Functional characterization of the 9q34.13 locus identifies *RAPGEF1* as modulating risk for melanoma and nevi via RAS activation

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Abstract

Genome-wide association studies identified a melanoma- and nevus count-associated locus on chromosome band 9q34.13. Fine-mapping and melanocyte expression data collectively suggest two potential causal genes with opposite association with risk: higher levels of Rap guanine nucleotide exchange factor 1 (*RAPGEF1*) and lower levels of uridine-cytidine kinase 1 (*UCK1*). Colocalization analyses and conditional TWAS suggest multiple causal *cis-*regulatory sequence variants in partial linkage disequilibrium (LD) to each other. Melanocyte capture-HiC and CRISPR-inhibition demonstrated regulatory interactions between fine-mapped variants and the *RAPGEF1* and *UCK1* promoters. Focusing on *RAPGEF1*, we demonstrate *RAPGEF1* expression promotes melanocyte growth and drives malignant transformation of human immortalized melanocytes. Following treatment with human EGF, *RAPGEF1* overexpression activated both RAP1 and RAS. Further, we show *RAPGEF1* expression is significantly enriched in melanomas lacking strongly activating RAS-MAPK mutations, suggesting that *RAPGEF1* may promote oncogenic RAS-MAPK signaling in melanomas. Furthermore, in these tumors, we provide preliminary evidence to support the prognostic relevance of *RAPGEF1* expression in patients lacking *RAS* or *BRAF* mutations. Together with other recent studies, these data suggest that germline variation influencing RAS activation may play a key role in nevus development and melanoma risk.

Introduction

Meta-analysis of genome-wide association studies (GWAS) for melanoma have identified 54 loci and 68 independent signals associated with melanoma risk [1]. Despite the success in identifying loci, the causal sequence variants and specific genes at these loci that influence risk and melanoma development remain to be established. Linkage disequilibrium (LD) typically results in numerous risk-associated variants, complicating efforts to identify causal variants. Further, most fine-mapped variants at complex trait loci identified by GWAS are not protein coding; instead most causal variants alter *cis*-regulatory activity of regulatory sequences including enhancers and promoters [2, 3]. Given that enhancers can act at distances of 1Mb or more [4, 5], numerous genes near risk-associated loci may be plausible causal candidates.

Identifying causal sequence variants and their gene targets is important to understanding potentially unappreciated key processes underlying risk, tumor development, and or tumor progression. Of known risk loci, the majority are also associated with heritable melanoma risk-associated traits [1], including hair, skin or eye color [1, 6], telomere length [1, 7-9], and number of melanocytic nevi [1, 10-12]. While these pleiotropic associations can provide clues to the likely causal genes at melanoma risk loci, establishing causal genes for most loci remains a challenge.

We and others have previously generated multiple genome- or GWAS-scale genomic, transcriptomic, epigenomic, chromatin conformation, and functional screening datasets in melanocytic cells or tissues relevant to melanoma risk [13-24]. Here we layer analyses of these datasets and characterize a melanoma risk and nevus count locus on chromosome band 9q34.13 [1] near the 3▯-end of RAP guanine nucleotide exchange factor 1 (*RAPGEF1*, also known as C3G) and establish the *RAPGEF1* gene as one of two potential causal candidate genes at this locus. We demonstrate that *RAPGEF1* expression promotes melanocyte proliferation, increases colony formation in immortalized melanocytes, and leads to both RAP1 and RAS activation. Finally, we demonstrate higher expression of *RAPGEF1* in melanoma tumors lacking RAS-pathway driver mutations consistently across multiple tumor sequencing datasets. These data suggest that germline modulation of RAS activity may play a key role in both nevus and melanoma development.

Methods

Genetic fine-mapping at the melanoma risk locus on chromosome band 9q34.13

We fine-mapped the melanoma risk locus on chromosome band 9q34.13 (a ±500 Kb

window surrounding rs3780269) to identify potential causal variants. We applied multiple

Bayesian fine-mapping methods including SuSiE (Sum of Single Effects) [25, 26], DAP-G [27,

28] (Deterministic Approximation of the Posteriors), and RSparsePro [29], which is robust to

mismatching LD between the GWAS and the LD-reference panels. We used default

parameters for each method. The min_abs_corr parameter was set to 0 for SuSiE.

Melanoma GWAS Z-score values for each variant were from the fixed-effect inverse variance

weighted meta-analysis of the full set of confirmed and self-reported melanoma cases and

control, as described previously by Landi and colleagues [1]. We used a pre-computed LD

matrix of 5,000 UK Biobank individuals as described previously [1]. While we allowed for a

maximum of five credible sets during fine-mapping, we only retained individual credible sets

where the cumulative posterior inclusion probability was > 0.85, and retained all variants in

the credible set where the individual variant posterior inclusion probability was > 0.005. To

inclusively fine-map variants, we also independently selected any variants with log

likelihood ratio (LLR 1:1000) relative to the GWAS lead variant rs3780269. Finally, to assess

variants that were not successfully genotyped or imputed in the GWAS (including notably

insertion/deletion variants that were not a part of the Haplotype Reference Consortium

imputation panel), we included any such variants that had an LD r^2 > 0.8 (1000 Genomes

Project, Phase 3, Version 5, EUR population) [30] with rs3780269 as identified by the LDlinkR

package [31-33].

To further assess the possibility of other potential independent signals in the region, we

conditioned on the lead GWAS variant (rs3780269) using the Genome-wide Complex Trait

Analysis (GCTA, v1.94.1) [34] COnditional and JOint association (COJO) module [35], using

default settings, a genomic window of ±1 Mb from the lead variant, and the UKBB LD matrix

described above.

Annotation of fine-mapped variants

We assessed all fine-mapped variants at this locus for overlap with melanocytic regulatory

elements (defined by chromatin states, or accessibility) using the BedtoolsR package

(intersect function) [36, 37] and epigenomic datasets from melanocytes and melanoma cell

line models. We classified genomic regions as enhancers or promoters using the Roadmap

project chromHMM data from two primary melanocyte cultures [17, 20]. Using this data,

enhancers were defined using the following chromHMM states from all chromHMM tracks

(primary: Enh, EnhG, EnhBiv; auxiliary: EnhG1, EnhG2, EnhA1, EnhA2, EnhWk, EnhBiv; and

imputed: TxEnh5, TxEnh3, TxEnhW, EnhA1, EnhA2, EnhAF, EnhW1, EnhW2, EnhAc).

Melanoma-specific enhancer annotations included 4_EnhA, 5_EnhM, 6_EnhW, 7_TxEnhM,

7_TxEnhW, and 9_TxWkEnhW, from a tumorigenic melanoma cell model [21]. Promoter

states were defined in melanocytes from imputed ChromHMM as PromU, PromD1, PromD2,

TssA, PromP, PromBiv, and Tx_Reg; and in melanoma cells as 1_TssA, 2_PromWkD, and

3_TssWkP. We analyzed our previously generated ATAC-seq data from five independent

melanocyte cultures [22], along with publicly available omni-ATAC-seq data from nine

melanoma cell lines derived from patient biopsies [19]. Any region annotated as enhancer

or promoter or that overlapped ATAC-peak in either melanocyte or melanoma cells was

considered *cis*-regulatory.

Allele-specific transcriptional activity of these variants was previously assessed using

Massively Parallel Reporter Assays (MPRA) in immortalized primary melanocytes and

UACC903 melanoma cells [23]. All fine-mapped variants for the melanoma risk signal were

included in the MPRA library design, however one failed data QC and was subsequently

excluded from analysis. Allelic impacts on transcriptional activity were assessed using a

linear regression model, accounting for strand and transfection batch as covariates. A

scrambled 145-bp sequence was used as a negative control and as a baseline to assess

relative enhancer activity of specific regions surrounding fine-mapped variants. Allele-

specific differences for each variant were tested relative to scrambled controls. Multiple

hypothesis testing was corrected using Benjamini-Hochberg false discovery rate.

We assessed the impact of fine-mapped variants on the protein-coding sequence Variant

effect predictor tool (https://grch37.ensembl.org/Homo_sapiens/Tools/VEP) [38] using

GENCODE version 19 protein-coding transcripts.

Cell culture

Frozen aliquots of melanocytes isolated from foreskin of healthy newborn males, mainly of

European descent, were obtained from the Specialized Programs of Research Excellence

(SPORE) in Skin Cancer Specimen Resource Core at Yale University following an established

protocol [39]. Melanocytes were grown in Dermal Cell Basal Medium (American Type

Culture Collection/ATCC PCS-200-030) supplemented with Melanocyte Growth Kit (ATCC

PCS-200-041) and 1% amphotericin B/penicillin/streptomycin (120-096-711, Quality

Biological) at 37°C with 5% CO_2. Immortalized C283T [18] melanocytes as well as C283T cells

stably expressing dCas9-KRAB [22] were generated as previously described and grown in

identical conditions to primary melanocytes. All cells tested negative for mycoplasma

contamination via MycoAlert PLUS Mycoplasma Detection Kit (LT07-710, Lonza).

Assessing micro-RNA binding for variant overlapping RAPGEF1 3'-UTR region

To assess whether melanoma GWAS risk variant rs3739497, which resides in the *RAPGEF1*

3️⃣ untranslated region (UTR), is involved in potential post-transcriptional regulation of

RAPGEF1 through micro-RNAs (miRNAs), we assessed predicted miRNA binding sites near

this variant. We queried several databases, including TargetScan [40], miRTarBase [41],

miRDB [42, 43], and mirCode [44]. Binding sites that overlapped or were located within ±50

nucleotides of the variant position were considered candidate variant-dependent miRNA

sites. Finally, to explore post-transcriptional regulation via sequence elements other than

miRNAs, we queried the 3'UTR variant region against the AREsite database [45] that

provides a genome-wide catalogue of AU-rich elements.

