Exome sequencing of desmoplastic melanoma identifies recurrent NFKBIE promoter mutations and diverse activating mutations in the MAPK pathway

A Hunter Shain1–3, Maria Garrido1–3, Thomas Botton1–3, Eric Talevich1–3, Iwei Yeh1–3, J Zachary Sanborn4, Jongsuok Chung5, Nicholas J Wang6,7, Hojabr Kakavand8,9, Graham J Mann8,9, John F Thompson8–10, Thomas Wiesner11, Ritu Roy2, Adam B Olshek2,12, Alexander Gagnon1–3, Joe W Gray6,7, Nam Huh5, Joe S Hur13, Klaus J Busam14, Richard A Scolyer8–10, Raymond J Cho3,16, Rajmohan Murali14–16 & Boris C Bastian1–3,16

Desmoplastic melanoma is an uncommon variant of melanoma with sarcomatous histology, distinct clinical behavior and unknown pathogenesis1–3. We performed low-coverage genome and high-coverage exome sequencing of 20 desmoplastic melanomas, followed by targeted sequencing of 293 genes in a validation cohort of 42 cases. A high mutation burden (median of 62 mutations/Mb) ranked desmoplastic melanoma among the most highly mutated cancers4. Mutation patterns strongly implicate ultraviolet radiation as the dominant mutagen5, indicating a superficially located cell of origin. Newly identified alterations included recurrent promoter mutations of NFKBIE, encoding NF-eB inhibitor ε (IkBe), in 14.5% of samples. Common oncogenic mutations in melanomas, in particular in BRAF (encoding p.Val600Glu) and NRAS (encoding p.Gln61Hys or p.Gln61Arg), were absent. Instead, other genetic alterations known to activate the MAPK and PI3K signaling cascades were identified in 73% of samples, affecting NF1, CBL, ERBB2, MAP2K1, MAP3K1, BRAF, EGFR, PTPN11, MET, RAC1, SOS2, NRAS and PIK3CA, some of which are candidates for targeted therapies.

Desmoplastic melanomas comprise 4% of all primary melanomas1. They most often occur in the chronically sun-exposed skin of older individuals. Clinically, they usually present as unpigmented scar-like indurations, delaying their detection. Histologically, they are primarily dermal-based tumors composed of a paucicellular proliferation of spindle-shaped cells situated within abundant desmoplastic stroma and can be easily misdiagnosed. Some desmoplastic melanomas grow extensively along or within nerves, a feature termed neurotropism.

Primary melanomas composed predominantly (>90%) of desmoplastic melanoma are classified as ‘pure’ desmoplastic melanoma, whereas primary melanomas that exhibit areas characteristic of desmoplastic melanoma amounting to <90% of the tumor (with the remainder being composed of non-desmoplastic melanoma) are classified as ‘mixed’ desmoplastic melanoma. Patients with the pure subtype of desmoplastic melanoma have a lower rate of nodal metastasis and better survival1,2,3,6,7.

Despite the fact that desmoplastic melanoma is a deadly and commonly misdiagnosed cancer, there are no known genetic drivers2. Several studies have failed to identify common pathogenic mutations4,5. This may be due to the small number of cases analyzed thus far as well as the technical limitations of studying tumors like desmoplastic melanoma that exhibit a low ratio of tumor to stromal cells. We sought to characterize the genome-wide mutational landscape of desmoplastic melanoma to identify genetic alterations that underlie the unique biology of this tumor and could serve as potential diagnostic biomarkers or therapeutic targets.

Low-coverage genome sequencing (13×) and high-coverage exome sequencing (89×) were performed on a discovery set of 20 fresh-frozen tumors and matched normal DNA samples (Supplementary Table 1). In addition, we sequenced neoplastic and non-neoplastic tissue from 42 formalin-fixed, paraffin-embedded primary desmoplastic melanomas as a validation cohort. Targeted sequencing of 293 genes (216×; see Supplementary Table 2 for the list of genes) that included the top candidates nominated from the discovery set was performed on the validation cohort. High-resolution array comparative genomic hybridization (array CGH; arrays with 180,000 to 1 million features) was performed on samples from both cohorts.

