Published in final edited form as:

Nat Genet. 2015 May ; 47(5): 486–495. doi:10.1038/ng.3275.

Transposon mutagenesis identifies genetic drivers of *Braf*^{V600E} melanoma

Michael B Mann^{1,2}, Michael A Black³, Devin J Jones¹, Jerrold M Ward^{2,12}, Christopher Chin Kuan Yew^{2,12}, Justin Y Newberg¹, Adam J Dupuy⁴, Alistair G Rust^{5,12}, Marcus W Bosenberg^{6,7}, Martin McMahon^{8,9}, Cristin G Print^{10,11}, Neal G Copeland^{1,2,13}, and Nancy A Jenkins^{1,2,13}

¹Cancer Research Program, Houston Methodist Research Institute, Houston, Texas, USA

²Institute of Molecular and Cell Biology, Singapore ³Department of Biochemistry, University of Otago, Dunedin, New Zealand ⁴Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA ⁵Experimental Cancer Genetics, Wellcome Trust Sanger Institute, Hinxton, UK ⁶Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut, USA ⁷Department of Pathology, Yale University School of Medicine, New Haven, Connecticut, USA ⁸Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, California, USA ⁹Department of Cell and Molecular Pharmacology, University of California, San Francisco, San Francisco, California, USA ¹⁰Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand ¹¹New Zealand Bioinformatics Institute, University of Auckland, Auckland, New Zealand

Abstract

Although nearly half of human melanomas harbor oncogenic *BRAF*^{V600E} mutations, the genetic events that cooperate with these mutations to drive melanogenesis are still largely unknown. Here we show that *Sleeping Beauty* (SB) transposon-mediated mutagenesis drives melanoma progression in *Braf*^{V600E} mutant mice and identify 1,232 recurrently mutated candidate cancer genes (CCGs) from 70 SB-driven melanomas. CCGs are enriched in Wnt, PI3K, MAPK and netrin signaling pathway components and are more highly connected to one another than predicted by

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

Correspondence should be addressed to N.A.J. (njenkins2@houstonmethodist.org).

¹²Present addresses: Global VetPathology, Montgomery Village, Maryland, USA (J.M.W.), National Heart Research Institute Singapore, Singapore (C.C.K.Y.) and Institute of Cancer Research, London, UK (A.G.R.)

¹³These authors contributed equally to this work.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

Accession codes

Whole-exome sequencing data are available at NCBI under BioProject accession PRJNA276299.

AUTHOR CONTRIBUTIONS

M.B.M., N.G.C. and N.A.J. designed the study and wrote the manuscript. N.G.C. and N.A.J. directed the research. M.W.B. and M.M. provided essential biological resources and contributed to the experimental design. J.M.W. and M.W.B. performed histological classification and diagnosis of tumors. M.A.B., J.M.W., A.G.R., M.W.B., M.M. and C.G.P. contributed to editing of the manuscript before submission. M.B.M., M.A.B., D.J.J., J.M.W., C.C.K.Y., A.J.D., A.G.R. and C.G.P. performed data analysis. M.A.B., J.Y.N., A.J.D., A.G.R. and C.G.P. provided essential statistical and bioinformatics resources.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

chance, indicating that SB targets cooperative genetic networks in melanoma. Human orthologs of >500 CCGs are enriched for mutations in human melanoma or showed statistically significant clinical associations between RNA abundance and survival of patients with metastatic melanoma. We also functionally validate *CEP350* as a new tumor-suppressor gene in human melanoma. SB mutagenesis has thus helped to catalog the cooperative molecular mechanisms driving *BRAF*^{V600E} melanoma and discover new genes with potential clinical importance in human melanoma.