Small RNA sequencing data from primary melanocytes and melanomas

To assess small RNA levels in primary human melanocytes (n=106), total RNA, including

microRNA (miRNA), was purified using Qiagen miRNeasy kit (#217004). Briefly, cells were

lyzed in Trizol and heated at 65C followed by chloroform extraction. The aqueous phase was

further purified using a miRNeasy column following manufacturer's instruction. miRNA

sequencing libraries were constructed using NEB Next Small RNA Library Prep kit (E7330s)

and the sequencing was performed on HiSeq2500. All samples show the proportion of Q30

or higher bases of at least 93%. 28-79 million reads were achieved for each sample with

overall alignment percentage between 67-85%. Sequencing reads were processed using the

mirMaster pipeline [46]. Quality control stage processing steps included removal of 3□

adapter sequences (AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC), trimming of low-

quality bases, and retention of reads that exceeded the default minimum length thresholds.

Reads were then aligned to the human genome (GRCh38) using Bowtie [47], allowing up to

five genomic alignments per read. miRNAs were quantified by aligning sequencing reads

against the mature and precursor miRNA reference sequences from miRBase v22.1 [48-53].

Alignments permitted up to one mismatch. Expression values were normalized across

samples using quantile normalization. For each miRNA, percentile rank of expression across

the dataset was calculated based on median expression values.

RAPGEF1 mRNA expression levels were available from the bulk RNA-seq data we previously

simultaneously generated from the same 106 melanocyte cultures [13]. The correlation of

RAPGEF1 mRNA expression levels with candidate miRNAs was measured using Spearman's

rank correlation.

Expression levels of micro-RNAs in TCGA melanoma samples [14] were accessed through

FirebrowseR (https://github.com/mxxdxxx/FirebrowseR).

Quantitative Trait Locus (QTL) analysis of melanocytic cells

We assessed FDR significant QTL signals near the GWAS risk locus using previously-published

melanocyte and melanoma datasets [9, 12]. Melanocyte expression QTL (eQTL), splice QTL

(sQTL), and methylation QTL (meQTL) datasets were generated from 106 primary

melanocyte cultures derived from individuals of European descent. Methylation probes

from meQTL data were assigned to genes based on CpG location within 1.5 kb of the TSS,

5□-UTR, 1st exon, gene body, or 3□-UTR of a gene. Tumor eQTL data were derived from

TCGA melanoma primary tumors as a part of the PancanQTLv2.0 eQTL study; only data from

nominally significant SNP-gene pairs (P<0.01) were made available [79]

(https://hanlaboratory.com/PancanQTLv2/).

GWAS colocalization using the melanocyte eQTL dataset was performed using the SuSiE +

Coloc [54] and SharePro tools [55]. SuSiE + Coloc performs fine-mapping of GWAS and QTL

signals via SuSiE, followed by colocalization via the Coloc tool. SharePro leverages linkage

disequilibrium (LD) to cluster correlated variants into effect groups, followed by

colocalization. GWAS summary data, and the *RAPGEF1* and *UCK1* melanocyte eQTL

summary data (± 500 Kb around the lead variant) were used as input for this analysis.

Default parameters were used except the sigma parameter, which represents the prior

probability of colocalization between traits. Since the region being assessed harbors

multiple GWAS/QTL signals with complex LD, we provide results using multiple sigma values,

ranging from most stringent (1×10^{-5}) to less stringent (1×10^{-4}, 1×10^{-3}). However given that

relaxing this value from the default of 1×10^{-5} increases the potential for false positive

findings, we formally tested colocalization using this default value. Colocalization probability

> 0.8 was considered strong evidence supporting colocalization, while a colocalization

probability < 0.2 was considered evidence against colocalization [55].

We also used these QTL datasets to perform transcriptome (melanocyte and TCGA

melanoma tumors) and methylome-wide (melanocyte) association studies (TWAS/MWAS).

To identify conditionally independent loci, we performed conditional TWAS analysis using

the FUSION tool [56].

Capture-HiC assay for linking risk variants to target genes

As described previously [22], we performed melanoma GWAS-region focused capture Hi-C

assays in primary human melanocytes to facilitate variant-to-gene mapping across all 68

melanoma genome-wide significant signals. Capture probes were designed by Arima

Genomics (San Diego, CA) using 2x tiling with least stringent masking and XTHSBoosting,

resulting in an Agilent SureSelect library (Santa Clara, CA) targeting all restriction fragments

generated by DpnII and MboI (recognition sites: GATC, GANTC) within each association

interval. We generated 15 capture Hi-C libraries from five independent primary melanocyte

cultures (C56, C140, C205, C24, and C27), with three technical replicates prepared per

culture. Barcoded libraries were pooled and sequenced on an Illumina NovaSeq platform

across one SP and one S1 flow cell, yielding ~5.7 billion 150 bp paired-end reads in total.

Sequencing reads were processed using the HiCUP pipeline [57] with alignment to the hg19

reference genome via Bowtie2 [58]. Technical replicates from each melanocyte culture were

combined prior to downstream analyses. Chromatin interactions were detected using the

CHiCAGO pipeline (v1.16.0) [59] at both individual restriction fragment-level and aggregated

four-fragment resolution, as previously described [22]. Interactions with CHiCAGO scores >5

were considered high-confidence visualized on the WashU [60, 61] and UCSC genome

browsers [62-66]. To mitigate cross-linking variability and restriction fragment boundary

effects, we expanded each fine-mapped variant into a ±500 bp window and evaluated

interactions from both the variant-overlapping and adjacent fragments. Genes were

nominated as candidate targets if a chromatin interaction was observed from a variant-

harboring (or adjacent) restriction fragment to the target gene promoter. To map *cis-*

regulatory targets of enhancer-promoter interactions, candidate genes initially nominated

based solely on physical chromatin interactions with fine-mapped variants were further

refined by evaluating whether these variants overlapped melanocytic regulatory regions.

CRISPRi validation of regulation of RAPGEF1 and UCK1 transcription

To validate regulatory interactions from risk variants to target genes, we performed CRISPR

interference (CRISPRi) assays in the immortalized human melanocyte cell line C283T stably

expressing dCas9-KRAB [22]. Briefly, C283T cells were infected with a lentiviral vector

pLX_311-KRAB-dCas9 (gift from John Doench, William Hahn, and David Root; Addgene

plasmid # 96918; http://n2t.net/addgene:96918; RRID: Addgene_96918) followed by

monoclonal cell selection. We confirmed stable expression and functional activity of dCas9-

KRAB in the clone used for CRISPRi.

Three different guide RNAs (gRNAs) were designed to target the genomic regions flanking

each variant site. The gRNA sequences were located ±50bps from the variant

(**Supplementary Table 1**). We used two non-targeting gRNAs (NTC1, NTC2) as controls.

gRNAs were ligated into the lentiviral vector pXPR-050 (gift from John Doench and David

Root, Addgene plasmid #96925; RRID: Addgene_96925). C283T immortalized melanocytes

[18] were infected with lentiviral particles encoding gRNA, and at 24h, 1.5 µg/mL of

puromycin was added for selection. After two days of puromycin selection, puromycin was

removed, and cells were harvested for RNA collection on the same day or one day after

puromycin removal. Total RNA was isolated with RNeasy Mini Kit (Qiagen) and cDNA was

generated with SuperScript IV VILO Master Mix (Thermo Scientific). Multiple infections were

performed with total ten biological replicates for each gRNA against the 3 different variants.

mRNA levels of either *RAPGEF1* or *UCK1* were measured by TaqMan assay (Thermo

Scientific; **Supplementary Table 1**) and normalized to *GAPDH* levels. qPCR triplicates

(technical replicates) were averaged and subsequently considered as a single data point.

Average $2^{\wedge(delta-Ct)}$ values for NTC1 and NTC2 were used for statistical comparisons to other

gRNAs, data in tables and graphs are represented as fold-change relative to the average of

NTC1 and NTC2. The statistical analysis was performed using a paired two-tailed t-test

comparing $2^{\wedge(delta-Ct)}$ values of individual guides to the average $2^{\wedge(delta-Ct)}$ of NTC1/NTC2.

Knock-down of RAPGEF1 and UCK1 expression by shRNA and qRT-PCR quantification of gene

expression

Pre-designed shRNAs against human *RAPGEF1* and *UCK1* genes were purchased from Sigma

(TRCN0000048129 and TRCN0000048130 for *RAPGEF1*, TRCN0000037763 and

TRCN0000199254 for *UCK1*; **Supplementary Table 1**) and expressed in lentiviral vector

backbone (pLKO). After viral infection and puromycin selection, total RNA was isolated with

the RNeasy Mini Kit (Qiagen) and cDNA was generated with SuperScript IV VILO Master Mix

(Thermo Scientific). Transcript levels were measured using TaqMan qPCR assays purchased

from Thermo Scientific. *RAPGEF1* and *UCK1* transcript levels were normalized to the levels

of *GAPDH*, and PCR triplicates were averaged and considered as one data point.

Overexpression of RAPGEF1 in human melanocyte culture

RAPGEF1 was cloned from a cDNA construct (OHS5893-202500127, ORFeome Collab,

Horizon Discovery) into pLX304 (a gift from David Root, Addgene plasmid # 25890;

http://n2t.net/addgene:25890) or pCW57.1 (a gift from David Root, Addgene plasmid #

41393; http://n2t.net/addgene:41393) lentiviral vectors. This clone is the isoform most

strongly expressed across neonatal melanocytes [13]. pCW57.1 contains a doxycycline

inducible promoter. Lentiviral vectors were co-transfected into HEK293 cells with the

pSPAX2, pMD2-G, and pCAG4-RTR2 packaging vectors. Virus was collected two days after

transfection and concentrated by Vivaspin Concentrators (76409-072, VWR). Cells were

incubated with virus for 24 h, followed by selection with blasticidin (5-10ug/ml) or

puromycin (1–2 µg/ml) for 2–4 days. For pCW57.1-RAPGEF1 infected cells, RAPGEF1

expression was induced by 0.2ug/ml doxycycline in melanocyte culture.

Gene-based CRISPR-Cas9 knockout screen

A gene-based pooled CRISPR-Cas9 cell proliferation knockout screen was previously

performed in an immortalized human melanocyte culture, C283T, as described by Xu and

colleagues [14]. Briefly, we designed 3,052 gRNAs covering a set of 288 genes, as well as

⬚200 non-targeting gRNAs. The 288 genes focused primarily on gene candidates from

genome-wide significant melanoma GWAS loci. DNA samples were collected from sgRNA

infected cells at different times after infection and gRNAs were sequenced and normalized

to counts of the integrated gRNA sequence from cells collected prior to Cas9 expression.