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1Department of Pathology, University of California, San Francisco, San Francisco, California, USA. 2Helen Diller Family Comprehensive Cancer Center, San Francisco, California, USA. 3Helen Diller Family Comprehensive Cancer Center, San Francisco, San Francisco, California, USA. 4Five3 Genomics, LLC, Santa Cruz, California, USA. 5Samsung Advanced Institute of Technology, Seoul, Korea. 6Department of Biomedical Engineering, Oregon Health and Sciences University, Portland, Oregon, USA. 7Knight Cancer Institute, Oregon Health and Sciences University, Portland, Oregon, USA. 8Melanoma Institute Australia, Sydney, New South Wales, Australia. 9Sydney Medical School, University of Sydney, Sydney, New South Wales, Australia. 10Royal Prince Alfred Hospital, Sydney, New South Wales, Australia. 11Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 12Department of Epidemiology and Biostatistics, University of California, San Francisco, San Francisco, California, USA. 13Samsung Electronics Headquarters, Seoul, Korea. 14Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 15Marie-Josée and Henry R. Kravis Center for Molecular Oncology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 16These authors contributed equally to this work. Correspondence should be addressed to B.C.B. (boris.bastian@ucsf.edu).
Desmoplastic melanomas have a substantial point mutation burden consistent with UV radiation–induced damage. The 62 tumors are ordered by their mutation burden (top) with the mutation types annotated (bottom). The dashed line corresponds to the mutation burden observed in sun-exposed non-desmoplastic melanoma: 15 mutations/Mb10,11. In the bottom panel, C>T mutations following a purine (*) or pyrimidine (**) base are distinguished. Tumors from patients older than 55 years of age had significantly more mutations than those from younger patients (P = 2 × 10−3, t test).

Using these approaches, we were able to determine point mutation and copy number information in 62 desmoplastic melanomas. The median number of mutations per megabase was 62 (Fig. 1), ranking desmoplastic melanoma among cancers with the highest known mutation burdens. This mutation rate contrasts with those for ‘conventional’ cutaneous melanomas (with approximately 15 mutations/Mb10,11) and the majority of solid cancers (with approximately 2 mutations/Mb4,5). In desmoplastic melanoma, 88% of the mutations were C>T transitions and favored dipyrimidines, implicating ultraviolet (UV) radiation as the dominant mutagen (Fig. 1). There were some notable exceptions to these patterns. Two tumors arose from sun-shielded sites and had the lowest mutation burdens; one of these occurred in a patient in whom we identified a germline CDKN2A mutation. Tumors arising in younger patients tended to have lower mutation burdens (P = 2 × 10−3, t test) (Fig. 1). Pure and mixed desmoplastic melanomas were genetically similar.

Overall, desmoplastic melanomas had fewer copy number alterations (CNAs) than other melanoma subtypes (Supplementary Fig. 1). Despite the low overall CNA burden, several samples had focal CNAs (Supplementary Figs. 2 and 3). Focal amplifications affected the following genes: EGFR, CDK4, MDM2, TERT and MAP3K1 (three cases each); MET, YAP1 and NFKBIE (two cases each); and CCND1, MYC and SOS2 (one case each). Immunohistochemistry for EGFR, CDK4, MDM2, MET, YAP1 and CCND1 confirmed increased protein expression in select cases (Supplementary Fig. 4). Focal deletions affected CDKN2A (11 cases) and NF1 (4 cases). Loss of p16 expression (encoded by CDKN2A) was confirmed by immunohistochemistry in select cases (Supplementary Fig. 5).

The high mutation burden made it difficult to identify potential driver mutations among the numerous somatic mutations. We searched for recurrent mutations clustering at specific base pairs (hotspots) and genes with a disproportionately high frequency of loss-of-function mutations to identify true driver mutations10–12.