Substantial sun exposure and numerous genetic factors, including skin type and family history, are the most important melanoma risk factors. Familial melanoma, which accounts for <10% of cases, is associated with mutations in *CDKN2A*¹, *MITF*² and *POT1* (refs. 3,4). In sporadic melanoma, mutations in *BRAF* and *NRAS* predominate and are mutually exclusive. The oncogenic *BRAF*^{V600E} allele is found in >70% of benign precancerous nevi and half of human melanomas. Preliminary results from a phase III clinical trial testing the selective BRAF inhibitor PLX-4032 (vemurafenib) in patients with *BRAF*^{V600E} mutations were remarkable with a majority of patients demonstrating clinical response to therapy; however, metastatic melanoma eventually recurred, and drug-resistant clones showed reactivation of the mitogen-activated protein kinase (MAPK) pathway⁵. Over 20% of melanomas harboring *BRAF*^{V600E} show intratumoral heterogeneity in *BRAF*^{V600E} protein expression, and neither *BRAF*^{V600E} expression nor tumor heterogeneity predicts relapse following BRAF inhibitor treatment⁶. Improving the long-term survival of patients with melanoma harboring *BRAF*^{V600E} mutant tumors will therefore depend on a systematic approach to identify and target genes and pathways that cooperate with *BRAF*^{V600E} in tumor development⁷.

Spontaneous melanoma formation is rare in laboratory mice; however, mice engineered to express key mutated oncoproteins, including *Braf*^{V600E}, recapitulate the genetic and histological hallmarks of human melanoma. In these models, increased MEK-ERK signaling initiates clonal expansion of melanocytes, which is limited by oncogene-induced senescence, resulting in dysplastic precancerous nevi. Additional cooperating mutations, including loss-of-function mutations in *Pten* or *Cdkn2a*^{8,9}, are required for melanoma progression¹⁰.

Despite years of intense study, there is an incomplete understanding of the genes and genetic networks that drive melanoma development. Sequencing of human melanoma genomes has shown that these tumors have an order of magnitude more somatic mutations than many other solid tumors^{11–22}. Ultraviolet radiation–induced DNA damage^{12,15,16,20–22} and intrinsic defective DNA repair mechanisms¹⁶ are thought to drive the high mutation rate, which poses a particular challenge in the identification of driver genes in melanoma. There is also evidence of extensive epigenetic alterations^{23,24} and inter- and intratumoral heterogeneity in human melanoma²⁵. This surprising complexity suggests that, in comparison to other solid tumors, many more melanoma genomes must be sequenced to find the low-penetrance mutations that cooperate with drivers such as *BRAF*^{V600E} to induce melanomagenesis. Furthermore, some key genes may not have somatic mutations (for example, if they are epigenetically altered or acquire copy number alterations) or are infrequently mutated (for example, *PTEN* and *MITF*). Unbiased, genome-wide melanoma

gene discovery methodologies are therefore needed to deconvolute the complexity of this deadly cancer.

RESULTS

SB drives melanoma development in *Braf*-mutant mice

To identify genes cooperating with *Braf*^{V600E}, we performed an SB mutagenesis screen in mice carrying a conditional *Braf*^{V600E} allele (*Braf*^{CA/+}) where wild-type *Braf* is expressed prior to Cre-mediated recombination and mutant *Braf*^{V600E} is expressed after Cre-mediated recombination. *Braf*^{CA/+} mice²⁶ were crossed to mice carrying an inducible *Tyr-crc*^{ERT2} transgene with melanocyte-specific expression²⁷ to create *Tyr-crc*^{ERT2/+}; *Braf*^{CA/+} mutant mice⁹ (hereafter, Braf mice; Supplementary Fig. 1a). Braf mice were then crossed to mice with an inducible SB transposon system^{28,29} to generate SB|Braf mice (Supplementary Fig. 1a)³⁰. The *Braf*^{CA} and SB transposase alleles were subsequently activated by topical application of 4-hydroxytamoxifen (4-OHT) to dorsal skin after birth⁹ (Supplementary Fig. 1b).