Prioritized gene hits critical for cell growth and/or survival were called and ranked by using

MAGeCK.

Cell proliferation assays

Cell proliferation was assayed using a BrdU flow kit (BD Pharmingen) according to the

manufacturer's protocol. Briefly, cells were labeled with 10 µM BrdU for 1-3 hours before

they were fixed, permeabilized, and subjected to DNase I treatment. Cells were then stained

with FITC-conjugated antibody to BrdU and 7-AAD, followed by flow cytometry analysis

using an AttuneNxT Cytometer (Thermo Scientific). For crystal violet staining, cells were

seeded at equal numbers after infection and drug selection and were stained with crystal

violet at different days after seeding. Crystal Violet was solubilized by methanol for

quantification as absorbance at 560nm.

Immunoblotting

For immunoblot analysis, total cell lysates were generated with RIPA buffer (Thermo

Scientific) and subjected to water bath sonication. Samples were resolved by 4–12% Bis-Tris

ready gel (Invitrogen) electrophoresis. The primary antibodies used were mouse antibody to

RAPGEF1 (MA5-26281, ThermoScientific), mouse antibody to RAS (05-516, Sigma), rabbit

antibody to RAP1 (ab181858, Abcam), mouse antibody to phospho-ERK(9106S, Cell Signaling

Technology), mouse antibody to Pan-AKT (2920s, Cell Signaling Technology), rabbit antibody

to total ERK(4695s, Cell Signaling Technology), rabbit antibody to Phospho-AKT(4060s, Cell

Signaling Technology), and mouse antibody to β-actin (A5316, Sigma).

Colony formation assays

Anchorage-independent growth was assayed with p'mel/BRAF^{V600E} cells (provided by H.

Widlund, Dana-Farber Cancer Institute). Briefly, 5,000 cells were mixed in HAMF12 medium

supplemented with 10% FBS containing 0.4% SeaKem LE agarose (Lonza) and plated on top

of a bottom layer with 0.65% agarose prepared in the same HAMF12 medium in a six-well

plate. After agarose is solidified, each well was covered with 0.5 ml of feeding medium,

which was refreshed twice a week. Colonies were counted and imaged 4-6 weeks after

seeding using Lionheart (Agilent) with total magnification of 4x. Colonies were counted from

six wells for each condition, and significant differences between samples were assessed by

unpaired two-tailed *t*-test assuming equal variance.

Zebrafish Melanoma Generation and Analysis

Zebrafish melanomas were generated by overexpressing human RAPGEF1 (cloned from a

cDNA construct, OHS5893-202500127, ORFeome Collab, Horizon Discovery; same *RAPGEF1*

isoform expressed in human melanocyte experiments) or an empty control vector (MCS) in

transformed melanocytes using the miniCoopR system [67]. This system involves injection of

a Tol2-based miniCoopR vector into the *Tg(mitfa:BRAF^{V600E}); p53(lf); mitfa(lf)* zebrafish

strain, where it rescues melanocyte development and simultaneously drives expression of a

gene of interest under the *mitfa* promoter. To visualize melanocytes, we co-injected

mitfa:Cas9, CRISPR gRNAs targeting *tyrosinase* (to block pigmentation), and *mitfa:mCherry*.

As melanocyte rescue is mosaic in this system, we screened these cohorts for fish that had

mitfa:mCherry melanocyte expression in either the RAPGEF1 cohort (n=35), or the MCS

control cohort (n=48). These cohorts were comprised of two separate clutches per condition

and were genotyped to confirm RAPGEF1 expression status. Starting at 13 weeks post-

fertilization, we monitored fish for precursor lesions—termed "cancer precursor zones"

(CPZ)—defined as patches of merged *mitfa:mCherry*-high melanocytes exceeding

established size and fluorescence thresholds. Screening continued every few weeks until 46

weeks post-fertilization. Kaplan-Meier survival analysis and log-rank (Mantel-Cox) tests

showed no significant differences in CPZ onset (p=0.1119) or tumor onset (p=0.211)

between groups. Tumor anatomical distribution did not differ substantially between groups.

RAPGEF1 functions upstream of BRAF in the MAPK pathway, and since our primary zebrafish

tumor model overexpresses oncogenic *BRAF*^{V600E}, this could obscure a role for RAPGEF1 or

represent a different biological context to that seen *in vitro*. To assess this, we used the

MAZERATI system (Modeling Approach in Zebrafish for Rapid Tumor Initiation) [68] to

generate zebrafish cohorts where rescued melanocytes expressed either *BRAFV600E/p53(lf)*

or *RAPGEF1/p53(lf)*. In this system, *mitfa:BRAFV600E* and *p53(lf)* mutations are not stably

expressed in the zebrafish strain background, but are delivered via plasmid injection at the

one-cell stage, allowing us to test whether RAPGEF1 alone can drive tumor initiation in the

absence of an activating BRAF mutation. While the *BRAFV600E/p53(lf)* cohort developed

tumors at the expected rate, *RAPGEF1/p53(lf)*– fish failed to develop precursor lesions or

tumors.

RAP1 and RAS activation assay

RAP1 activation was measured using the RAP1 activation assay kit from Abcam (ab212011)

and RAS activation was measured using GST-fusion Raf-1 RBD beads (Sigma, #14863) or RAF-

RBD Protein GST Beads (RF02-A, Cytoskeleton). Cells were grown in supplement-free ATCC

medium for 24 hours before treated with 50ng/ml human EGF protein (R&D, Cat# 236-EG-

200) at the indicated time. Whole cell extract was taken to be pulled down by the Raf-RBD

beads for RAS activation assay or by the RalGDA RBD beads from the Abcam kit for

measuring RAP1 activation. Beads were then lysed in SDS loading buffer, resolved by 4–12%

Bis-Tris ready gel (Invitrogen) electrophoresis, and blot with RAS (Sigma) or RAP1 (Abcam)

antibody. Only the active GTP-bound form of RAS or RAP1 can be pulled down by the beads

and the intensity of RAS or RAP1 activation was determined by comparing the pulled-down

signal with that from the whole cell extract.

<u>*Assessing RAS-MAPK alterations across multiple melanoma cohorts*</u>

We assessed *RAPGEF1* expression association with activating mutations in RAS-MAPK

signaling pathway in melanoma tumors. We analyzed cutaneous melanoma tumors across

six cohorts: TCGA n=363 [14], Leeds n=503 [69-71], Lund n=146 [72], Liu n=84 [73], Van

Allen n=38 [74], Riaz n=39 [75] (**Supplementary Tables 2-8**). Briefly, the Leeds, Lund, and

TCGA cohorts included very few samples treated with immunotherapy (TCGA n=23 [76];

Leeds n=12 [70]; and Lund n=4 [72]), while the other three cohorts consisted of patients

treated with immunotherapy following tumor collection. Our sample inclusion criteria

required melanoma samples that had both mutation and gene expression data available

across the cohorts. Cohort details are provided in the **Supplementary Materials**.

<u>*Assessing RAPGEF1 expression association with RAS-pathway*</u>

We tested several models classifying RAS-pathway wild-type and mutant tumors, focusing

initially on the most commonly-mutated oncogenic driver genes and subsequently adding in

less-commonly altered genes. Specifically, within the Leeds sample set (n = 503 total), only

specific mutations in *BRAF* (V600|K601) and *NRAS* (Q61|G12|G13) were assessed in a

subset of the tumors (n=234), while for the remaining a full set of putative MAPK driver

mutations [77] were assessable from sequencing data, so our initial model (Model 1)

defined the mutant subgroup as those samples with these specific *BRAF* (V600|K601) and

NRAS (Q61|G12|G13) hotspot mutations. Additional models were as follows: Model 2

mutant group, any driver mutation in *BRAF* or *NRAS*; Model 3 mutant group, any driver

mutation in *BRAF*, *NRAS*, *KRAS*, or *HRAS*; Model 4 mutant group, any driver mutation in

BRAF, *NRAS*, *KRAS*, *HRAS*, or *NF1* ("triple wild-type); and Model 5, any putative driver

mutation in the 44 genes defined from the TCGA Pan-Cancer analysis [77] (**Supplementary**

Table 9). Outside of the Leeds dataset, all cohorts had full mutation information for *BRAF*,

NRAS, *KRAS*, *HRAS*, and *NF1*. As described above, Leeds and Lund cohorts had targeted

sequencing of select cancer-related genes and were not fully assessed for all genes included

in Model 5. In this case for Model 5, we classified wild-type and mutant subgroups based on

the available gene sequencing data from each dataset. Methods for annotating individual

mutations as drivers are described in the **Supplementary Material**. We assessed an

association of *RAPGEF1* levels with RAS-MAPK wild-type and mutant subgroups using a

linear regression model, adjusting for age, sex, tumor purity, and tumor stage when

available.

Survival analysis

We conducted survival analyses across multiple melanoma cohorts using the Cox

proportional hazards models to assess the association between *RAPGEF1* expression and

patient outcomes in tumors wild-type for *BRAF* (V600|K601) or *NRAS* (Q61|G12|G13)

hotspot mutations. For each cohort, *RAPGEF1* expression was dichotomized into high- and

low-expression groups based on the median expression value of the full dataset.

Associations between *RAPGEF1* expression and survival were then evaluated within the

wild-type tumor subgroup using the Cox models. To ensure comparability across datasets,

all survival times were uniformly converted to days. We performed melanoma-specific

survival in the TCGA, Leeds, and Lund cohorts, and overall survival in the Liu, Van Allen, and

Riaz cohorts as they lacked melanoma-specific death data. Because stage I tumors were

profiled exclusively in the Leeds cohort and are associated with a high survival rate (93-97%)

[78], we excluded these tumors from the survival analysis to ensure comparability between

the Leeds cohort and other melanoma cohorts, which predominantly included more

advanced-stage tumors. For these analyses, we adjusted for covariates specific to each

cohort as follows: TCGA: Age, Tumor site; Leeds: Age; Lund: Age; Liu: Cosmic aging; Van

Allen: Age. Across all cohorts, the WT tumor subgroup included only a small number of

samples, and when the number of events is low relative to the number of covariates, the

Cox model can encounter monotone likelihood issues, resulting in extremely large or

unstable coefficient estimates. To address this, we applied Firth's correction.

