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RARE VARIANT, GENE-BASED ASSOCIATION STUDY OF HEREDITARY MELANOMA USING WHOLE EXOME SEQUENCING

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ABSTRACT

Background. Extraordinary progress has been made in our understanding of common variants in many diseases, including melanoma. Since the contribution of rare coding variants is not as well characterized, we performed an exome-wide, gene-based association study of familial cutaneous melanoma (CM) and ocular melanoma (OM) using 12,033 jointly processed cases and controls.

Methods. Whole exome sequencing was performed followed by large-scale joint variant calling using GATK. Plink/SEQ was used for statistical analysis of genetic variation. Four models were used to estimate association among different types of variants. Functional validation was performed using somatic, cellular and tumorigenesis assays.

Results. Strong signals were detected for CDKN2A (p=6.16E-08) and BAP1 (p=3.83E-06) in the CM (n=273) and OM (n=99) cohorts, respectively. Eleven genes which exhibited borderline association (p<10^{-4}) were independently validated using the TCGA melanoma cohort (379 CM, 47 OM) and a matched set of 3,563 European controls with CDKN2A (p=9.31E-03), BAP1 (p=0.026) and EBF3 (p=4.75E-04), a novel CM risk locus, all showing evidence of replication. EBF3 was then evaluated using germline data from a set of 160 familial melanoma cases and 4,769 controls from the U.K. (joint p=3.29E-05). Somatically, loss of EBF3 expression correlated with progression, poorer outcome and high MITF tumors. Functionally, induction of EBF3 in melanoma cells reduced arrested cell growth in vitro, retarded tumor formation in vivo and reduced MITF levels.

Conclusions. The results of this large rare variant germline association study further define the mutational landscape of hereditary melanoma and implicate EBF3 as a possible CM predisposition gene.

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BACKGROUND

In 2016, an estimated 76,380 Americans will develop cutaneous melanoma (CM) and 10,130 will succumb to this disease{Siegel, 2016 #4144}. Of all cancers in the U.S., melanoma ranks fifth in incidence among men and seventh among women{Siegel, 2016 #4144}. The incidence of ocular melanoma (OM) is significantly lower with an estimated 2,500 cases annually in the United States{Jovanovic, 2013 #4151}. It has been reported that 10% of melanoma patients report a family history of melanoma{Greene, 1985 #1870;Kraemer, 1983 #940}. The risk for melanoma is increased 2-fold for 1st degree relatives{Ford, 1995 #1591} and approximately 5-fold if two or more 1st-degree relatives are affected. Twin studies have estimated the heritability of melanoma to be 58%, which is significantly higher than the 33% for cancers overall{Mucci, 2016 #4145}. Thus, there is evidence for a strong genetic influence on melanoma risk.

As with many cancers, the history of melanoma genetics can be divided into the pre- and post-genomic era. Prior to the advent of high-density human genomic maps, linkage efforts implicated familial melanoma loci on 1p36{Bale, 1989 #14} and 9p21{Cannon-Albright, 1992 #18}. Subsequent interval gene screening led to the identification of deleterious germline alterations of CDKN2A in a subset of 9p21-linked families. In the ensuing years, linkage efforts across multiple families have produced additional candidate loci on chromosomes 1p36{Bale, 1989 #14}, 9q21{Jonsson, 2005 #3587}, 5q31{Falchi, 2006 #3854} and 1p22{Gillanders, 2003 #2213} without isolation of specific disease causing mutations. Rare germline mutations in CDK4, BAP1, MITF, TERT (promoter), POT1, ACD and TERF2IP have also been reported in both ocular and cutaneous melanoma families though, collectively, they account for less than 5 percent of all hereditary melanoma cases{Soura, 2016 #4176;Soura, 2016 #4175}.

Common variant association studies (i.e. genome wide association studies (GWAS)) recently culminated in an analysis of 15,990 CM cases and 26,409 controls, which substantiated 20 genome-wide significant loci{Law, 2015 #4150}. The general synthesis from GWAS and other

candidate association studies is that loci associated with pigmentation (MC1R, TYR, ASIP, OCA2 and SLC45A2), nevus count (CDKN2A-MTAP, PLA2G6 and TERT) and DNA repair (PARP1 and ATM) represent core drivers of a risk phenotype that has been delineated by epidemiologic studies{Elwood, 1984 #1760;Holman, 1984 #1772;MacKie, 1989 #1738;Gallagher, 1990 #1766;Bliss, 1995 #2191;Holly, 1995 #2866;Naldi, 2005 #2865}.