Several tumors harbored the following oncogenic hotspot mutations known to occur in other cancers: ERBB2 (p.Ser310Phe) (n = 4); MAP2K1 (p.Pro124Ser or p.Pro244Leu); PTPN11 (p.Glu76Ala or p.Glu76Lys); PPP6C

![Figure 1](image1)

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![Figure 2](image2)

Nomination of driver mutations in desmoplastic melanoma. (a) The number of specific mutations detected (y axis) is stratified by the occurrence of these mutations across samples (x axis). For example, 3,364 mutations occurred only once, whereas there were 151 mutations that occurred in 2 samples each and a single mutation that occurred 9 times, affecting the NFKBIE genetic locus. There was also a secondary hotspot in NFKBIE, 15 bp from the more common mutation site, which was mutated in two samples. (b) Quantile-quantile plot of observed loss-of-function burdens compared to expected loss-of-function burdens. The solid and dashed lines correspond to false discovery rates (FDRs) of 1.0 and 0.5, respectively. The most significant genes are labeled. (c) Tumor-suppressor candidates have a higher proportion of damaging mutations and fully clonal mutant allele frequencies (MAFs), undergoing loss of heterozygosity in some cases. Left, fraction of mutation categories for each candidate compared to all mutations. Right, normalized mutant allele frequencies (NMAFs) of mutations in candidate tumor suppressors compared to all mutations. Red vertical bars represent average MAFs.
Noonan syndrome

Germline

missense mutations predicted to be damaging were nominated as candidate driver mutations. Mutations that had undergone loss of heterozygosity were particularly scrutinized. Taking these criteria into account, we identified genes for which the burden of loss of function exceeded what would be expected by chance by comparing the actual burdens to the loss-of-function burdens generated from permutation data (Supplementary Methods).

Briefly, truncating mutations (nonsense, splice site or frameshift) were enriched for loss-of-function mutations (Online Methods).

To identify tumor-suppressor candidates, we looked for genes that were enriched for loss-of-function mutations (Online Methods). Briefly, truncating mutations (nonsense, splice site or frameshift) and missense mutations predicted to be damaging were nominated as candidate driver mutations. Mutations that had undergone loss of heterozygosity were particularly scrutinized. Taking these criteria into account, we identified genes for which the burden of loss of function exceeded what would be expected by chance by comparing the actual burdens to the loss-of-function burdens generated from permutation data (Fig. 2b).

The genes implicated by this approach were MAPK31, FBXW7, RB1, IDH1, ARID1A, EZH2, PIK3CA, NRAS, Braf, BRAF, PTEN, FBXW7, NFKBIE, CBL and CBL-PTB. Although genetic alterations of MAP3K1 have not been reported in melanoma, a Sleeping Beauty screen in a melanoma mouse model driven by BrafV600E identified Map3k1 as an oncogene in melanoma. In that study, tumors with Map3k1 insertions arose exclusively in melanocytes that failed to activate the conditional BrafV600E allele in control mice with wild-type Braf, indicating that Map3k1 activation can substitute for Braf activation. Map3k1 amplifications may therefore represent an equivalent driver mutation in desmoplastic melanoma.

Another implicated gene involved in the mitogen-activated protein kinase (MAPK) pathway was MAP3K1 (refs. 18,19). We observed highly focal amplifications of MAP3K1 in three tumors (Fig. 3b). Although genetic alterations of MAP3K1 have not been reported in melanoma, a Sleeping Beauty screen in a melanoma mouse model driven by BrafV600E identified Map3k1 as an oncogene in melanoma. In that study, tumors with Map3k1 insertions arose exclusively in melanocytes that failed to activate the conditional BrafV600E allele in control mice with wild-type Braf, indicating that Map3k1 activation can substitute for Braf activation. Map3k1 amplifications may therefore represent an equivalent driver mutation in desmoplastic melanoma.

FBXW7 encodes an E3 ubiquitin ligase responsible for MYC and cyclin E1 degradation. Truncating or damaging missense mutations of FBXW7 mapping to the critical WD domains are common in several cancers. In our cohort, 11% of tumors harbored nonsense or damaging missense mutations, often involving the WD domains, whereas synonymous and conservative missense mutations were recurrently mutated.

The most recurrent mutational hotspot, observed nine times, affected the NFKBIE gene (Fig. 2a). NFKBIE also harbored recurrent mutations at another nearby position in two additional instances (Fig. 2a).