Meta-analysis of tumor data

We performed meta-analysis of the *RAPGEF1* expression association with RAS-MAPK

subgroups, as well as the association of *RAPGEF1* expression with survival across the

analyzed melanoma cohorts as follows: Let   be the z-score for cohort . Let $^+$ be the number

of subjects with the mutation and $^-$ be the number of subjects of wild type, then the

effective sample size $= \frac{+-}{++-}$. We choose weights $= \sqrt{/}\sum_{=1}^{5}$ and perform meta-analysis

as $= \sum_{=1}^{5}$. One can show that $\sim(0,1)$ under $_0$ and has optimal statistical power assuming

equal effect sizes (after normalization).

460   Results

Fine-mapping of the 9q34.13 melanoma risk signal

In order to functionally characterize the melanoma risk locus on chromosome band 9q34.13,

which is also associated with nevus count, but not hair color, tanning ability, or telomere

length [1], we first assessed whether the locus harbors a single or multiple risk signals.

Conditional GWAS analysis using the lead variant at this locus (rs3780269, P_{GWAS}=1.92 x 10^{-8},

rs3780269-G OR=0.94) revealed no evidence of a secondary signal within +/- 1Mb of the

main GWAS association signal (**Supplementary Table 10-11**, **Supplementary Figure 1A**).

Multiple Bayesian fine-mapping methods (SuSiE, DAP-G, and RSparsePro) [25-29] similarly

converged on a single and similar credible causal set of variants (CCVs; **Supplementary**

Table 12).

To facilitate variant-to-gene mapping, we defined a credible causal set by taking the union

of CCVs identified using Bayesian fine-mapping approaches. Within this set, the GWAS lead

variant (rs3780269) was consistently assigned the highest posterior probability of being

causal across methods (DAP-G PIP = 0.52, RSparsePro PIP = 0.58, SuSiE PIP = 0.55). We also

included as CCVs variants identified by fine-mapping directly from GWAS summary statistics

by assessing log-likelihood ratio (LLR) relative to the lead variant. Finally, to account for

additional variants not genotyped or imputed in the GWAS, we performed LD-based fine-

mapping (r^2 > 0.8 with the lead variant). These methods collectively identified 14 potential

causal variants (**Figure 1A, Supplementary Table 12**).

None of these variants mapped to protein-coding sequence or canonical splice junctions

(**Supplementary Table 13**), nor was there a significant or colocalizing splice QTL (sQTL) for

rs3780269 (see **Supplementary Material**). One fine-mapped variant, rs3739497 (P_{GWAS} = 1.9

× 10^{-6}; OR = 0.95; risk allele = A; protective allele = G; r^2 to GWAS lead = 0.88), is located in

binding site for two microRNAs (miRNAs; *miR-124-3p* and *miR-506-3p*) in the 3⬚

untranslated region (UTR) of *RAPGEF1*, however we found no evidence that levels of

RAPGEF1 were correlated with either in melanocytes, making it unlikely this variant impacts

either miRNA binding or *RAPGEF1* levels (see **Supplementary Material**).

The 9q34.13 melanoma risk region physically interacts with and regulates RAPGEF1 and

UCK1

Several melanoma credible set variants overlap active enhancer or accessible chromatin

regions in human melanocytes or melanoma cell models (**Figure 1B, Supplementary Table**

14), while the lead variant is located within a region broadly annotated as an enhancer

across multiple cell types (ENCODE *cis*-regulatory element EH38E2732514)[79], suggesting a

cis-regulatory function. Consistent with this annotation, previous massively parallel reporter

assays (MPRA) of fine-mapped risk variants [23] showed the regions surrounding several

had significantly different transcriptional activity compared to scrambled DNA sequences in

melanocytes and melanoma cells (scramble vs alt comparisons in **Supplementary Table 15**),

indicating enhancer and/or repressive function (**Supplementary Figure 2**).

Capture-HiC-based variant-to-gene mapping [22] of the full region of association in primary

melanocytes identified chromatin interactions between enhancers harboring fine-mapped

variants (**Supplementary Table 14**) and the promoters of *RAPGEF1 and UCK1* (**Figure 1A**;

Supplementary Table 16), as well as to *NTNG2* (which is lowly-expressed in melanocytes

and melanoma). CRISPR-inhibition (CRISPRi) of regions surrounding select variants

interacting with *RAPGEF1* and *UCK1* promoters (**Figure 1B**) in immortalized melanocytes

confirmed a *cis*-regulatory effect of the region surrounding the GWAS lead variant

(rs3780269) for both *RAPGEF1* (2/3 guide RNAs; 0.85–0.88□fold relative to non□targeting

controls, P = 0.0031 and P = 0.0018 respectively; paired Student's t-test) and *UCK1* (2/3

gRNAs 0.78–0.82□fold, P = 0.0025 and P = 0.0021 respectively; paired Student's t-test), as

well as rs3818580 for *UCK1* (2/3 guides; P = 0.025 and P = 0.031) (**Figure 1C**). However,

melanocyte and melanoma MPRA data for credible set variants [23] did not find variants

with significant allelic differences in transcriptional activity (ref versus alt columns in

Supplementary Table 15), so while elements in this region regulate both genes, these data

do not implicate a specific variant.

Figure 1. Fine-mapping and epigenomic annotations of melanoma GWAS and melanocyte eQTLs for the 9q34.13 melanoma risk locus. A. Genetic variants fine-mapped for the melanoma GWAS are annotated, along with Bayesian fine-mapped variant credible sets (CS) from SuSiE and DAP-G for human primary melanocyte eQTLs for *RAPGEF1* and *UCK1*. Roadmap chromatin states (ChromHMM) from primary melanocytes are shown; red and orange denote annotated promoter, yellow and light green enhancer. Chromatin

accessibility from DNAse I hypersensitivity sequencing (DHS) and ATAC-sequencing (ATAC)
of melanocytes and melanoma cell models is shown. Chromatin interactions between the
entire region of association (Capture-HiC baits) in human melanocytes is shown; fine-
mapped variants interact with the promoters of both *RAPGEF1, UCK1*, and *NTNG2* (not
shown on figure). **B**. Zoomed in view of the fine-mapped variants. The locations of small
guide RNAs used for CRISPR-inhibition (CRISPRi sgRNAs) experiments are shown. **C**. CRISPR-
inhibition (CRISPRi) validation of *RAPGEF1 and UCK1* as *cis*-regulatory targets at 9q34.13.
Guide RNAs were designed to target three fine-mapped variants within an ~11.3 kb interval.
Each guide was individually tested for effects on *RAPGEF1 and UCK1* expression relative to
the average of two non-targeting guides in immortalized melanocytes stably expressing
dCas9-KRAB via a TaqMan quantitative RT-PCR assay. Whiskers show minimum and
maximum values. *P*-values were calculated using a two-sample two-sided paired t-test
comparing $2^{\wedge(delta-Ct)}$ values from individual guides to those from the average of the two non-
targeting guides with 10 biological replicates in total.

RAPGEF1 and UCK1 are cis-regulatory targets at the 9q34.13 melanoma risk locus in

melanocytic cells

Transcriptome wide association studies (TWAS) using eQTL data from human primary

melanocytes (n=106) [13] as well as primary melanoma tumors from TCGA (n=103) [14, 15,

80] collectively implicate two genes in the region, with melanoma risk positively associated

with predicted melanocyte and melanoma expression of *RAPGEF1* ($P_{melanocyte}$ = 4.8 x 10^{-5};

$P_{melanoma}$ = 4.7 x 10^{-5}) and negatively associated with melanocyte *UCK1* ($P_{melanocyte}$ = 1.8 x 10^{-5};

Supplementary Table 17; *UCK1* was not included as a predictable gene from the melanoma

dataset). Consistent with TWAS, which considers the collective effects of multiple sequence

variants, the risk allele of rs3780269 itself is individually associated with higher *RAPGEF1* in

both melanocytes and melanomas ($P = 1.4 \times 10^{-11}$ and $P = 5 \times 10^{-4}$, respectively;

Supplementary Figure 3A, Supplementary Table 18, Supplementary Table 19,

Supplementary Table 20) and negatively but not significantly associated with *UCK1* ($P =$

0.22; **Supplementary Figure 3A**, **Supplementary Table 18**).

In contrast to melanoma GWAS fine-mapping results which suggest a single signal,

colocalization and fine-mapping of *RAPGEF1* and *UCK1* eQTL signals point to potentially

distinct *cis*-regulatory variants for *RAPGEF1* and *UCK1*, each in partial LD with rs3780269.

Melanocyte eQTL results show different lead SNPs for *RAPGEF1* (rs13295485, P_{eQTL} = 2.8 x

10^{-20}, P_{GWAS} = 4.8 x 10^{-5}) and *UCK1* (rs7867616, P_{eQTL} = 2.8 x 10^{-20}; P_{GWAS} = 3.3 x 10^{-5};

Supplementary Tables 21, 22), each in partial LD with rs3780269 (1000G EUR r² = 0.50, D' =

0.74 for rs13295485; r^2 = 0.17, D' = 0.66 for rs7867616) and each other (r^2 = 0.12, D' = 0.5).

Analyses of GWAS and eQTL signals using multiple Bayesian approaches (SuSiE + COLOC [54]

and SharePro [55]) do not provide support for colocalization of these eQTLs with the

melanoma GWAS (**Supplementary Tables 23, 24; Supplementary Figure 3B, C**). Fine-

mapping the *RAPGEF1* eQTL in melanocytes reveals multiple sets of CCVs collectively

containing over 100 variants and spanning the entire gene and promoter (**Figure 1A**,

Supplementary Table 25); critically several overlap the set of melanoma CCVs

(**Supplementary Table 12**). The single fine-mapped *UCK1* eQTL credible set variant overlaps

annotated *UCK1* promoter (rs7867616, **Figure 1A**, **Supplementary Table 26**) but not the

melanoma credible set. Consistent with the eQTL data, we note a strikingly similar pattern in

melanocyte methylation QTL (meQTL) data (see **Supplementary Material**), where a

methylome-wide association study identified significant CpG methylation probes in both

genes and individual meQTL signals mirror eQTL signals for the respective genes. These data

collectively reflect a complex *cis*-regulatory structure underlying the 9q34.13 association

signal.