To overcome complications caused by local transposon hopping³¹ and achieve genome-wide coverage for SB mutagenesis, we used three different transgenic lines that carried transposon concatamers on different chromosomes (Supplementary Fig. 1d). SB transposons contain an internal promoter and downstream splice acceptor site, which can activate expression of proto-oncogenes, and splice acceptor sites in both orientations and a bidirectional polyadenylation sequence, which can inactivate expression of tumor-suppressor genes (TSGs). In total, 13 cohorts of mice, carrying different allele combinations, were aged for the development of cutaneous melanoma (Supplementary Figs. 1b,c and 2).

By the time the mice reached 8–10 weeks of age, we observed hundreds of hyperpigmented nevi on 4-OHT-treated skin. By 25 weeks of age, all painted surfaces appeared uniformly black (Fig. 1a), whereas, at necropsy, imaging the underside of the dorsal pelt showed many individual clones of pigmented nevi (Fig. 1b and Supplementary Fig. 3a–c). All SB|Braf mice developed nevi, and 80% developed one to four tumor masses on 4-OHT-treated dorsal skin, with an average latency of 36 weeks (Fig. 1c). Tumor penetrance and latency were similar for all three transposon transgenic lines, and the data were therefore combined for subsequent analyses (Supplementary Table 1). In contrast, Braf mice developed nevi but no melanomas, whereas SB-only mice did not develop nevi or melanomas (Supplementary Fig. 1). Taken together, these data show that SB cooperates with *Braf*^{V600E} to drive melanoma progression.

SB|Braf mice developed amelanotic melanomas with nerve sheath differentiation (Fig. 1d–f and Supplementary Fig. 3d–f), and the tumors had morphological characteristics ranging from spindle-shaped to round, plump cells with abundant pale eosinophilic cytoplasm. Individual tumors were pleomorphic and often showed all morphological patterns, suggesting that there was extensive inter- and intratumoral heterogeneity. Melanomas were generally dermal or subcutaneous and did not show junctional melanocytic proliferation. In one instance, melanoma cells invaded the body wall (Supplementary Fig. 3g–i). Tumor cells generally expressed S-100 and sometimes contained melanin (Fig. 1g and Supplementary

Fig. 3j) or normal melanocyte antigens (Fig. 1h and Supplementary Fig. 3k). SB transposase protein was expressed within tumor cells (Fig. 1i and Supplementary Fig. 3l).

Common transposon insertion sites in melanomas

We sequenced the SB insertion sites from 77 melanomas using the 454 Splink method^{32,33} (Supplementary Fig. 1d, Supplementary Table 1 and Supplementary Note), generating 184,371 mapped reads corresponding to 35,908 non-redundant transposon insertions. We then identified common transposon insertion sites (CISs) using the Gaussian kernel convolution (GKC)³³ and gCIS³⁴ methods, as described³⁵ (Supplementary Figs. 4 and 5, and Supplementary Note). The concordance between the genes identified by both methods was high ($P = 8.67 \times 10^{-204}$; Supplementary Fig. 6a–c), and the methods together identified 1,232 SB|Braf CIS genes (Supplementary Table 2).

In previous studies, Karreth *et al.*³⁶ used SB mutagenesis to identify 36 statistically significant CISs that acted as putative *Pten* competing endogenous RNAs (ceRNAs). Our screen identified 72% (26/36) of these *Pten* ceRNAs³⁶; however, we did not observe a significant enrichment for these ceRNAs among our CIS genes ($P > 0.05$). In a separate study, Ni *et al.*³⁷ identified five CIS genes using *PiggyBac* transposon mutagenesis. Our screen identified three of these genes and paralogs of the other two genes.

More than ~95% of CCGs were predicted to be TSGs (Supplementary Fig. 7a,b, Supplementary Table 2 and Supplementary Note), similar to findings in other reported SB studies³⁵, and many of these genes are likely to function as haploinsufficient TSGs. This observation is consistent with studies showing that loss-of-function mutations in hundreds of genes, many of which likely function as haploinsufficient TSGs, can provide a growth advantage to telomerase-immortalized human mammary epithelial cells^{38,39}. These data support the notion that cumulative haploinsufficiencies in many TSGs maximize proliferative fitness in cancer cells.