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absent (Fig. 3c). FBXW7 mutations are also present, albeit at a much lower frequency, in published melanoma exome sequencing studies\textsuperscript{10,26–29}. Therefore, FBXW7 is a tumor suppressor across multiple melanoma subtypes with a higher frequency of mutations in desmoplastic melanoma.

IκBε (encoded by NFKBIE) inhibits downstream nuclear factor (NF)-κB signaling by sequestering NF-κB transcription factors in the cytoplasm\textsuperscript{30}. The NFKBIE locus was focally amplified in two samples (Fig. 3d) and showed recurrent mutations at several nearby hotspots, implicating NFKBIE as a candidate oncogene. The clustered mutations were not found in the Catalogue of Somatic Mutations in Cancer (COSMIC) database or in The Cancer Genome Atlas (TCGA) studies of any cancer, including sequencing studies of melanomas mostly from intermittently sun-damaged skin. We sequenced NFKBIE in an extension cohort of diverse melanomas and found six non-desmoplastic melanomas with NFKBIE mutations (Online Methods). Similar to desmoplastic melanomas, the NFKBIE-mutant non-desmoplastic melanomas did not harbor BRAF or NRAS mutations and had a very high mutation burden with evidence of UV radiation–induced mutational damage.

Overall, we found 20 clustered mutations in NFKBIE in 15 tumors (Fig. 4). Mutations from all cases were validated using at least two sequencing assays (Supplementary Figs. 7–9 and Supplementary Table 5). Remarkably, 5 of the 15 tumors had 2 mutations each, and in all 5 cases the mutations affected opposing alleles (Fig. 4 and Supplementary Fig. 8). Four of the ten tumors with only a single NFKBIE mutation had undergone loss of heterozygosity at the second allele, as evidenced by elevated frequencies of the mutant alleles (Fig. 4 and Supplementary Table 5). Collectively, these results strongly suggest selection for biallelic NFKBIE mutations.

There are two gene models for NFKBIE. The main mutational hotspot resided in the coding region of the long isoform and the promoter of the short isoform (Fig. 4, NFKBIE genes tracks). We mined Illumina Human BodyMap 2.0 RNA sequencing (RNA-seq) data to determine the tissue distribution of the NFKBIE isoforms. The short isoform of NFKBIE was ubiquitously expressed in all tissues, whereas...
the long isoform was restricted to brain tissue (Fig. 4, NFKBIE genes track). Similarly, RNA-seq data from the melanoma TCGA project exclusively showed expression of the short isoform (Fig. 4, melanoma TCGA RNA-seq track). We identified two non-desmoplastic melanoma cell lines that harbored NFKBIE hotspot mutations (M257 and M375; Fig. 4, mutations track). Using isoform-specific RT-PCR (Supplementary Fig. 10a) and immunoblotting (Supplementary Fig. 10b,c), we found that these cell lines also only expressed the short isoform. In conjunction, these data indicate that the mutational hotspot of NFKBIE is present in the promoter region of the relevant isoform (Fig. 4, promoter track), in an area that is highly conserved across multiple species (Fig. 4, conservation tracks), and is predicted to affect the binding sites for 32 transcription factors, including the consensus binding motifs for GABPA and ELF1 (Fig. 4, transcription factor tracks).

Many melanoma cell lines have been reported to show nuclear localization of NF-kB transcription factors, suggesting that NF-kB signaling is active in melanoma31-34. By contrast, we found that NF-kB nuclear translocation was absent in the two NFKBIE-mutant cell lines, M375 and M257 (Supplementary Fig. 10d). This result is consistent with the proposed gain-of-function role for NFKBIE resulting in inactivation of NF-kB signaling. However, future studies will be necessary to dissect the specific mechanism by which these promoter mutations modulate signaling.