Contributions of RAPGEF1 and UCK1 expression to the melanoma association signal

To further reinforce *RAPGEF1* and or *UCK1* as strong causal candidates regulated by a

complex pattern of *cis*-regulatory signals, we performed additional conditional GWAS

analyses. Conditioning on predicted melanocyte *RAPGEF1* and *UCK1* expression each

partially attenuated the GWAS signal for lead SNP rs3780269 (P_{GWAS} = 1.9 x 10^{-8}, *RAPGEF1*

$P_{conditional}$ = 1.0 x 10^{-4}; *UCK1* $P_{conditional}$ = 2.1 x 10^{-5}; **Supplementary Table 27, 28**). When both

genes were included in a joint model, both are significant (**Supplementary Table 29**) and the

signal for rs3780269 is dramatically attenuated ($P_{conditional}$ = 0.002; **Supplementary Table 30**).

We observe similar results when instead conditioning on respective lead eQTL SNPs

(*RAPGEF1* $P_{conditional}$ = 4.9 x 10^{-3}, *UCK1* $P_{conditional}$ = 6.33 x 10^{-5}; **Supplementary Table 31, 32;**

Supplementary Figure 1B, 1C), suggesting a contribution of *cis*-regulatory variants for both

genes to the melanoma risk signal. We note that conditioning only on the fine-mapped

RAPGEF1 eQTL SNP with the highest PIP identified by DAP-G itself resulted in complete

attenuation of the GWAS signal ($P_{conditional}$ = 0.29, **Supplementary Table 33, Supplementary**

Figure 1D), perhaps suggesting a critical role for *RAPGEF1*. Taken together, these results

point to multiple causal variants in partial LD to rs3780269 respectively influencing two

plausible effector genes, *RAPGEF1* and *UCK1*, with both genes having opposing effects

correlated with melanoma susceptibility: decreased *UCK1* expression and increased

RAPGEF1 expression are linked to risk.

RAPGEF1 and UCK1 both are critical for melanocyte growth and overexpression of RAPGEF1

stimulates melanocyte growth

Given the opposing effects for *RAPGEF1* and *UCK1* on melanoma risk, we assessed whether

their expression impacts melanocyte growth and/or survival. We previously included

RAPGEF1, but not *UCK1*, in a small pooled CRISPR proliferation screen in human

melanocytes, noting the set of guides targeting *RAPGEF1* as the third most depleted

amongst all genes screened ($P = 4.97 \times 10^{-6}$) [18] suggesting a strong positive role in

melanocyte proliferation and or viability. Here, we validated this finding, silencing *RAPGEF1*

using two independent shRNAs in immortalized human melanocytes and assessing cell

proliferation by crystal violet staining and BrdU incorporation assays. Knockdown of

RAPGEF1 using both guides reduced melanocyte number as assessed by crystal violet assay

(**Figure 2A, 2B**). Consistent with these data, BrdU flow cytometry revealed a significant

decrease in DNA synthesis after targeting with both shRNAs, indicating a block in cell cycle

progression upon *RAPGEF1* knockdown (**Figure 2C**). Parallel knockdown of *UCK1* similarly

resulted in reduced proliferation in immortalized melanocytes, as evidenced by both assays

(**Figures 2D-F**), mirroring the effects observed with *RAPGEF1* suppression. Critically, while a

positive role for *RAPGEF1* in melanocyte proliferation is consistent with observation that

increased melanocyte *RAPGEF1* is associated with melanoma risk, the essential role of *UCK1*

in melanocyte proliferation contrasts with eQTL and TWAS findings which show that

increased *UCK1* expression is protective against melanoma. While we cannot exclude a

potential role for *UCK1* in melanoma susceptibility at the 9q34.13 locus via means other

than influencing melanocyte proliferation, the strong concordance between the essential

function of *RAPGEF1* in melanocyte proliferation and its elevated expression linked to

increased melanoma risk led us to prioritize *RAPGEF1* for further investigation.

We then assessed whether increased *RAPGEF1* levels contributed to increased proliferation

in immortalized melanocytes using a doxycycline induced promoter construct. Upon

doxycycline treatment, cells showed significantly enhanced growth by crystal violet staining

compared to untreated controls (**Figure 2G, H**), demonstrating that increased *RAPGEF1*

expression stimulates melanocyte growth and could contribute to melanoma development.

In summary, *RAPGEF1* expression influences melanocyte proliferation, with elevated levels

driving increased cell growth that could in turn contribute to nevus and or melanoma

development.

Figure 2. RAPGEF1 and UCK1 positively regulate melanocyte proliferation. A. Crystal
violet assays following *RAPGEF1* knockdown in immortalized melanocytes. Equal numbers of
cells either infected with two *RAPGEF1* shRNAs or vector control were seeded after infection
and drug selection. Cells were fixed and stained with crystal violet at multiple timepoints
after seeding. Crystal violet was then solubilized from stained cells and measured by OD560.
OD560 values were normalized to the reading at D1, and the mean of six biological

replicates and SEM were plotted. *P*-value was calculated from two-way ANOVA; **B.**
Quantitative RT-PCR for *RAPGEF1* in immortalized melanocytes following infection with
shRNAs targeting *RAPGEF1* relative to empty vector control. RNA was collected and assayed
at D5 after infection. **C.** FACS analysis of immortalized melanocytes infected with either
empty vector or *RAPGEF1* shRNA at D5 after infection. BrdU and 7-AAD staining were used
to characterize the cell cycle status of different cells. The images shown here are from a
representative experiment from three biological replicates. **D.** Crystal violet assays following
UCK1 knockdown in immortalized melanocytes. Equal numbers of cells either infected with
two *UCK1* shRNAs or vector control were seeded after infection and drug selection. Cells
were fixed and stained with crystal violet at multiple timepoints after seeding. Crystal violet
was then solubilized from stained cells and measured by OD560. OD560 values were
normalized to the reading at D1, and the mean of six biological replicates and SEM were
plotted. *P*-value was calculated from two-way ANOVA. **E.** Quantitative RT-PCR for *UCK1* in
immortalized melanocytes following infection with shRNAs targeting *UCK1* relative to empty
vector control. RNA was collected and assayed at D7 after infection **F.** FACS analysis of
immortalized melanocytes infected with either empty vector or *UCK1* shRNA at D7 after
infection. BrdU and 7-AAD staining were used to characterize the cell cycle status of
different cells. The images shown here are from a representative experiment from three
biological replicates. **G.** Immortalized melanocytes infected with a dox-inducible vector
(pCW57.1) to express *RAPGEF1* were seeded after infection and drug selection. Dox-treated
cells were treated with 200ng/mL dox at seeding (D0) and cells were fixed and stained with
crystal violet at multiple timepoints after seeding. Crystal violet was then solubilized from
stained cells and measured by OD560. OD560 values were normalized to the reading of D1
and the mean of 12 biological replicates and SEM were plotted. *P*-value was calculated using
two-way ANOVA. **H.** Western blot for RAPGEF1 in dox-treated immortalized melanocytes.
Whole cell extract was collected 48 hours post-dox treatment at D7 after infection.

RAPGEF1 overexpression triggers malignant transformation of human melanocytes

Having established that *RAPGEF1* overexpression enhances melanocyte growth, we next

investigated whether *RAPGEF1* functions as a potential oncogene capable of promoting

malignant transformation. While RAPGEF1 has diverse roles in cancer depending on the

cellular context, tumor type, and stage, its function as a GEF for the small GTPase protein

RAP1 is central to its potential oncogenic activity. Mechanistically, RAP1 can initiate and

maintain MAPK/PI3K signaling, which can trigger malignant transformation of cells. To

further evaluate whether *RAPGEF1* can stimulate malignant transformation by affecting

melanocyte growth, we examined the potential effect of RAPGEF1 on anchorage-

independent growth of TERT-immortalized human melanocytes (p'mel cells) by soft-agar

colony formation assay. While p'mel cells themselves do not efficiently form large colonies

in soft-agar, stably expressing BRAF^{V600E} in p'mel together with other oncogenes lead to

colony formation [81, 82]. NRAS and its constitutively active mutants have been shown to

promote malignant cellular transformation in multiple cells of different origins [83, 84] while

the direct role of RAP1 in cellular transformation has not been well-reported. Here, we

observed enhanced colony formation (>70 µm) in p'mel cells stably expressing both

BRAF^{V600E} and constitutively activated NRAS, specifically NRAS^{G12V} or NRAS^{Q61R} ($P < 0.0001$, P

< 0.0001, respectively; **Figure 3A, B**) relative to those expressing BRAF^{V600E} alone. Not

surprisingly, enhanced colony formation was also observed in p'mel cells stably expressing

both BRAF^{V600E} and a synthetic constitutively activated RAP1A^{G12V} mutant ($P < 0.0001$). By

introducing RAPGEF1 overexpression in p'mel cells, we observed that RAPGEF1 similarly

cooperated with *BRAF*^{V600E} to enhance colony formation ($P < 0.0001$). The NRAS^{Q61R} mutant

induced a stronger colony formation effect than the NRAS^{G12V} mutant ($P < 0.0001$),

consistent with *NRAS* mutations at codon 12 (such as G12V) generally exhibiting

intermediate oncogenic potency compared to the stronger activating mutations at codon 61

[85]. The number of colonies formed upon *RAPGEF1* overexpression was lower than those

induced by NRAS^{Q61R} ($P < 0.0001$) and RAP1A^{G12V} ($P = 0.0002$), but on a more similar level to

wild-type RAP1A ($P = 0.10$) or NRAS^{G12V} ($P = 0.32$; **Figure 3A, B**). We note that we did not

observe such cooperation in a BRAF^{V600E}-expressing zebrafish model

(*Tg(mitfa:BRAF(V600E));p53$^{-/-}$*) [67], where overexpression of human RAPGEF1 did not

accelerate formation of cancer precursor zones (CPZs; $P = 0.11$) or tumors ($P = 0.21$), nor

was RAPGEF1 overexpression sufficient to drive tumor formation in fish that did not

overexpress BRAF^{V600E}. Overall, these data suggest that *RAPGEF1* possesses an intermediate

level of oncogenic potential, contributing to colony formation in melanocytes but

insufficient to drive tumorigenesis in the absence of a strong oncogenic mutation *in vivo*.