While GWAS have now been successfully deployed in a large spectrum of disorders, the systematic pursuit of rare disease-causing variants is just emerging. Given the high cost of sequencing in the past, linkage analysis provided a robust method to leverage recombination for positional information. For relatively common endpoints, linkage has been largely unsuccessful as, beyond rare examples of single pedigrees that are sufficiently large and carry a near-fully penetrant mutation to generate significant linkage, the polygenic nature of common disease precludes gene localization. To overcome the fact that rare variants are distributed across many genes, we and others have proposed methodologies for gene discovery through rare variant association studies (RVAS{Zuk, 2014 #4138;Samocha, 2014 #3959}). One common approach is to group the individual variants into sets (e.g. gene-based association) and compare the aggregate frequency distribution in cases vs. controls. Using this framework, we set out to comprehensively map the mutational landscape of cutaneous and ocular melanoma by performing whole exome sequencing followed by a rare variant association analysis in over 8,000 individuals (cases and PCA-matched European non-cancer controls). Our results indicate that CDKN2A and BAP1 remain the leading predisposition loci in CM and OM respectively, though a number of additional promising candidates emerged. One of these, EBF3, was subsequently replicated in two additional cohorts of germline melanoma samples and credentialed as a tumor suppressor in a set of functional assays.

METHODS

Patient cohorts. Details could be found in Supplementary Methods.

Exome sequencing, variant processing and calling. Whole exome libraries were prepared using a modified version of Agilent's Exome Capture kit and protocol, automated on the Agilent Bravo and Hamilton Starlet. Libraries were then prepared for sequencing using a modified version of the manufacturer's suggested protocol, automated on the Agilent Bravo and Hamilton Starlet, followed by sequencing on the Illumina HiSeq 2000. We used an aggregated set of samples consented for joint variant calling resulting in 37,607 samples (germline from 292 CM patients, 101 OM patients, 379 TCGA CM patients, 47 TCGA OM patients, 24,612 controls and 12,158 other individuals not used for association studies). All samples were sequenced using the same capture reagents at the Broad Institute and aligned on the reference genome with BWA{Li, 2009 #4178} and the best-practices GATK/Picard Pipeline, followed by joint variant calling with all samples processed as a single batch using GATK v 3.1-144 Haplotype Caller{Van der Auwera, 2013 #4181;DePristo, 2011 #4180;McKenna, 2010 #4179}. The resulting dataset had 7,094,027 distinct variants. Haplotype Caller, which was used for the ExAC database (http://exac.broadinstitute.org/gene), was also used to detect indels. Selected mutations in CDKN2A and BAP1 were confirmed with Sanger sequencing.

We performed principal component analysis (PCA) on common (MAF>5%) autosomal independent SNPs to filter out all non-European samples with Eigenstrat{Price, 2006 #4183}. Relatedness analysis among Europeans was conducted with PLINK{Purcell, 2007 #4184} as suggested in the PLINK best practices{, #4185}. We used VEP{McLaren, 2010 #4186} for functional annotation of the DNA variants. Common and rare variants analyses were conducted using PLINK/SEQ, which allows indexing of the large datasets. A burden test was used for rare protein truncating variants. In addition, the VT and C-alpha tests were chosen as an adaptive

burden test and variance-component test, respectively, to complement each other and to boost the power of rare missense and protein truncating variation association detection{Lee, 2014 #4187}. See details in supplementary methods.

Statistical Methods. Gene-based association was performed using 3 distinct, but related, analytical frameworks. In the first analysis, a burden test was applied to all rare (MAF<1%) protein truncating (PT) variants since the functional impact is presumed to be severe and most directly inferred. Then, to expand on all rare variants (missense and PTV), a second analysis using both the C-alpha and variable threshold (VT) tests was employed. A third analysis applied the burden test to examine "ultra-rare" (MAF<.0001; ExAC database http://exac.broadinstitute.org/gene/) variants as these may represent the most highly penetrant alleles. In the case of a single-model association test – the null statistic was represented by the uniform distribution of p-values. Since four different test statistics (i.e. VT, C-alpha, burden of PTVs and burden of ExAC filtered variants) were applied and the lowest p-value was chosen, the null distribution was constructed by choosing the smallest p-value from 4 null single-statistic models (4 sets of uniform p-values). This process simulates the procedure of selecting the best p-value out of 4 different test statistics that was used for gene-association testing thus making it a more conservative approach. Genome-wide significance was determined by Bonferroni correction (0.05 /17,337 genes tested, i.e. $p<2.88E-06$).

EBF3 validation. EBF3 validation and expression analysis can be found in Supplementary Methods.