Many of the individually nominated driver genes are components of critical signaling pathways in melanoma and cancer in general. The RTK-RAS-MAPK/phosphoinositide 3-kinase (PI3K) signaling cascade harbored genetic alterations predicted to lead to its activation in 73% of tumors (Fig. 5). Concordantly, immunohistochemistry for phosphorylated ERK was ubiquitously positive in desmoplasmic melanomas (Supplementary Fig. 11), confirming the importance of MAPK pathway activation in this melanoma type as well as in conventional melanoma. This similarity with other melanoma types, the genes affected in the MAPK pathway in desmoplasmic melanoma differed distinctly from those found in other melanoma types. Most notably, the most common MAPK-activating mutations in melanoma, BRAF mutation encoding p.Val600Glu and NRAS mutation encoding p.Gln61Gly or p.Gln61Arg, were completely absent in our cohort. Only a single tumor had an NRAS mutation encoding p.Gln61His, a rare type of RAS mutation in cutaneous melanoma. Three tumors had BRAF mutations encoding p.Gly466Glu, p.Gly469Glu and p.Asp594Asn substitutions; these mutations inactivate BRAF kinase activity but paradoxically activate MAPK signaling via activation of c-Raf35,36. Intriguingly, the multifarious mutations in the RTK-Ras-MAPK/PI3K signaling cascade did not appear to be mutually exclusive, indicating that some of these alterations may cooperate in pathway activation (Fig. 5a).

Other pathways with recurrent mutations (Fig. 5) included the p53 and Rb pathways, mostly resulting from inactivation of CDKN2A, TP53 and RB1. Also, the SWI/SNF chromatin-remodeling complex, a tumor suppressor in many malignancies37, harbored inactivating mutations of ARID2 and ARID1A. TERT was activated in 90% of samples, mostly through promoter mutations13,14 but also through amplification. Both the promoter mutations and amplification would be expected to increase the expression of TERT.

The distinct landscape of genetic alterations in desmoplasmic melanoma mirrors the unique clinical behavior of this tumor type. Some of the alterations found may have clinical implications. For example, there are small molecule inhibitors directly targeting the products of several oncogenes, such as MET, EGFR, ERBB2, IDH1, MAP2K1, PIK3CA and CDK4. Furthermore, the exorbitantly high...
mutation burden found in desmoplastic melanoma makes it a promising candidate for immune checkpoint blockade therapy. Finally, the mutational patterns observed in desmoplastic melanoma indicate the ontogeny of this neoplasm. Although desmoplastic melanomas typically present as intradermal cutaneous neoplasms, the overwhelming UV mutational signature in most indicates that they arose from a cell in or near the epidermis that accumulated most of its mutation burden before dermal invasion.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Raw array CGH copy number data have been deposited in the Gene Expression Omnibus (GEO) under accession GSE55150. Exome and targeted sequencing data have been deposited in the database of Genotypes and Phenotypes (dbGaP) under accession phs000977.v1.p1.

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AUTHOR CONTRIBUTIONS

A.H.S., R.M. and B.C.B. designed the study. M.G., I.Y., H.K., G.J.M., J.E.T., T.W., K.J.B., R.A.S., R.M. and B.C.B. provided cases. A.H.S., M.G., I.Y., K.I.B., R.A.S., R.M. and B.C.B. evaluated and/or microdissected cases. J.C., N.J.W., A.G., J.W.G., N.H., J.S.H., R.J.G. and B.C.B. sequenced samples. A.H.S., M.G., R.R., A.B.O., E.T. and B.C.B. analyzed copy number data. A.H.S., J.Z.S., R.J.C. and B.C.B. analyzed sequencing data. A.H.S. and R.M. carried out immunohistochemistry. A.H.S. and T.R. performed Sanger sequencing, RT-PCR, immunoblot and cell culture work. A.H.S. and B.C.B. wrote the manuscript. All authors reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Sample collection and microdissection. The study protocol was approved by the University of California, San Francisco (UCSF) Committee on Human Subjects, the Memorial Sloan Kettering Cancer Center institutional review board and human biospecimen utilization committee, and the Sydney Local Health District. Informed consent was obtained from all patients involved. Twenty fresh-frozen desmoplastic melanomas and matching blood samples were acquired from the Memorial Sloan Kettering Cancer Center (n = 10) and the Melanoma Institute of Australia (n = 10), comprising the discovery set. Forty-two archived formalin-fixed, paraffin-embedded desmoplastic melanomas and unrelated non-lesional ‘normal’ samples were microdissected from the archives of the UCSF Dermatopathology Service, comprising the validation cohort. There was no source of non-lesional tissue for 4 of the 42 validation samples. All cases were initially diagnosed as desmoplastic melanoma and confirmed by an independent review. Tumors were assessed by hematoxylin and eosin, and adjacent unstained sections were manually microdissected by a pathologist to enrich for neoplastic cells. For desmoplastic melanomas of the mixed subtype, best efforts were made to microdissect only the desmoplastic components. Clinical characteristics are summarized in Supplementary Table 1.

