNA analysis. Chromatogram showing sequence from cDNA of (A) healthy family member 14 and (B) a *NEK11* p.Arg374Ter carrier (family member 18). Arrows indicate the *NEK11* p.Arg374Ter mutation site. (C) Allele frequency of *NEK11* WT and *NEK11* p.Arg374Ter (MT) detected by ddPCR *NEK11* mutation assay using DNA and cDNA from family member 18. WT, wildtype.

of any other potentially damaging variants found by WES in this family to either be causal or contributing to the melanoma-risk of this family was considered. Since not enough scientific evidence was available to support a contributing role, these variants were not investigated further.

*NEK11* has been initially characterised as a DNA-damage response kinase with two isoforms, the full-length isoform consisting of 645 residues (*NEK11*-FL) and the short isoform consisting of 470 residues (*NEK11*-S).<sup>37</sup> A regulatory effect during IR-induced G2/M cell-cycle arrest has been described, that is, *NEK11* was shown to be involved in phosphorylation of *CDC25A* triggering its degradation and ultimate blocking of progression into mitosis.<sup>27 38 40</sup> *NEK11* has been described to (de) regulate G2/M cell-cycle arrest in colorectal carcinoma and low expression was observed at late-advanced stages of the disease.<sup>27 40</sup> Furthermore, decreased *NEK11* mRNA levels have also been associated with drug resistance in ovarian cancer cells<sup>41</sup> supporting that *NEK11* may prevent metastatic progression in ovarian cancer. Collectively, these results point towards a putative tumour suppressive role of *NEK11*.

*NEK11* expression follows a cell-cycle dependent manner with a peak at G2/M phase<sup>38</sup> and mRNA expression is found in the brain, uterus and lungs with moderate expression in melanoma (median expression=6).<sup>42 43</sup> No significant difference in expression between benign nevi and melanomas can be observed;<sup>34</sup> however, cutaneous melanoma patients with higher

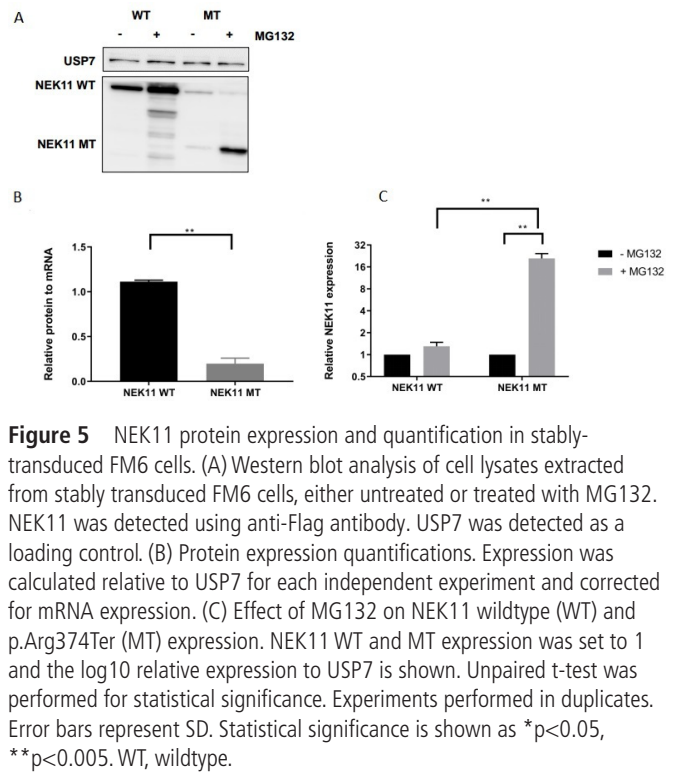


**Figure 4** Expression of NEK11 wildtype and p.Arg374Ter in U2OS cells. (A) Lysates of U2OS cells transiently transfected with *NEK11* wildtype (WT), *NEK11* p.Arg374Ter (MT) and *pLV-empty* expression plasmids were either untreated or treated with MG132. NEK11 was detected with anti-Flag antibody. GAPDH was determined as a loading control. (B) Expression was calculated relative to GAPDH for each independent experiment and corrected for mRNA expression of *NEK11*. Data shown represent mean expression from three independent experiments. Error bars represent SD. Statistical significance is shown as \* $p < 0.05$ , \*\* $p < 0.005$ .

*NEK11* expression have slightly improved survival, although this association is not statistically significant.<sup>35</sup> Moreover, *NEK11* has been suggested to play a role in the G1/S checkpoint in association with *NEK2*; however, the exact mechanism remains unknown.<sup>39-44</sup> Therefore, these data suggest that *NEK11* could be regarded an interesting target to validate as a high-penetrance melanoma susceptibility gene.

Genetic analysis confirmed LOH in the melanoma tissue of a mutation carrier. Expression of *NEK11* MT and *NEK11* WT allele is detected in lymphocytic RNA indicating that the mutant transcript is not degraded by NMD, confirmed by mRNA expression analysis in transfected U2OS cells.