RESULTS

Cohort and overview

For this study, germline DNA from "genetically-enriched" cases (individuals with familial melanoma or multiple primary melanomas) of CM and OM were identified and subjected to whole exome sequencing. For the discovery set (Fig 1a), a total of 273 CM (M/F 128/145), 99 OM (M/F 46/53) and 7,629 (M/F 5451/2178) European non-cancer control passed quality control and were included in the subsequent analysis.

   In order to ensure ancestral matching, we performed principal component analysis (PCA; Fig. 1b) between the cases and control cohorts in the primary and TCGA replication cohorts. Our analysis was then restricted to European cluster of samples only. Examination of common synonymous variants (MAF>5%) revealed a null-distribution of the test statistic between cases and controls. There was an average of 25,633 SNPs per sample, which is within the expected range for a typical European germline exome{DePristo, 2011 #4180}.

   The first replication cohort included 379 CM and 47 OM cases from TCGA and 3,563 European non-cancer controls. These additional 3,989 samples were jointly processed through the same alignment and variant calling pipeline as the initial discovery set and subjected to the same quality control standards. For EBF3, a second replication was performed using allele counts derived from 160 familial melanoma cases (i.e. 77 cases (66 families) from Leeds, U.K., 56 cases (9 families) from Sydney, Australia and 27 cases (6 families) from Queensland, Australia) and 4,769 non-cancer controls from the UK10K population project; whole genotypes were not available for the U.K. replication cohort and thus were not matched by PCA.

Mutational landscape of cutaneous and ocular melanoma

   We first interrogated our melanoma cases for rare PT mutations among known melanoma predisposition genes and their associated complexes or pathways (i.e. RB, telomerase/shelterin,

BAP1; Supplementary Table 1 and Supplementary Table 2). As expected, the most significant associations were for CDKN2A (p=6.16E-08 with CM) and BAP1 (p=0.005 for CM and p=3.83E-06 for OM). We detected 5 rare POT1 mutations (p=.002), including novel nonsense (p. Ser522X) and splice donor (chr7:124475332 C/T) mutations and a previously reported p.D224N variant. For MC1R, we examined red hair color (RHC) variants and observed 126, 23 and 6 individuals among CM cohort with single heterozygous, double heterozygous and homozygous genotypes, respectively; analogous counts for controls were 2,269, 251 and 60 (Supplementary Table 3; p=1.00E-08; cumulative allele odds ratio=1.79, 95% CI: 1.46 to 2.16).

The top cutaneous melanoma risk candidates which emerged from each of the 3 analytical frameworks are shown in Supplementary Table 4 (full rankings and details shown in Supplementary Tables 5, 6 and 7). Figure 2a shows the P_{min} Manhattan plot for all 17,337 genes that were analyzed for association. CDKN2A exhibited the strongest association (P_{min} = 6.16E-08, Supplementary Table 4) and was the only locus to reach genome-wide significance. Other candidates which were near, but not reaching, genome-wide significance were considered novel and which were subjected for further study included ACTR8 (P_{min}=2.18E-05, C-alpha), ECHD1 (P_{min}=3.73E-05, C-alpha), COL11A2 (P_{min}=5.42E-05, PTV burden) and EBF3 (P_{min}=8.22E-05, C-alpha). Similar analyses for ocular melanoma are also shown in Supplementary Table 4 (also Supplementary Tables 8, 9 and 10). BAP1 was the leading risk gene (P_{min}=3.83E-06; Supplementary Table 4 and Fig 2b) though it did not quite reach genome-wide significance. Other borderline candidates that were subjected to further analysis include IAH1 (P_{min}=2.27E-05, C-alpha), NHLRC3 (P_{min}=6.88E-05, C-alpha), RSRC1 (P_{min}=1E-04, C-alpha), PAPOLG (P_{min}=1E-04, C-alpha).

All CM and OM loci with a significance level of $p \leq 1.00E-4$ (6 CM genes, 5 OM genes) were then subjected to replication with germline exome sequences from the TCGA (CM, n=379; OM, n=47) and another independent cohort of 3,563 matched European controls jointly processed with the discovery set (Table 1; Fig S1). For CM, CDKN2A (p=9.31E-03; PTV burden) and EBF3 (p=4.75E-