Sequencing, variant calling and copy number analysis. DNA was extracted using Qiagen DNeasy kits and prepared for sequencing using the NuGen Ovation library preparation kit (0331-32) according to the manufacturers’ protocols. Agilent SureSelect Exome V4 + UTR (5190-4638) or NimbleGen SeqCap EZ (06588786001) libraries were used for capture of the whole exome or 293 targeted genes (Supplementary Table 2), respectively. Multiplexed samples were sequenced on the Illumina HiSeq 2500 platform. Initial alignment was performed with Burrows-Wheeler Aligner (BWA), followed by indel realignment, deduplication and recalibration by the Genome Analysis Toolkit (GATK). Point mutations were called using MuTect, and indels were called using the UnifiedGenotyper and SomaticIndelDetector in comparison with non-lesional normal samples. Somatic variants were annotated by Oncotator.

For the four samples in the validation cohort without non-lesional normal tissue (Supplementary Table 1), somatic mutations were inferred by searching for variants from the reference genome that had not been reported in the 1000 Genomes Project or the ESP5400 National Heart, Lung, and Blood Institute (NHBLI) Exome Sequencing Project. To rule out private germline SNPs, mutations with MAFs near 50% were removed. Because of stromal cell contamination, most somatic events clustered at MAFs less than 50%.

High-resolution array CGH on Agilent arrays with 180,000, 244,000 or 1 million features was performed for 44 samples in the cohort (Supplementary Table 1). Copy number was inferred from sequencing data in all 62 samples using the software package CNVkit. Segmented copy number calls43 from array CGH and derived from CNVkit are included in Supplementary Table 6.

Melanoma subtype comparisons. Genome-wide copy number profiles were compared for desmoplastic melanomas and other melanoma subtypes40 (Supplementary Fig. 1). To do this, we needed to accurately call gains and losses in the presence of varying stromal cell contamination across samples. Toward this goal, we first used the scaled median absolute deviation (MAD) of the sequential order difference as implemented in the matrixStats package in R to estimate the sample-specific experimental variation. Next, we declared a sample MAD away from the median segmented values of the autosomal probes in that sample. Using this approach, the frequencies of gains and losses are displayed for each melanoma subtype in the top panels of Supplementary Figure 1.

We also explored whether other subtypes of melanoma had CNAs significantly different from those in desmoplastic melanoma. We tested the association between CNAs and melanoma subtype for each probe by performing two-sided Fisher’s exact tests on trichotomous gain/loss/normal data. We next calculated q values from these P values with the Bioconductor q value package instituted in R. Significantly different regions of CNA are displayed in the lower panels of Supplementary Figure 1.

Calling TERT promoter mutations. TERT promoter mutations were discovered midway through the study. The region was tiled and sequenced for several of the latter samples in the study, but it was not directly sequenced for many of the initial samples. However, as the mutations resided near the first exon of TERT, there was low coverage of the TERT promoter even when it was not specifically tiled. For the discovery cohort, we could also supplement this coverage with whole-genome sequencing reads. As there was a range of sequencing coverage and neoplastic cellularity, we calculated our power to call mutations in this locus for each tumor. Using a binomial test, we determined the likelihood that a true mutant sample with a given coverage and tumor cellularity could yield fewer than two mutant reads, thus yielding a false negative. We report the TERT promoter mutation status for all samples that could be called with 90% and 95% confidence (Supplementary Fig. 6).

Point mutation and copy number validation. The accuracy of mutation calling was assessed by resequencing a subset of mutations detected in the discovery phase to extremely high depth (>900× coverage). For validation, we selected 336 mutations spanning 6 samples. In total, 329 of 336 (97.9%) mutations were confirmed. These are noted in an extra column in Supplementary Table 3. In addition, 11 NFkBIE mutations were validated using Sanger sequencing (Supplementary Fig. 7). Finally, every sample exhibited specific mutational signatures that would not be detectable if the majority of mutations were artifacts.