To investigate whether mutagenic mechanisms other than SB mutagenesis might contribute to SB|Braf melanomagenesis, we used whole-exome sequencing to characterize eight SB|Braf melanomas (six tumor-normal pairs and two tumor-only samples) (Online Methods and Supplementary Table 3). Whole-exome sequencing identified 52 genes with significant single-nucleotide variant (SNV) or indel alterations (Bonferroni-corrected P value < 0.05 ; Fig. 2a and Supplementary Tables 4–7), 6.5 ± 4.7 protein-altering point mutations per tumor genome (far fewer than reported for mouse small-cell lung carcinoma⁴⁰), and there was no evidence for a mutation signature (Fig. 2b,c). We identified nine indel mutations (Supplementary Table 8) resulting from TACAG or TACTG insertions, which are the canonical SB mutagenic footprints resulting from SB remobilization⁴¹. We also identified 177 copy number variation (CNV) regions from the 6 tumor-normal genomes, including 130 CNV losses and 47 CNV gains (Supplementary Table 9). No genes mapping to these CNVs were recurrently mutated; as a result, their relevance remains unclear (Supplementary Table 10). We did, however, observe recurrent focal deletions at three loci, which harbored four CISs—*Cep350*, *Cdkn2a-E130I14P18Rik* and *Slc9a9* (Supplementary Fig. 8a–f). We also observed recurrent focal amplification in five genomes on distal chromosome 12, a region that contains *Adam6a* and *Adam6b* (Supplementary Fig. 8a–f). Finally, we identified 455 SB

insertions that occurred within or at the junctions of exons (Supplementary Table 11). *Cep350* was mutated in five of the eight genomes and was the only significantly mutated CIS gene identified in these melanoma genomes ($q = 2.59 \times 10^{-269}$; Supplementary Table 11). Together, our data suggest that SB mutagenesis is the predominant driving force in SB|Braf tumors, with less than the expected contributions from other types of variation normally observed in tumors without induction of SB (ref. 40 and M.B.M., M.A.B., N.G.C. and N.A.J., unpublished data).

Comparative oncogenomic filtering

SB|Braf CISs were selectively enriched among the genes listed in the Cancer Gene Census⁴² database ($n = 60$, $P = 1.77 \times 10^{-5}$; Supplementary Tables 2 and 12a) and the Catalogue of Somatic Mutations in Cancer (COSMIC) database (for genes that have >10 reported mutations in human tumors) ($n = 478$, $P = 6.65 \times 10^{-45}$; Supplementary Tables 12b and 13). The orthologs of four CIS genes (*Cdkn2a*, *Gnaq*, *Mitf* and *Pten*) also have known causal roles in human melanoma, the human orthologs of eight CIS genes (*Csmc3*, *Epha6*, *ErbB4*, *Adgrv1* (*Gpr98*), *Grm8*, *Lrp1b*, *Pkhd1* and *Ptprd*) are recurrently mutated in human melanoma^{15–22} and the human orthologs of five CIS genes (*Ankhd1*, *Cct3*, *Gna12*, *Scamp2* and *Slc12a7*) contribute to melanoma-specific fusion transcripts¹¹. In addition, of the 106 genes containing intragenic rearrangements in 2 or more melanomas¹², 33% were CCG orthologs (*RBFOX1* (*A2BP1*), *CSMD1*, *FHIT*, *MACROD2* and *MAGI2*; $P = 4.97 \times 10^{-11}$; Supplementary Table 12c). Together, these data suggest a key role for SB|Braf CCGs in human melanomagenesis.