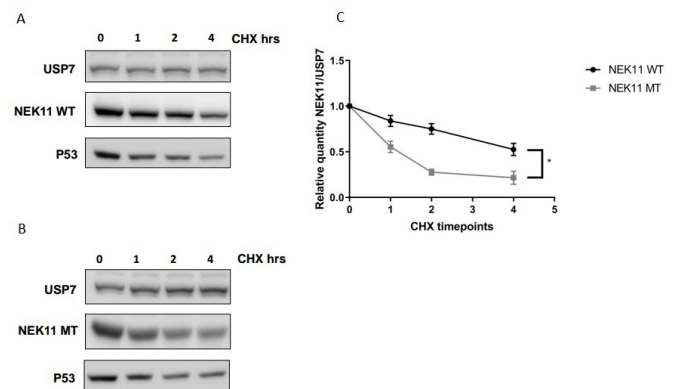
The oncogenicity of the *NEK11* p.Arg374Ter mutation could be caused by two possible scenarios. First, a gain-of-function mutation, as the non-catalytic C-terminal domain was shown to have an autoinhibitory effect on protein function;<sup>44</sup> thus, loss of this domain could activate the kinase activity. Alternatively, the mutation might lead to the synthesis of a non-functional truncated protein, for example, by loss of coiled-coil domain motifs (online supplementary figure S2a). Here, we provide data strongly suggesting a LOF of the *NEK11* p.Arg374Ter mutation. Collectively, our results implicate that the truncated *NEK11* protein has a very short half-life, implying that the mutant protein is not significantly expressed in cells and reflects a loss-of-function. Since loss of *NEK11* abrogates the G2/M cell cycle



**Figure 5** NEK11 protein expression and quantification in stably-transduced FM6 cells. (A) Western blot analysis of cell lysates extracted from stably transduced FM6 cells, either untreated or treated with MG132. NEK11 was detected using anti-Flag antibody. USP7 was detected as a loading control. (B) Protein expression quantifications. Expression was calculated relative to USP7 for each independent experiment and corrected for mRNA expression. (C) Effect of MG132 on NEK11 wildtype (WT) and p.Arg374Ter (MT) expression. NEK11 WT and MT expression was set to 1 and the log10 relative expression to USP7 is shown. Unpaired t-test was performed for statistical significance. Experiments performed in duplicates. Error bars represent SD. Statistical significance is shown as \* $p < 0.05$ , \*\* $p < 0.005$ . WT, wildtype.

arrest on DNA damaging agents and can induce apoptosis,<sup>25</sup> it is very well possible that LOF results in genomic instability with the possible selection of cells with increased survival and proliferation, stimulating the acquirement of additional mutations and the development into a tumour.

Unfortunately, our analyses of *NEK11* p.Arg374Ter mutation were restricted by the limited availability of relevant (tumour) tissue, as we only had access to melanoma tissue and lymphocytic RNA from one affected family member. Analysis of tumour tissue from more affected family members could strengthen the case for *NEK11* as a novel melanoma-susceptibility gene. Moreover, the *NEK11* p.Arg374Ter mutation had 14 submissions in



**Figure 6** NEK11 protein half-life analysis in FM6 cells using CHX treatments. Time-course CHX treatments of FM6 cells expressing (A) *NEK11* WT and (B) *NEK11* p.Arg374Ter (MT). USP7 was detected as a loading control and P53 as a positive control. (C) Quantification of NEK11 WT and MT corrected for USP7 expression over different time-points of CHX treatments. Error bars represent SD. Pearson R squared correlation value was 0.82. Statistical significance is shown as \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ . CHX, cycloheximide; WT, wildtype.



dbSNP, although frequency of the alternate allele was extremely low (0–0.00003) and was not found in the Genome of The Netherlands (GoNL) database.<sup>30 45 46</sup> In a recent study, >300 000 UK WES/WGS non-melanoma data sets were analysed for non-synonymous PTVs;<sup>47</sup> however, no mutations were identified in *NEK11*, further strengthening *NEK11* to be a novel but rare melanoma-susceptibility gene and p.Arg374Ter as a potential pathogenic mutation.

As to why this family is predisposed to develop melanoma and not a different tumour type, we cannot conclude based on data from a single family. The increased risk of only one or a few tumour types is common in monogenic tumour predisposition syndromes.<sup>48</sup> Furthermore, the absence of any *NEK11* mutation in 488 Dutch familial melanoma cases<sup>49</sup> warrants screening for *NEK11* mutations in melanoma families worldwide in order to confirm the importance of *NEK11* as a melanoma-susceptibility gene.

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**Contributors** RvD and NG formulated research goals and aims. WES analysis was performed by NKH. Experiments were performed by EC, MVJ, AT, MVE. Pdv supervised Digital PCR analyses. AJ supervised functional analyses in cell lines. Statistical analysis was performed by EC. Overall progress was supervised by RvD, AJ and NG.

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