CNAs were initially inferred from array CGH data. We additionally inferred CNAs from sequencing data using the CNVkit software suite41. The segmentation output from both approaches is included in Supplementary Table 6. Every single featured CNA in Figure 3 and Supplementary Figures 2 and 3 was confirmed by both methods.

Nominating gene candidates for validation sequencing. Whole-genome or whole-exome sequencing was not feasible on the validation cohort of desmoplastic melanomas because of the low tissue yields and fragmenting resulting from formalin fixation. The top candidate cancer-related genes (n = 293 genes; listed in Supplementary Table 2) were nominated from the discovery set (n = 20 samples) for targeted sequencing in a validation cohort (n = 42 samples). We included genes with >2 new hotspot mutations in the discovery set. Any gene with a COSMIC hotspot mutation, even in a single sample, was also included. From the discovery set, we computed a loss-of-function burden for each gene and included the top 50 candidates for further validation sequencing. Focally deleted and amplified genes were also included for validation sequencing. Finally, a curated list of known cancer-related genes was also included42.

Calculating the loss-of-function burden. To identify genes with an elevated loss-of-function mutational burden, we developed a computational method to compare the frequency and zygosity of the loss-of-function mutations affecting each gene to the values expected by chance. A brief description of the computation follows.

Step 1. The overall nonsynonymous: synonymous mutation ratio is calculated. In this study, this ratio was 2.13:1.

Step 2. The number of synonymous mutations mapping to each gene is multiplied by the overall nonsynonymous: synonymous mutation ratio, as calculated in step 1, and subtracted from the total number of mutations mapping to that gene. Genes for which the resulting number is positive are considered to be enriched for nonsynonymous mutations and were selected for further analysis.

Step 3. Within each gene, sample and mutation type, the nonsynonymous mutation counts are multiplied by their NMAFs. The NMAF is calculated by dividing the MAF of a mutation by the median MAF of all mutations in a sample. NMAF effectively approximates the clonality and zygosity of a given mutation. Thus, weighing mutations by their NMAFs favored the identification of fully clonal mutations that had undergone loss of heterozygosity over subclonal or heterozygous mutations.

Step 4. For genes with multiple nonsynonymous mutations in a single sample, the NMAFs of all mutations in that specific gene for that single sample are summed and capped at 2. The cap at 2 represents the maximum number of alleles that a single sample can lose. The rationale behind this approach is that multiple mutations mapping to the same gene in a single sample are likely to affect both alleles, but can logically affect no more than both alleles. This approach avoids placing undue emphasis on large genes that accumulate...
high counts of incidental mutations. To illustrate the usefulness of this methodology, in this study, TTN, the largest gene in the genome, harbored over 100 mutations in a single sample; this correction credited that single sample with 2 hits to TTN (inactivating both alleles) rather than 100+ hits.

**Step 5.** For each gene, we sum the rescaled and capped mutation burdens across all samples, yielding a preliminary measure of the number of alleles affected by mutations in a gene across all samples. This preliminary measure is referred to as the number of hits.

**Step 6.** Determine the deleterious skew of nonsynonymous mutations in comparison to all mutations in all samples. The frequency of truncating (nonsense, frameshift or splice-site) and missense (probably damaging, possibly damaging or benign) mutations is calculated across all mutations in all samples. For the present study, the breakdown was 8.1% for truncating mutations and 29.6%, 13.6% and 16.7% for missense variants that were probably damaging, possibly damaging and benign, respectively; the remaining variants were synonymous mutations.

**Step 7.** Determine the truncation mutation burden for each gene. The frequency of truncating mutations affecting a specific gene across all samples is calculated and divided by the genome-wide average. For example, if a gene has 5 truncating mutations out of 10 total mutations, then the truncating factor would be 0.5/0.081 = 6.17. This factor has a wide range of values.

**Step 8.** Determine the missense–probably damaging burden for each gene. The frequency of probably damaging mutations affecting a gene is calculated as follows: 1 + (fraction of probably damaging mutations in a gene) × (genome-wide fraction of probably damaging mutations). For example, if a gene has 5 probably damaging missense mutations out of 10 mutations in total, then this factor would be as follows: 1 + 0.5 × 0.296 = 1.204. This factor has a relatively small range of values.