04; C-alpha) both remained significant while, for OM, BAP1 also remained significant (p=2.64E-02; PTV burden). As EBF3 represents a potentially novel risk gene, we performed a second replication with this gene by interrogating whole exome and genome sequence data for 160 familial melanoma cases from 81 melanoma-prone kindreds. We identified a Leeds family with a p.N455S mutation which was detected in 1 of 2 affected members and a Sydney family with a p.G21S variant that was present in 2 of 6 affected members. Similar evaluation of 4,769 individuals from the UK10K cohort revealed 21 EBF3 mutation carriers in this control set. In aggregate (Table 1), a joint burden test across all EBF3 cohorts resulted in a significant association between EBF3 and cutaneous melanoma (9/811 cases and 42/15,961 controls; OR= 4.64 [95% CI 2.21- 9.72], p=3.29E-05, Mantel-Haenszel chi-square test); to avoid a bias counting of carriers, we censored one of the two p.G21S carriers in the Sydney family while accounting for all non-carriers (i.e. 3 mutations out of 160 cases were identified in the U.K. replication but 2 carriers out of 159 individuals used for Mantel-Haenszel test). Taken together, these results confirm the known contribution of CDKN2A and BAP1 as strong risk loci for CM and OM, respectively, and nominate EBF3 as a novel risk gene for CM. In addition, the recovery of these known hereditary melanoma loci further verified the technical and methodological pipeline used in this RVAS.

Although the study was not designed to compare genes which selectively confer risk for either, or both, CM and OM, we did compare the P_{min}'s for each gene relative to their melanoma type (Fig 2c). There was some evidence that LCE1E can confer risk for both CM and OM (P_{min}=1.59E-04 for OM and 2.56E-03 for CM) though neither reached genome-wide significance.

Functional validation of EBF3

Among the secondary candidates, none has been previously linked to cancer predisposition. Since EBF3 ranked in 3 of 4 framework analyses, was replicated in the TCGA and European cohorts and has been reported to possess tumor suppressive activity in a number of non-melanoma cancers{Liao, 2009 #4204;Tao, 2015 #4192}, we decided to examine EBF3's in greater detail.

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Among melanoma cases, there were 4 mutations in our discovery set (Fig S2; one p.Q137R, one p.A409T and two p.N484S), 3 mutations in the TCGA cohort (one p.P594L and two p.G459C) and 2 variants in the European melanoma panel (p. N455S and p. G21S). Among the EBF3 variants in our cases, 2 have been previously observed - p.N484S (MAF= 3.00E-05; European/Non-Finnish) and p.G21S (MAF=2.21E-04; European/Non-Finnish) albeit at a much lower rate. The p.Q137R mutation falls within the DNA binding domain, the p.A409T mutation lies in the third helix of the HLHLH domain and the p.N484S alteration sits in and the C-terminal Pro/Ser/Thr-rich (PST) region (Fig S2). As EBF3 shows low sequence homology to other protein families and have no known biological connection to melanoma, we set out to perform proof-of-concept validation for EBF3 as a tumor suppressor using available somatic data and empirical cell-based assays.

Cancer susceptibility loci, including those for melanoma (e.g. CDKN2A and BAP1), often function as tumor suppressor genes and thus exhibit structural damage and/or expression loss during malignant progression. To this end, we surveyed the TCGA melanoma tumors for evidence of somatic copy-number loss. As shown in Figure 3a, EBF3 harbored significantly more deletions than other genes (Wilcoxon test, p=2.00E-151), which may, in some cases, reflect loss of the entire 10q arm. There is evidence of concurrent shallow deletions of EBF3 and PTEN (also on 10q) though deep deletions of EBF3 are uncommon and independent of PTEN while expression levels of the two genes appear to be also uncorrelated (Fig S3). On a mutation level, there were there were 28 missense alterations identified in the TCGA melanoma cohort with no loss-of-function variants (Supplementary Table 11).

With regards to expression, primary melanomas exhibited lower EBF3 RNA levels when compared to either normal skin or benign nevi (Fig 3b; melanoma vs nevus, p=0.005) suggesting that loss of EBF3 may contribute to melanoma progression. Diminished EBF3 expression was also associated with heightened tumor aggressiveness. Using microarray data from a panel of 125 stage III melanoma tumor specimens obtained at Lund University and RNA-seq data from 470 TCGA tumor specimens, we found that lower EBF3 levels showed a statistically significant

correlation with poorer overall survival in the Lund data set (Fig 3c; p=0.02) and a marginal trend toward a worsened outcome among the TCGA tumors (p=0.10).