A

B

Figure 3. Colony formation in soft agar of p'mel cells stably expressing $BRAF^{V600E}$ **infected**
with lentivirus to express either RAPGEF1, $NRAS^{G12V}$**,** $NRAS^{Q61R}$**, RAP1A, or** $RAP1A^{G12V}$**. A.**
Immortalized melanocytes (p'mel cells) expressing constitutively active $BRAF^{V600E}$ were
infected with empty pLX304 vector or constructs expressing different proteins and seeded in
soft-agar plates after blasticidin selection. Images were taken between week 4-5 after
plating, and colonies were counted from two sets of triplicates (n = 6), with counts from
each individual replicate shown as a dot. Whiskers show mean with SEM. P-values were
calculated using two-tailed, unpaired Student's t-test. Significance is shown only for
comparisons with the empty vector control and RAPGEF1 overexpression. **B**. Representative
images of colonies are shown. The same magnification (4x) was applied for all the images.

RAPGEF1 overexpression in serum-starved melanocytes potentiates activation of both RAS

and RAP1

Since melanocyte growth and malignant transformation effects were noted for RAPGEF1

overexpression, we hypothesized that its overexpression promotes oncogenic activity via

activation of its known target RAP1. To test this, we overexpressed RAPGEF1 in melanocytic

cells using a tetracycline-inducible system, and RAP1 activation was assessed by pulldown

with RalGDS RBD beads, which selectively bind to the GTP-bound active form of RAP1.

Under standard melanocyte culture conditions, RAP1 activation remained minimal

regardless of RAPGEF1 overexpression, suggesting that cell growth supplement factors may

suppress or mask RAPGEF1 related effects (data not shown). However, when cells were

grown in supplement-free medium for 24 hours and then stimulated with human Epidermal

Growth Factor (hEGF), a weak, transient increase in RAP1 activation was detected in cells

not treated with doxycycline (dox), while dox-treated RAPGEF1 overexpressing cells showed

enhanced activation, with a marked increase in active RAP1 at 15 minutes following hEGF

treatment (**Figure 4A**). These results are consistent with previous studies showing that

RAPGEF1 acts as a GEF for RAP1 in mammalian cells.

RAP1 belongs to RAS small G-protein family which also contains NRAS, HRAS, KRAS and

many other RAS proteins. Almost all known GEFs for RAS family G-proteins contain a CDC25

homology domain as the catalytic domain, yet each GEF acts on only a small subset of the

RAS family members with a high selectivity [86]. While RAPGEF1 has been established as a

major GEF for RAP1, its role as a GEF of other RAS family proteins is controversial. *In vitro*

GEF assays using RAPGEF1 containing only CDC25 domain showed no activation of any RAS

proteins [86] whereas full-length RAPGEF1 displayed activation of R-RAS both *in vitro* and in

living COS7 cells [87]. At the same time, it was reported that RAP1 activation by RAPGEF1

might also contribute indirectly to RAS activation [88]. Given that the RAS signaling pathway

is a key regulator of melanoma development, RAS alterations are common in melanoma,

and that nearly every nevus harbors a mutation in BRAF or RAS, we asked whether RAPGEF1

overexpression affects RAS activation in melanocytes as we observed for RAP1. Compared

to controls, RAPGEF1 overexpression led to RAS activation even in the absence of hEGF,

with stronger RAS activation observed in RAPGEF1 overexpressing cells following hEGF

stimulation (**Figure 4B**). We also assessed downstream signaling activity following RAS

activation, noting increases in ERK and AKT activation correlating with levels of activated

RAS (**Figure 4B**). Notably, although RAPGEF1 overexpression enhanced both RAP1 and RAS

activation in response to hEGF, the kinetics of activation between RAS and RAP1 is clearly

different, with strong RAS activation from 2 to 15 minutes in comparison to noticeable

active RAP1 only at 15 minutes post hEGF treatment. These data raise the possibility that

the activation of RAS and RAP1 by RAPGEF1 may not happen concurrently, and may be

subjected to spatio-temporal regulation [89]. These data support a model in which

dysregulated RAPGEF1 expression amplifies not only RAP1, but also RAS signaling in

melanocytes, driving abnormal melanocyte proliferation and survival and contributing to

melanogenesis and tumor development.

Figure 4: RAPGEF1 potentiates RAP1 and RAS activation in serum starved melanocytes.
C283T immortalized melanocytes with doxycycline-inducible expression of *RAPGEF1* were
grown in supplement-free medium for 24hrs and subsequently treated with human EGF

(hEGF) protein for the indicated time. **A**. Active GTP-bound RAP1 was pulled down by
RalGDS-RBD beads from whole-cell extract. Pull-down and whole-cell (total RAP) fractions
were compared by Western blotting with an antibody against RAP1. **B**. Active GTP-bound
RAS was pulled down by Raf-RBD beads, with the pull-down and whole-cell (total RAS)
fractions compared by Western blotting with an antibody against RAS. Active and total AKT
and ERK were compared via Western blotting. Figures are representative experiments from
three biological replicates.
759
760

Elevated RAPGEF1 levels are associated with tumors lacking activating mutations in RAS-

MAPK signaling

Hyperactivation of the RAS-MAPK signaling is a hallmark of melanoma [90, 91]. Most

melanomas acquire activating mutations in _BRAF_ or _NRAS_, or loss-of-function mutations in

_NF1_, which result in constitutive RAS-MAPK pathway activation [92, 93]. In tumors lacking

these canonical drivers, rare mutations in other RAS-MAPK pathway members have been

reported to sustain RAS-MAPK signaling [14, 94-96]. In melanocytes, RAPGEF1

overexpression induced RAS activation in the absence of hEGF treatment. Based on this, we

hypothesized that _RAPGEF1_ expression may act as an alternative contributor to driving RAS-

MAPK activation in melanoma tumors without known or strongly-activating RAS-MAPK

activating alterations. To test this, we analyzed RAPGEF1 expression in six publicly available

melanoma cohorts that included both mutation and gene expression profiling data (TCGA

melanoma n=366 [14], Leeds n=503 [69-71], Lund n=146 [72], Liu n=84 [73], Van Allen n=38

[74], Riaz n=39 [75]; **Supplementary Tables 2-8**). Since mutational data for specific strongly-

activating _BRAF_ (codons V600 and K601) and _NRAS_ (codons Q61, G12, and G13) alterations

across the widest range of tumors across all datasets (a subset of the Leeds cohort was only

assessed for these specific mutations), we initially focused on defining the RAS-MAPK wild-

type and mutant groups based on these specific alterations. After adjusting for clinical and

technical covariates (age, sex, tumor stage, and tumor purity), we consistently observed

significantly elevated _RAPGEF1_ expression in wild-type tumors compared to the mutant

subgroup across five of six cohorts (TCGA P = 3.4 x 10^{-4}; Leeds P = 0.01; Lund P = 0.007; Liu P

= 0.004; Van Allen P = 0.02), with the direction of effect in the non-significant Riaz cohort

consistent with the other five (P = 0.2) (**Supplementary Table 34; Figure 5A**). Meta-analysis

across these datasets showed a highly significant difference (P_{meta} = 6.6 x 10^{-10}). We also

expanded to consider models cumulatively adding additional _BRAF_ and _NRAS_ driver

mutations (Model 2), *HRAS* and *KRAS* driver mutations (Model 3), loss-of-function *NF1*

mutations (Model 4), and driver alterations in other RAS-MAPK pathway members using

definitions from the TCGA Pan-Cancer analysis [77] (Model 5; models listed in

Supplementary Table 9). *RAPGEF1* expression remained consistently higher in the wild-type

subgroups in meta-analyses of each model, with the strongest association in models

considering driver mutations specifically in *BRAF* and *NRAS* (Model 2; $P_{model2} = 4.54 \times 10^{-10}$;

$P_{model3} = 1.09 \times 10^{-8}$, $P_{model4} = 2.73 \times 10^{-6}$, $P_{model5} = 1.96 \times 10^{-3}$; **Supplementary Table 34**).

Taken together, the consistent enrichment of *RAPGEF1* expression in tumors lacking RAS-

MAPK activating alterations, coupled with its ability to induce RAS, RAP, and MAPK

activation in melanocytes, supports a model in which *RAPGEF1* contributes to RAS-MAPK

pathway activation in the absence of strongly-activating canonical driver mutations.

RAPGEF1 expression is associated with prognosis in tumors lacking RAS-MAPK activating

alterations

We next evaluated whether *RAPGEF1* expression has prognostic significance in melanoma

tumors lacking RAS-MAPK activating alterations. Since *BRAF* (V600|K601) and *NRAS*

(Q61|G12|G13) hotspot alterations were profiled across most cohorts; we limited survival

analysis to tumors that were wild-type for these alterations. To minimize potential

confounding due to treatment-specific effects, we analyzed datasets comprised primarily of

patients not treated with immunotherapy separately from those focused on

immunotherapy-treatment. The Leeds, Lund, and TCGA cohorts predominantly included

patients who had not received immunotherapy, whereas the Liu, Van Allen, and Riaz cohorts

comprised immunotherapy-treated patients. *RAPGEF1* expression was dichotomized into

high and low groups based on a cohort-wide median cut-off for each cohort. In the analysis,

non-immunotherapy-treated WT tumors from TCGA (n = 92), Leeds (n = 70), and Lund (n =

26) cohorts, higher *RAPGEF1* expression subgroup was associated with poorer prognosis

compared to *RAPGEF1* low expression group (TCGA HR = 1.5, Leeds HR = 1.7, Lund HR = 2.8;

Supplementary Table 35, Figure 5B). Though these associations are below significance

threshold within individual cohorts, a meta-analysis of these cohorts is significant (P=0.049)

for *RAPGEF1* overexpression predicting shorter survival (HR = 1.6; **Supplementary Table 35;**

Figure 5B). In contrast, for cohorts of patients with WT tumors treated with immunotherapy

(Liu = 26, Van Allen = 16, Riaz = 14), the *RAPGEF1* high expression group was associated with

improved prognosis (Liu HR = 0.4, Van Allen HR = 0.2, Riaz HR = 1.03) and was significant

upon meta-analysis of these cohorts confirming a significant association between higher

RAPGEF1 expression and longer survival (HR = 0.4, *P* = 0.01; **Supplementary Table 35, Figure**

5B). The opposing effects of *RAPGEF1* expression association between relatively non-treated

and treatment cohorts is noteworthy, however given the low sample size across most

cohorts, these preliminary findings should be validated in larger, independent cohorts to

confirm and extend these observations.