**Step 9.** Calculate the loss-of-function burden as the product of the number of hits and mutation burdens calculated in steps 5–8: multiply the normalized number of hits (step 5) by the truncating mutation burden (step 7) and the probably damaging missense mutation burden (step 8). The factors in steps 7 and 8 have the potential to magnify the value calculated in step 5 if the mutations affecting a gene are disproportionately damaging as compared to the genome average.

**Step 10.** Determine significance by simulation. Repeat steps 1–9 on sample, gene and mutation permuted data (1,000 permutations recommended). Calculate FDRs by comparing the observed loss-of-function burdens to the permuted data.

**NFKBIE PCR amplicon sequencing.** Deep PCR amplicon sequencing was performed to validate a subset of the mutations in NFKBIE (Supplementary Fig. 7). PCR amplicon (1 µg) was prepared for sequencing as described above and directly sequenced (without undergoing target capture). The resulting reads were aligned to the genome but were not deduplicated.

**Discovery of NFKBIE-mutant cases in non-desmoplastic melanoma.** As part of ongoing efforts unrelated to this study, our group (B.C.B.) is sequencing diverse pigmented lesions. Incidentally, we uncovered NFKBIE promoter mutations in six non-desmoplastic melanomas that were not explicitly part of this study. We have reported these mutations and the clinical features of those samples here (Fig. 4 and Supplementary Tables 1 and 5); however, the full body of work will be described in its entirety in future publications.

**Inferring tumor cellularity.** Tumor cellularity was inferred for each case and is listed in Supplementary Table 1. Tumor cellularity was estimated by doubling the median MAF of the somatic mutations from a given sample. This calculation assumes that the median MAF approximates the frequency of a clonal, heterozygous mutation. CNAs and subclonal mutations could skew this approximation. CNAs were infrequent in desmoplastic melanoma, so they would not be expected to affect our calculation. However, to more broadly confirm that CNAs and subclonal mutations had little effect on this calculation, we manually inspected the distribution of MAFs in each sample. For each sample, we observed a predominant cluster of MAFs centered on the median.

It is also possible to infer tumor cellularity from CNAs. We attempted to do this, but most samples were not adequate for analysis because of the scarcity of CNAs characteristic of desmoplastic melanoma. Notably, CNA-derived estimates did agree well with our MAF-derived estimates for the subset of samples that were adequate for analysis.

**RNA sequencing analysis of TCGA data.** NFKBIE transcription was analyzed from RNA-seq data for 60 melanoma TCGA samples. Only the short isoform of NFKBIE was expressed. The track in Figure 4 was created by combining all 60 bam files into a single bam file and visualizing the cumulative read depth.

**Immunohistochemistry and immunoblotting.** Immunohistochemistry was performed using antibodies to the following proteins: p16 (Ventana, 9517), p53 (Dako, GA6166), cyclin D1 (Thermo Fisher, RM-914-S), CDK4 (Invitrogen, AHZ0202), EGFR (Ventana, 790–4347), MDM2 (Invitrogen, 182403), Rb (BD Biosciences, 554136), Met (clone c-28), YAP1 (Cell Signaling Technology, 4912) and phosphorylated ERK p44/42 (clone D13, 14.4E).

The antibodies for immunoblotting were to the following proteins: NFKBIE (Santa Cruz Biotechnology, sc-7155), RELA/p65 (Cell Signaling Technology, 8242), p50/p105 (Cell Signaling Technology, 3035), c-REL (Cell Signaling Technology, 4727), PARP (Cell Signaling Technology, 9542) and HSP60 (Santa Cruz Biotechnology, sc-1722). Small interfering RNAs (siRNAs) to NFKBIE were from Dharmacon (ON-TARGETplus Human NFKBIE siRNA SmartPool). Cell fractionation was performed using the Subcellular Protein Fractionation Kit for Cell Culture (Thermo Scientific, 78840). The M257 and M375 cell lines were provided by A. Ribas and have been confirmed to be free of mycoplasma.

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