To better understand the molecular context of EBF3 function, we examined EBF3 levels across known molecular subtypes of melanoma. Strikingly, EBF3 appears to be inversely correlated with the MITF-anchored classes, i.e. EBF3 expression was lowest in the MITF-hi ("Pigmentation") subtype from the Lund cohort and highest in the "MITF-lo" subtype from the TCGA set (Fig 3d). Given this intriguing relationship, we searched for interactions between levels of EBF3 and melanocyte lineage genes. There was a statistically significant inverse relationship between EBF3 and MITF expression levels in both the Lund (Spearman r, -0.36, 95% CI: -0.47 to -0.24, p<0.0001) and TCGA (Spearman r, -0.30, 95% CI: -0.40 to -0.18, p<0.0001, Supplementary Table 12) tumor sets. Moreover, examination of several known MITF targets (Fig 3e; TYR, MLANA) and upstream regulators of MITF (TCF4, SOX10, PAX3) all revealed a consistent pattern whereby lineage genes which positively correlated with MITF (i.e. TYR, MLANA, SOX10 and PAX3) were negatively correlated with EBF3 while TCF4, a known negative regulator of MITF, was positively correlated with EBF3. These results reveal a reciprocal relationship between EBF3 and MITF and raise the possibility that EBF3 may antagonize MITF. To test this hypothesis, we induced EBF3 in two MITF-expressing melanoma lines (G-mel and UACC-62) using a Tet-responsive system and found a concomitant reduction of MITF in both cell lines (Fig 3f).

We next sought to phenotypically credential EBF3 in cellular and animal experiments. As shown in Figure 4a, there was dose-dependent suppression of cellular proliferation with escalating levels of EBF3 in 3 melanoma cell lines; exposure of cells with the Tet-GFP control vector to doxycycline had no measurable effect on survival (data not shown).   A375 and LOX cells were then subjected to a 3-D matrigel spheroid formation assay and, for both lines, there was a significant reduction in colony volume with the introduction of EBF3 (Fig 4b). Finally, overexpression of EBF3 in A375 melanoma cells led to significant suppression of tumor growth in nude mice (Fig 4c). Taken

together, these results suggest that EBF3 is a veritable tumor suppressor in melanoma, a function

consistent with the role as a predisposition gene.

CONCLUSIONS

The contribution of rare coding mutations to disease risk, such as melanoma, remains a bourgeoning but largely unexplored domain in human genomics. To the best of our knowledge, this is the first exome-based, rare variant association study in melanoma. Several technical advancements, which were introduced to permit the robust assembly of the large exome sequence dataset include the joint variant calling and an advanced quality-check protocol.

Our results indicate that in the landscape of hereditary melanoma, CDKN2A and BAP1 exhibit the strongest association with CM and OM, respectively. By design, we did not exclude these samples from analysis but blindly included the cases in the entire pipeline as "positive" controls; we were reassured that our methodology did recover these genes. There were additional risk loci which approached genome-wide significance (Supplementary Table 2 and 4; $P_{min}<1E-04$), one of which, EBF3, was further replicated in the TCGA and European melanoma cohorts.

We propose that EBF3 has many of the features of melanoma predisposition gene based on multiple lines of investigation: (i) the enrichment for germline EBF3 variants among individuals with melanoma across disparate cohorts, (ii) the presence of deletions at the EBF3 locus in tumor specimens, (iii) the increased clinical aggressiveness associated with EBF3 loss, (iv) the reciprocal interaction between EBF3 and the known lineage oncogene MITF and (v) the direct inhibitory effects of EBF3 on cellular growth and tumor formation. In two families where multiple affected cases were available for analysis, EBF3 mutations were identified only in a fraction of the cases (1/2 and 2/6 affected members) suggesting that these mutations are moderate risk alleles, which is similar to the risk conferred by the MITF(E318K) variant and consistent with EBF3's calculated odds ratio of ~4-5.

EBF3 belongs to a family of transcription factors (EBF1-4) known to be involved in B cell differentiation and the pathogenesis of several tumor types{Liao, 2009 #4204;Tao, 2015 #4192}. Although speculative, it is conceivable that the role of EBF proteins in immune cell

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specification{Medina, 2004 #4332;Liao, 2009 #4331} could explain, in part, the observed association between EBF3 and the "high immune" melanoma subclass (Fig 3d). The EBF3 gene is located on the chromosome 10q26.3 and encodes a 596 amino acid protein with a conserved N-terminal DNA binding region, an IPT/TIG domain, an unusual helix-loop-helix-loop-helix (HLHLH) motif and a C-terminal PST domain{Siponen, 2010 #4212}. Functionally, the EBF transcription factors bind to DNA with a consensus sequence of 5′-CCCNNGGG-3′ as homo- or heterodimers and can interact with p300{Liao, 2009 #4331}. Our mutations do not appear to cluster in any single domain though genotype/functional experiments are currently underway.