Branching tumor evolution complicates efforts to implement personalized medicine and suggests that targeted therapies might be better directed against genes mutated at the trunk of the cancer evolutionary tree, as these genes are mutated in a high proportion of tumor cells⁴³. On the basis of sequencing read counts, we identified 21 genes that appeared to be drivers of early progression in *Braf*^{V600E} melanomas (Fig. 3a). These genes included the orthologs of two human melanoma drivers (*Cdkn2a* and *Gnaq*), three non-melanomic cancer drivers (*Crebbp*, *Pten* and *Lpp*; Cancer Gene Census, $P = 1.54 \times 10^{-4}$), a gene essential for Wnt signaling in colorectal cancer (*Tnik*)⁴⁴, two regulators of Hedgehog signaling (*Gli3* and *Tead1*) and a centrosomal protein that recruits FOP-FGFR1 to centrosomes in myeloproliferative disease (*Cep350*)⁴⁵. Notably, 70% of all tumors had an insertional mutation in at least one of these 21 genes (Fig. 3b).

We next employed several complementary cross-species oncogenomic approaches to gain further insight into the biological and clinical relevance of the SB|Braf CIS genes in human melanoma. We compiled a list of genes with sequence alterations in 347 human melanomas identified by sequencing whole genomes^{14,18,23}, exomes^{13,17,20–22,24} or RNA⁴⁶ (Supplementary Table 14). We focused on genes that were mutated in 5% of melanomas and considered all mutations in each gene as a single mutation event to avoid the over-representation of genes containing more than one mutation. Overall, 3,577 genes were mutated in 5% of melanomas, and each gene had 18 somatic mutation events. We found a significant enrichment of SB|Braf CIS genes among these genes ($P = 6.76 \times 10^{-21}$; Supplementary Table 14). Remarkably, 8.7% of the SB|Braf CIS genes were among the top

10% of the most frequently mutated genes in human melanoma (34 somatic mutations per gene, $n = 104$; $P = 4.20 \times 10^{-11}$) (Table 1).

We also compared our list of CIS genes to melanoma susceptibility loci defined by genome-wide association studies (GWAS) and CNV associations (Supplementary Table 2). Twenty-two CIS genes mapped to 16 GWAS loci^{46–53} (Supplementary Table 2). An additional 398 CIS genes mapped to recurrent chromosomal imbalances in human melanoma genomes: 220 genes mapped to chromosome losses on 1p, 8p, 9p, 10p, 14q, 16q, 17p and 22q, whereas 178 genes mapped to chromosome gains on 3p, 7p, 7q, 8q and 17q (ref. 54; Supplementary Table 15).

Long noncoding RNAs (lncRNAs) are also important genetic elements that may become deregulated by somatic mutations and contribute to disease^{55–57}. We identified 113 CCGs with at least one lncRNA transcript embedded within the coding sequence (Supplementary Table 16) and significant enrichment of CIS genes with genes harboring one or more lncRNAs^{58,59} ($P = 9.46 \times 10^{-29}$; Supplementary Table 12d). These data suggest that there might be additional layers of gene regulation in these regions, perhaps epigenetic in nature, which control TSG activity.

Several statistical approaches have been developed to identify high-confidence driver genes in tumor genomes^{60–62}. We identified 27 CIS genes within JISTIC-defined susceptibility loci⁶³, including 5 deleted (12 CIS genes) and 9 amplified (15 CIS genes) regions, from 123 human short-term melanoma cultures⁶¹ ($P = 0.014$; Supplementary Table 12e). In addition, 49 CIS genes were JISTIC-defined candidate drivers in melanoma ($P = 1.16 \times 10^{-3}$), and 8 were CONEXIC-defined modulators in melanoma ($P = 1.69 \times 10^{-5}$; Supplementary Table 12f), including the known melanoma drivers *Mitf*, *Grb2* and *Tbc1d16*. Furthermore, 16 CIS genes mapped to regions of focal deletion (10 CIS genes from 8 loci) or amplification (6 CIS genes from 5 loci) from 31 human melanoma cell lines containing a *BRAF*^{V600E} mutation⁶² ($P = 1.69 \times 10^{-5}$; Supplementary Table 12g). Taken together, >300 SB|Braf CIS genes map to susceptibility regions in human melanoma (Supplementary Table 2).