Figure 5: *RAPGEF1* is consistently higher in NRAS/BRAF-wild type tumors across six
independent melanoma tumor cohorts. **A**. We assessed mutation and RNA expression data
from six melanoma tumor cohorts: TCGA, Leeds, Lund, Liu, Riaz, and Van Allen cohorts.
Within each cohort, melanoma tumors were classified into two groups: mutant and wild-
type (WT) based on the presence of any *BRAF* V600 or K601 or *NRAS* Q61, G12, or G13
codon somatic mutations; tumors were designated wild-type otherwise. For each
cohort, *RAPGEF1* expression distribution is visualized using boxplots. In each boxplot, the
central black line represents the median expression value, upper whisker extends from the
third quartile (Q3) to the largest value within 1.5x inter-quartile range (IQR) above Q3, while
the lower whisker spans from the first quartile (Q1) to the smallest value within 1.5 × IQR
below Q1. The interquartile range (IQR) is defined as the difference between Q3 and Q1.
Because expression data were normalized using a cohort-specific normalization
method, *RAPGEF1* expression values are not directly comparable between cohorts. **B**.
Meta-analysis across melanoma cohorts assessing *RAPGEF1* as a marker of survival in
tumors wild type for *BRAF* codon V600 and K601 and *NRAS* codon Q61, G12, and G13
somatic mutations. To minimize potential confounding due to treatment-specific effects, we
analyzed datasets comprised primarily of patients not treated with immunotherapy (TCGA,
Lund, and Leeds cohorts) separately from those focused on immunotherapy-treatment (Van
Allen, Riaz, and Liu cohorts). The variable n denotes the number of samples analyzed in the
WT group for each cohort. Within each cohort, *RAPGEF1* expression was dichotomized into
high and low groups based on the median value. The low *RAPGEF1* expression group is the
reference group in Cox models and is depicted by the grey line on the plot. Each point
represents the estimated log HR for a given cohort; horizontal lines denote the 95%
confidence interval around the point estimate. The meta-analysis label on the plot denotes
the log HR estimate from the meta-analysis.

Discussion

GWAS of melanoma and melanoma risk-associated phenotypes have successfully identified

numerous loci associated with risk, but the causal sequence variants and genes underlying

most have yet to be established. Here, we characterize a GWAS locus at 9q34.13 associated

with melanoma risk as well as nevus count [1] and identify a likely role for germline

variation regulating RAS and RAP signaling via modulation of expression of the guanine

nucleotide exchange factor *RAPGEF1*. Given that both nevi and melanomas originate from

melanocytes, we focused on quantitative trait locus (QTL) data from melanocytic cells,

demonstrating an association between higher *RAPGEF1* expression and the risk allele via

both colocalization and TWAS/MWAS approaches. We show that RAPGEF1 promotes both

RAP1 and RAS activation in melanocytes and contributes to colony formation in

immortalized melanocytes. Notably, we also observe consistently higher *RAPGEF1* levels in

melanomas lacking canonical, strongly-activating RAS-MAPK pathway mutations, suggesting

that *RAPGEF1* could potentially contribute to pathway activation in these wild-type tumors.

Analyses of this locus using multiple melanocytic genomic datasets (eQTL, meQTL) revealed

a complex pattern of LD between the melanoma GWAS signal and two partially-correlated

melanocyte eQTLs in the region for *RAPGEF1* and *UCK1*, complicating efforts to firmly

identify the causal sequence variant or variants underlying melanoma risk at this locus.

While conditional analysis of this GWAS locus indicates the presence of only a single signal,

fine-mapping of the GWAS and respective melanocyte eQTLs reveal mostly distinct sets of

potential causal variants for each, all in LD with each other, possibly indicating the presence

of functional variants in each credible set contributing to risk, or alternatively that the GWAS

signal may simply be an efficient tag for haplotypes containing causal *UCK1* and *RAPGEF1*

cis-regulatory variants. We demonstrated that the region harboring the GWAS signal itself

physically interacts with the promoters of and is a functional regulatory sequence for both

genes. Conditioning on predicted melanocyte expression of both *RAPGEF1* and *UCK1*

together does not fully attenuate the GWAS signal, suggesting a potential functional role for

the GWAS signal itself. Still, past MPRA work assessing fine-mapped melanoma GWAS

variants in both melanocytes and melanoma failed to identify variants with allele-specific

cis-regulatory activity, perhaps indicating the lack of causal variants amongst the GWAS

credible set or alternatively that there may be undiscovered variation strongly associated

with the signal, or both. Further work will be required to identify the causal *cis*-regulatory

variants in this region.

Similarly, the complex LD between GWAS and eQTL signals in this region complicate efforts

to firmly establish a single gene as the only likely causal gene underlying melanoma risk at

this locus. eQTL and meQTL analyses establish both *RAPGEF1* and *UCK1* as *cis*-regulatory

targets and strong candidate melanoma risk genes where higher *RAPGEF1* expression and

lower *UCK1* are associated with risk. Conditional analysis of the GWAS based either on

predicted *RAPGEF1* and *UCK1* expression, or alternatively using lead eQTL SNPs for both

genes showed that *RAPGEF1* expression potentially contributes more to the melanoma risk

signal than *UCK1*, but these data need to be interpreted cautiously given potential

differences in the populations from the GWAS and the relatively small sample of

melanocytes and melanomas used in QTL analyses.

We performed gene knockdown experiments in melanocytes to assess potential phenotypes

mediated by both genes. Knockdown of either gene resulted in dramatically decreased

melanocyte proliferation. Given the opposing directions of effect for melanocyte eQTLs

relative to melanoma risk, we chose to focus on *RAPGEF1* in melanocytes where higher

expression associated with risk and an observed positive effect on melanocyte growth is

consistent with a role promoting tumor formation or growth. Nonetheless, we cannot fully

exclude a role for *UCK1*, a member of the pyrimidine salvage pathway that is crucial for DNA

replication and repair mechanisms. We observed that RAPGEF1 overexpression in an

immortalized melanocyte model that expresses oncogenic BRAF (V600E) results in efficient

formation of large (>70um) colonies, where BRAF^{V600E} expression itself is not sufficient.

These data are consistent with that observed for other genes with oncogenic potential in

melanocytes in this model [81, 82]. While colony formation in melanocytes expressing

constitutively active mutants of NRAS (Q61R) and RAP1A (G12V) was considerably higher

than that for RAPGEF1, colony formation was similar between RAPGEF1 overexpressing

melanocytes and those expressing the less common and potent G12V mutant of NRAS, as

well as overexpression of wild-type RAP1A, highlighting the oncogenic activity of RAPGEF1.

RAPGEF1 is a guanine nucleotide exchange factor that facilitates the exchange of GDP for

GTP, thereby activating small GTPases, through interactions mediated by SH3 domains of its

partner proteins. Almost all known GEFs for RAS family G-proteins contain a CDC25

homology domain as the catalytic domain, yet each GEF acts on only a small subset of the

RAS family members with a high selectivity [86]. While SOS proteins are widely-

expressed GEFs activating RAS proteins, RAPGEF1 has been established as a major GEF for

RAP1 [97, 98]. Given the similarity in RAPGEF1 colony formation results as compared to

NRAS^{G12V}, and that RAPGEF1 has previously been implicated in activation of R-RAS [87] as

well as indirect activation of RAS [88], we assessed the effect of RAPGEF1 expression on

both RAP1 and RAS activation in serum starved melanocytes. Strikingly, RAPGEF1

overexpression contributed to both RAP1 and RAS activation upon hEGF treatment, as well

as leading to constitutive activation of RAS itself in the absence of hEGF suggesting RAPGEF1

contributes to RAS activation. Temporally, we observe that RAS activation in response to

hEGF appears to precede activation of RAP1, with a noticeable increase in active RAS at 2

minutes compared to that for RAP1 at 15 minutes.

While a role for direct activation of RAS by RAPGEF1 has not been established, some GEFs

can indeed act on both RAS and RAP1 proteins. GRP3, a GEF abundantly expressed in brain,

increased GTP/GDP-bound ratios of both HRAS and RAP1A in 293T cells [99]. CalDAG-GEFIII,

one of the four CalDAG-GEFs, promoted nuclear exchange of HRAS, R-RAS and RAP1, and

shows the widest substrate specificity among the known GEFs [100]. RasGRP2, an

alternatively spiced form of CalDAG-GEF1, selectively catalyzes nucleotide exchange on N-

and K-RAS, but not H-RAS. RasGRP2 also activates RAP1, albeit less potently than CalDAG-

GEF1, which preferentially activates RAP1 [101]. The selectivity of a substrate by a GEF is

largely determined by the amino acid residues at the interface between the GEF and the

GTPase, yet this interaction can be highly context-dependent and subjected to spatio-

temporal regulation [89, 102]. During *Drosophila* eye and wing development,

overexpression of membrane-tagged full-length *Drosophila* RAPGEF1 phenotypically mimics

overactivation of the RAS1–MAPK pathway, suggesting that *Drosophila* RAPGEF1 is involved

in RAS activation *in vivo*. These effects can be suppressed by both reduction in the gene

dose of RAS1–MAPK pathway components or RAP1, as well as overexpression of a

membrane-tagged version of *Drosophila* RAPGEF1 lacking the CDC25 catalytic domain.