For melanoma, there is a single report of EBF1 SNPs being correlated with survival among stage III and IV patients{Fang, 2015 #4177}. In our study, EBF3 showed strong association in all analytical dimensions except the LoF burden test. Examination of this gene in the ExAC database revealed an extreme intolerance for LoF variation (pLI=1.0) thereby suggesting that full loss of EBF3 function may undergo strong negative selection. The precise mechanism of EBF3 action in melanoma remains to be elucidated though one recent report suggests that EBF3 might play a role in cell migration, and possibly proliferation, in a subset of melanoma lines{Chatterjee, 2016 #4334}. There is precedence for transcription factors in cancer-predisposition. Perhaps the most relevant is a low prevalence SNP in the MITF transcription factor (i.e. p.E318K) which alters a sumoylation site and which confers risk for both cutaneous melanoma and renal cell carcinoma{Yokoyama, 2011 #3783;Bertolotto, 2011 #3786}. Germline mutations in other transcription factors such as TP53 and RUNX1 also produce strong cancer phenotypes in Li-Fraumeni syndrome (OMIM #151623) and familial AML (OMIM #601399), respectively.

The design of our study incorporates several classic RVAS strategies{Zuk, 2014 #4138} though there are also several limitations. Full exome sequencing is still more expensive compared to genotyping but the costs are converging. Although we enriched for genetic causation by focusing on rarer familial and multiple tumor cases, statistical power is still significantly lower than those reported for common variant GWAS. Recognizing this risk, we subjected all available cases to our

analytical pipeline including the CDKN2A and BAP1 families and were reassured that significant association signals were readily detected for these loci.

Despite limitations, the analyses in this report represent a major first step towards understanding the landscape of rare mutations in hereditary melanoma. The statistical methodologies which were blindly deployed in the case/control design unequivocally recovered several anticipated signals (i.e. CDKN2A and BAP1). Moreover, several sub-threshold loci have been nominated for future studies including a proof-of-concept validation of one such gene, EBF3.

REFERENCE LIST

1.	Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66(1):7-30.

2.	Jovanovic P, Mihajlovic M, Djordjevic-Jocic J, et al. Ocular melanoma: an overview of the current status. Int J Clin Exp Pathol 2013;6(7):1230-44.

3.	Greene MH, Clark WH, Jr., Tucker MA, et al. High risk of malignant melanoma in melanoma-prone families with dysplastic nevi. Ann Intern Med 1985;102(4):458-65.

4.	Kraemer KH, Greene MH, Tarone R, et al. Dysplastic naevi and cutaneous melanoma risk. Lancet 1983;2:1076-7.

5.	Ford D, Bliss JM, Swerdlow AJ, et al. Risk of cutaneous melanoma associated with a family history of the disease. The International Melanoma Analysis Group (IMAGE). Int J Cancer 1995;62(4):377-81.

6.	Mucci LA, Hjelmborg JB, Harris JR, et al. Familial Risk and Heritability of Cancer Among Twins in Nordic Countries. JAMA 2016;315(1):68-76.

7.	Bale SJ, Dracopoli NC, Tucker MA, et al. Mapping the gene for hereditary cutaneous malignant melanoma-dysplastic nevus syndrome to chromosome 1p. New England Journal of Medicine 1989;320:1367-1372.

8.	Cannon-Albright LA, Goldgar DE, Meyer LJ, et al. Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-p22. Science 1992;258:1148-1152.

9.	Jonsson G, Bendahl PO, Sandberg T, et al. Mapping of a novel ocular and cutaneous malignant melanoma susceptibility locus to chromosome 9q21.32. J Natl Cancer Inst 2005;97(18):1377-82.

10.	Falchi M, Spector TD, Perks U, et al. Genome-wide search for nevus density shows linkage to two melanoma loci on chromosome 9 and identifies a new QTL on 5q31 in an adult twin cohort. Hum Mol Genet 2006;15(20):2975-9.

11.   Gillanders E, Hank Juo SH, Holland EA, et al. Localization of a novel melanoma susceptibility locus to 1p22. Am J Hum Genet 2003;73(2):301-13.

12.   Soura E, Eliades PJ, Shannon K, et al. Hereditary melanoma: Update on syndromes and management: Genetics of familial atypical multiple mole melanoma syndrome. J Am Acad Dermatol 2016;74(3):395-407.

13.   Soura E, Eliades PJ, Shannon K, et al. Hereditary melanoma: Update on syndromes and management: Emerging melanoma cancer complexes and genetic counseling. J Am Acad Dermatol 2016;74(3):411-20.

14.   Law MH, Bishop DT, Lee JE, et al. Genome-wide meta-analysis identifies five new susceptibility loci for cutaneous malignant melanoma. Nat Genet 2015;47(9):987-95.

15.   Elwood JM, Gallagher RP, Hill GB, et al. Pigmentation and skin reaction to sun as risk factors for cutaneous melanoma: Western Canada Melanoma Study. Br Med J (Clin Res Ed) 1984;288(6411):99-102.

16.   Holman CD, Armstrong BK. Pigmentary traits, ethnic origin, benign nevi, and family history as risk factors for cutaneous malignant melanoma. J Natl Cancer Inst 1984;72(2):257-66.

17.   MacKie RM, Freudenberger T, Aitchison TC. Personal risk-factor chart for cutaneous melanoma. Lancet 1989;2(8661):487-90.

18.   Gallagher RP, McLean DI, Yang CP, et al. Suntan, sunburn, and pigmentation factors and the frequency of acquired melanocytic nevi in children. Similarities to melanoma: the Vancouver Mole Study. Arch Dermatol 1990;126(6):770-6.

19.   Bliss JM, Ford D, Swerdlow AJ, et al. Risk of cutaneous melanoma associated with pigmentation characteristics and freckling: systematic overview of 10 case-control studies. The International Melanoma Analysis Group (IMAGE). Int J Cancer 1995;62(4):367-76.

20.   Holly EA, Aston DA, Cress RD, et al. Cutaneous melanoma in women. II. Phenotypic characteristics and other host-related factors. Am J Epidemiol 1995;141(10):934-42.

21.   Naldi L, Altieri A, Imberti GL, et al. Sun exposure, phenotypic characteristics, and cutaneous malignant melanoma. An analysis according to different clinico-pathological variants and anatomic locations (Italy). Cancer Causes Control 2005;16(8):893-9.

22.   Zuk O, Schaffner SF, Samocha K, et al. Searching for missing heritability: designing rare variant association studies. Proc Natl Acad Sci U S A 2014;111(4):E455-64.

23.   Samocha KE, Robinson EB, Sanders SJ, et al. A framework for the interpretation of de novo mutation in human disease. Nat Genet 2014;46(9):944-50.

24.   Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25(14):1754-60.

25.   Van der Auwera GA, Carneiro MO, Hartl C, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics 2013;11(1110):11 10 1-11 10 33.

26.   DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011;43(5):491-8.

27.   McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20(9):1297-303.

28.   Price AL, Patterson NJ, Plenge RM, et al. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 2006;38(8):904-9.

29.   Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81(3):559-75.

30.   http://pngu.mgh.harvard.edu/purcell/plink/.

31.   McLaren W, Pritchard B, Rios D, et al. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. Bioinformatics 2010;26(16):2069-70.

32.   Lee S, Abecasis GR, Boehnke M, et al. Rare-variant association analysis: study designs and statistical tests. Am J Hum Genet 2014;95(1):5-23.

33.   Liao D. Emerging roles of the EBF family of transcription factors in tumor suppression. Mol Cancer Res 2009;7(12):1893-901.

34.   Tao YF, Xu LX, Lu J, et al. Early B-cell factor 3 (EBF3) is a novel tumor suppressor gene with promoter hypermethylation in pediatric acute myeloid leukemia. J Exp Clin Cancer Res 2015;34:4.

35.   Medina KL, Pongubala JM, Reddy KL, et al. Assembling a gene regulatory network for specification of the B cell fate. Dev Cell 2004;7(4):607-17.

36.   Siponen MI, Wisniewska M, Lehtio L, et al. Structural determination of functional domains in early B-cell factor (EBF) family of transcription factors reveals similarities to Rel DNA-binding proteins and a novel dimerization motif. J Biol Chem 2010;285(34):25875-9.

37.   Fang S, Wang Y, Chun YS, et al. Association of Common Genetic Polymorphisms with Melanoma Patient IL-12p40 Blood Levels, Risk, and Outcomes. J Invest Dermatol 2015;135(9):2266-72.

38.   Yokoyama S, Woods SL, Boyle GM, et al. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. Nature 2011;480(7375):99-103.

39.   Bertolotto C, Lesueur F, Giuliano S, et al. A SUMOylation-defective MITF germline mutation predisposes to melanoma and renal carcinoma. Nature 2011;480(7375):94-8.