These findings indicated that membrane localization of dysregulated RAPGEF1 can trigger

activation of RAP1 and RAS resulting in the activation of MAPK [102]. We did perform mass

spectrometry analysis of proteins pulled-down by RAPGEF1 antibody in RAPGEF1

overexpressing immortalized melanocytes grown either in regular melanocyte medium or

under serum-starved and hEGF-treated conditions, but did not identify any small GTPases

(including RAP1; data not shown). While the traditional affinity-purification Mass

spectroscopy (AP-MS) methods often require stable protein interactions in cell extracts,

small GTPases undergo dynamic conformational changes as they cycle between active and

inactive states and the transient nature of their interactions with GEFs making it difficult to

be captured. A typical example is SOS1, a major GEF of RAS protein, is historically

challenging to be captured with AP-MS [103, 104]. Interestingly, using live-cell proximity-

dependent biotin labelling of proteins (BioID) followed by Mass Spec analysis, Kovalski and

colleagues captured an interaction between HRAS and RAPGEF1 in bladder cancer cells,

supporting the notion that RAPGEF1 may indeed function as a GEF of HRAS [104]. Further

work will be required to assess whether RAPGEF1 directly acts on RAS in melanocytes,

however our data clearly demonstrate a role for activation of RAS by RAPGEF1 in

melanocytes whether directly or indirectly.

Most melanoma tumors harbor somatic RAS-MAPK pathway activating alterations, including

oncogenic mutations in RAS genes, BRAF, or loss of NF1, and likely do not need to rely on

more subtle germline influence on pathway-activating genes. Still, a minority of melanomas

lack strongly-activating RAS and BRAF mutations or alterations in other directly-involved

pathway members, suggesting other mechanisms likely contribute to pathway activation in

a subset of tumors. Given our observation that RAPGEF1 activates RAS in melanocytic cells,

we evaluated whether *RAPGEF1* expression differs between tumors with RAS-MAPK

activating mutations and those that are wild-type. Across six melanoma cohorts, we

consistently observed elevated *RAPGEF1* expression in wild-type tumors. These data are

consistent with a potential role for RAPGEF1 contributing to RAS-MAPK pathway activation

in these tumors, whether via an effect on RAS, RAP, or both. We also assessed whether

RAPGEF1 expression in wild-type tumors could serve as a prognostic marker. Across

melanoma cohorts where most patients had not received any form of immunotherapy

treatment, *RAPGEF1* overexpression predicted poor prognosis. Conversely, in cohorts where

all patients were treated with immunotherapy, elevated *RAPGEF1* expression predicted

improved survival. Interestingly, rare somatic mutations in *RAPGEF1* have been observed in

non-Hodgkin's lymphomas that disrupt RAPGEF1 autoinhibition and lead to constitutive

RAP1 activation, suggesting a plausible role for RAPGEF1 in promoting cancer progression

[105]. Nonetheless, given the relative rarity of RAS-MAPK wild-type tumors and the

relatively small sample sizes in these patient cohorts, this association need to be treated

cautiously and more rigorously validated.

Rare germline variation in RAS pathway genes is known to impact nevus development,

specifically strong activating germline mutations that cause developmental syndromes

termed "RASopathies" [106]; patients with select RASopathies have higher number of

melanocytic nevi [107-109]. Together with recent data potentially implicating other RAS-

pathway members in melanoma/nevus risk loci, our data contribute to emerging evidence

that common germline variation in genes regulating RAS pathway activity may also play a

prominent role in melanoma risk and nevus development. We previously used a GWAS

region-focused capture-HiC assay to nominate potential target genes from other melanoma

GWAS loci [22], with *CBL* and *SHC1* nominated at separate loci. *CBL* encodes a negative

regulator of receptor tyrosine kinases (and in turn, RAS signaling). We previously validated

that the risk allele at the respective locus was associated with decreased *CBL* expression and

demonstrated regulation of *CBL* by the region of the risk signal [22]. *SHC1*, which was

nominated by the observation of active enhancer-promoter interactions from regions

surrounding fine-mapped risk variants, encodes an adaptor protein that transduces signals

from activated receptors to RAS by recruiting GRB2-SOS complexes, thereby promoting RAS

activation and downstream RAS-MAPK signaling [110, 111]. In addition, the gene encoding

another RAP GEF (*RAPGEF5*) is located at a melanoma risk locus identified by Landi and

colleagues (rs12539524) [1], raising the possibility that other such GEFs could play a similar

role. Lastly, a new GWAS of nevus count that nominated *NRAS* itself as a potential causal

gene at a novel locus marked by rs7537232 [112]. While nearly every nevus acquires an

oncogenic mutation in either *BRAF* or *NRAS*, our data suggest that germline variation

regulating RAS activity may confer a growth or survival advantage prior to acquisition of a

strongly activating somatic mutation.

We note several limitations of our study. Firstly, we have not positively identified the causal

variant or variants underlying risk at this locus. Efforts to do so statistically are hampered by

partial LD between QTL and melanoma risk signals, as well as a very large number of

potential causal variants for melanocyte *RAPGEF1* expression. Nonetheless, the evidence

implicating *RAPGEF1*, and perhaps *UCK1*, at this locus is strong. As noted above, we cannot

rule out a role for *UCK1* in melanoma risk, further work will be required to assess potential

phenotypes associated with variation in *UCK1* levels in melanocytes. Critically, while we

have established that RAPGEF1 dysregulation leads to RAS activation, we have not

demonstrated that RAPGEF1 acts as a direct GEF for RAS. We show a strong association of

RAPGEF1 levels with mutation status in melanoma tumors, however further work is

required to establish whether and or to what degree RAPGEF1 actively contributes to RAS-

MAPK pathway activation in tumors. Finally, the melanoma survival datasets are each

relatively small, with even smaller numbers of RAS-MAPK pathway wild-type tumors; further

assessment of associations of *RAPGEF1* with survival in patients with wild-type tumors is

needed.

1020

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Declaration of Interests

The authors declare no competing interests.

Web Resources

- NIH Biowulf Cluster, http://hpc.nih.gov
- PLINK: https://www.cog-genomics.org/plink/
- GCTA-COJO : https://yanglab.westlake.edu.cn/software/gcta/
- RSparsePro: https://github.com/zhwm/RSparsePro_LD
- SuSiE: https: https://stephenslab.github.io/susieR/reference/susie_rss.html
- DAP-G: https://github.com/xqwen/dap

•   AREsite2: http://nibiru.tbi.univie.ac.at/AREsite2/welcome

•   Ensembl variant Effect Predictor:

https://useast.ensembl.org/info/docs/tools/vep/index.html

•   TWAS FUSION: http://gusevlab.org/projects/fusion/

•   COLOC: https://github.com/chr1swallace/coloc

•   SharePro: https://github.com/zhwm/SharePro_coloc

•   ENCODE: https://www.encodeproject.org/encyclopedia/

•   CHiCAGO: https://www.functionalgenecontrol.group/chicago

•   HiCUP: https://www.bioinformatics.babraham.ac.uk/projects/hicup/

•   LDlink: https://ldlink.nih.gov/?tab=home

•   UCSC browser: https://genome.ucsc.edu

•   WashU Epigenome Browser: http://epigenomegateway.wustl.edu

•   Bedtools: https://bedtools.readthedocs.io/en/latest/

•   Bedtoolsr: http://phanstiel-lab.med.unc.edu/bedtoolsr.html

•   ROADMAP epigenomic project:

https://egg2.wustl.edu/roadmap/web_portal/chr_state_learning.html

•   TCGA melanoma cohort:

https://www.cbioportal.org/study/summary?id=skcm_tcga_pan_can_atlas_2018

•   Van Allen cohort data:

https://www.cbioportal.org/study/summary?id=skcm_dfci_2015)

•   Liu Cohort data: https://www.cbioportal.org/study/summary?id=mel_dfci_2019)

•   Riaz cohort data: https://github.com/riazn/bms038_analysis/tree/master/data

1071

Data and Code Availability

1072

Small RNA-seq data generated from 106 melanocyte cultures is currently being deposited on

ArrayExpress. Melanocyte ATAC-seq and capture-HiC data are publicly available

(ArrayExpress accession: E-MTAB-15079, E-MTAB-15048). Melanocyte genotyping, RNA-

sequencing, and methylation data are previously published [13, 16] and accessible through

NCBI database of Genotypes and Phenotypes (dbGaP) under accession dbGaP:

phs001500.v2.p1.

MPRA are previously published [23]; raw sequencing can be accessed through NCBI Gene

Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under the accession

GEO: GSE210356.

Summary data for 2020 melanoma GWAS were obtained from dbGaP (dbGaP:

phs001868.v1.p1), with the exclusion of self-reported data from 23andMe, Inc. and UK

Biobank. The full GWAS summary statistics for the 23andMe discovery dataset will be made

available through 23andMe to qualified researchers under an agreement with 23andMe

that protects the privacy of the 23andMe participants. Please

visit https://research.23andme.com/collaborate/#dataset-access/ for more information and

to apply to access the data. Summary data from the remaining self-reported cases are

available from the corresponding authors of that manuscript (Matthew

Law, matthew.law@qimrberghofer.edu.au; Mark Iles, m.m.iles@leeds.ac.uk; and Maria

Teresa Landi, landim@mail.nih.gov).

Melanoma tumor data for TCGA melanoma cohort was accessed using cBioportal database

https://www.cbioportal.org/study/summary?id=skcm_tcga_pan_can_atlas_2018.

Leeds Melanoma cohort gene expression and mutation profiling dataset is available on

European Genome-phenome Archive (study ID: EGAS00001002922, dataset ID:

EGAD00010001561, dataset ID: EGAD00001008360). Lund Melanoma gene expression

dataset is available on NCBI Gene Expression Omnibus (GSE65904); mutation profiling data

was available from Cirenajwis *et al.*, Supplementary Table 3 [72]. Liu and Van Allen cohort

dataset was accessed using cBioportal (Liu cohort:

https://www.cbioportal.org/study/summary?id=mel_dfci_2019) (Van Allen cohort:

https://www.cbioportal.org/study/summary?id=skcm_dfci_2015) Riaz cohort mutation and

expression dataset was accessed using

https://github.com/riazn/bms038_analysis/tree/master/data

1104

Author Contributions

1106   R.T., M.X, H.S., J.Yon, S.A.-S., M.L., T.R., H.S., G.D., L.J., T.M, T.Z., R.C., E.L., K.F., J.Yin, R.H.,

1107   T.Z., G.J., D.T.B., J.N.-B., J.N., M.M.I., M.T.L., M.H.L., T.A., J.C., J.S., and K.M.B. contributed to

1108   the research activities described in this manuscript. K.M.B. led and supervised the research

1109   described. M.M. provided additional supervision and mentorship. R.T., M.X., and K.M.B.

1110   wrote the manuscript.

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