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Figure 1. Study cohorts. (a) Cutaneous melanoma and ocular melanoma cases used in analysis. Genetically-enriched probands as defined in the Supplementary Methods. (b). Principal component analysis using the PCA module in PLINK; cases showing close matching with European controls. (c) Histogram of common synonymous SNPs between cases and controls and observed:expected ratios. MPM, multiple primary melanoma; CM, cutaneous melanoma; OM, ocular melanoma; PCA, principal component analysis; SNPs, single nucleotide polymorphisms; TCGA, The Cancer Genome Atlas; repl, replication study

Figure 2. Mutational landscape of melanoma. (a). Manhattan plot showing gene-based associations across all loci. Genome-wide significance is indicated by solid line. Genes which show near-significant associations fall within the shaded region (p<10E-4). (b). Calculated -$\log_{10}$(P values) for genes in both CM and OM analyses. BAP1 and CDKN2A clearly show strong preferential associations with OM and CM, respectively.

Figure 3. EBF3 validation. (a). Copy number profile of EBF3 (shaded red box) and various melanoma drivers among TCGA tumor specimens. Median EBF3 copy number is significantly lower than all other genes (p=2.0E-151). (b). EBF3 expression is lower in a panel of benign nevi compared to primary melanomas (p=0.005). (c). Loss of EBF3 expression is associated with worse overall survival in 125 metastatic stage III melanoma patients from Lund University Medical Center (p=0.04) and 349 TCGA metastatic melanoma patients (p=0.10). (d) EBF3 expression is significantly different between the molecular subtypes of melanoma with the lowest levels in the MITF-hi (pigmentation; Lund) subclass and highest levels in the MITF-lo subclass (TCGA). (e) Spearman correlations for both EBF3 and MITF and melanocyte lineage genes in the Lund and

TCGA data sets. (f) Induction of EBF3 in G-mel and UACC-62 cells using a Tet-responsive promoter shows a reduction of MITF protein levels. Dox, doxycycline.

Figure 4. Functional accreditation of EBF3. (a). Dose dependent arrest of the LOX, UACC-62 and A375 cell lines with EBF3 upregulation by doxcycyline. (b). 3D spheroid formation is dramatically reduced by the induction of EBF3. (c). Constitutive overexpression of EBF3 in A375 cells led to a profound suppression of tumor growth in nu/nu mice. Vec, vector; Dox, doxycycline; *** p<0.001

Table 1- Melanoma Case/Control and TCGA Replication (Variants with MAF<0.01)

		Primary Cohort			TCGA Replication Cohort			U.K. EBF3 Replication		Joint Odds Ratio#
Gene	Method	CM CASES (N=273)	CONTROLS (N=7629)	P-value	TCGA CM (N=379)	CONTROLS (N=3563)	P-value	Sanger CM (N=159)†	UK10K (N=4769)	
CDKN2A	PTV burden	5	0	**6.16E-08***	2	0	**9.31E-03**	-	-	-
ACTR8	C-alpha	7	56	2.18E-05	5	26	0.143	-	-	-
ECHDC1	C-alpha	6	32	3.73E-05	1	14	0.833	-	-	-
COL11A2	PTV burden	4	3	5.42E-05	0	4	0.6	-	-	-
DIP2B	C-alpha	21	272	7.15E-05	4	78	0.2	-	-	-
EBF3	C-alpha	4	14	8.22E-05	3	7	**4.75E-04**	2	21	**4.64 (95%CI: 2.21-9.72)**
		OM CASES (N=99)	CONTROLS (N=7629)	P-value	TCGA OM (N=47)	CONTROLS (N=3563)	P-value			
BAP1	PTV burden	4	3	3.83E-06	1	1	**0.0264**			
IAH1	C-alpha	6	68	2.27E-05	0	31	-			
NHLRC3	C-alpha	10	183	6.88E-05	0	84	-			
RSRC1	C-alpha	5	44	1.00E-04	0	18	-			
PAPOLG	C-alpha	6	47	1.00E-04	0	21	-			

Abbreviations: CM, cutaneous melanoma; OM, ocular melanoma; TCGA, The Cancer Genome Atlas; *, genome-wide significant;

† Melanoma kindreds of British ancestry were included to approximate the UK10K control collection. The U.K. replication included 77 cases (66 families) from Leeds, U.K., 56 cases (9 families) from Sydney, Australia and 27 cases (6 families) from Queensland, Australia. A p.N455S mutation was found in one Leeds family (1/2 cases positive for mutation) and a p.G21S mutation was identified in one Sydney family (2/6 cases positive for mutation).

#, Mantel-Haenszel chi-square test p value = 3.29E-05. In the joint burden analysis, we undertook a more conservative stance and censored the second carrier from the Sydney family while including all the non-carriers. Thus, 3 mutations were detected in 160 total cases but only 2 mutation carriers from 159 cases were used in the calculation.