Conserved altered pathways in melanoma

To gain insight into the biological function of CIS genes, we used the Ingenuity Pathway Analysis (IPA), Database for Annotation, Visualization and Integrated Discovery (DAVID)⁶⁴, Kyoto Encyclopedia of Genes and Genomes (KEGG)⁶⁵ and GeneSetDB⁶⁶ analysis platforms. We found significant enrichment of CIS genes in many cancer-related signaling pathways and biological processes, including Wnt/ β -catenin, transforming growth factor (TGF)- β , phosphoinositide 3-kinase (PI3K) and MAPK signaling and biological processes regulating ubiquitin-mediated proteolysis, tight junctions, the cell cycle and axonal guidance (Supplementary Table 17a,b). GeneSetDB analysis confirmed enrichment of CIS genes in TGF- β , Wnt, PI3K and MAPK signaling, in addition to *ErbB4*, *Rac1*, *RhoA*, *netrin*, *Kit* receptor, *hepatocyte growth factor (HGF) receptor* and *epidermal growth factor (EGF) receptor* signaling (Supplementary Fig. 9 and Supplementary Table 17c). Collectively, 70% of SB|Braf melanomas carried mutations in genes that function in protein kinase A (PKA) or C (PKC) signaling, 66% carried mutations in Wnt/ β -catenin signaling, 67% carried mutations in MAPK signaling and 57% carried mutations in Pten-PI3K

signaling (Supplementary Fig. 10a). Among the 21 SB|Braf drivers of early progression (Fig. 3a), 3 function in axon guidance (*Gli3*, *Gnaq* and *Myo10*), two function in Wnt/TGF- β signaling (*Crebbp* and *Gsk3b*), two function in PI3K-Akt signaling (*Pten* and *Phlpp1*), 3 function in PKA signaling (*Crebbp*, *Gli3* and *Tnik*) and 2 function in Rac or Rho signaling (*Pten* and *Gli3*) (Supplementary Fig. 10b). Twelve genes with roles in glutamate receptor signaling (GO:0007215) were also collectively altered in >50% of SB|Braf melanomas. Although this finding did not reach statistical significance (false discovery rate (FDR)-adjusted $P = 0.1580$), it does provide further evidence that glutamate receptor signaling may have a role in melanoma²².

SB|Braf CIS genes were also enriched for transcription factors ($n = 158$, $P = 7.67 \times 10^{-3}$; Supplementary Tables 12h and 18). Using DAVID, we identified 15 transcription factors whose binding sites were enriched in the promoters of SB|Braf CIS genes (Supplementary Table 19). Remarkably, these 15 transcription factors were predicted to bind to 91% of CIS loci (Supplementary Fig. 10). CIS genes were also enriched for genes encoding phosphoproteins (59%, $n = 717$; $P = 5.51 \times 10^{-88}$) and genes known to undergo alternative splicing (41%, $n = 499$; $P = 1.81 \times 10^{-46}$; Supplementary Table 20). Collectively, these results suggest that CIS genes function in large signaling networks that can be deregulated at multiple levels and identify a number of signaling networks that should be further evaluated in human melanoma.

CIS interaction networks in melanoma

To further explore the potential interaction networks among CIS genes, we searched for functional links between these genes using a database of human molecular interactions (Online Methods). CIS genes were more functionally connected to one another than expected by chance (empirical $P = 0.0043$); 1,281 functional links could be drawn between CIS genes, and 352 CIS genes had a functional biological connection to one or more other CIS genes (Supplementary Fig. 11a and Supplementary Table 21), suggesting that SB mutagenesis enriches for mutations affecting functionally interacting proteins. Fifteen highly connected CIS genes had 19 functional connections with other CIS genes (Fig. 4a–c and Supplementary Fig. 11b).

Approximately 47% of SB|Braf melanomas (Fig. 4d) and >30% of human melanomas had one or more SB insertions or somatic mutations, respectively, in genes that function in netrin signaling, a pathway involved in mediating axonal guidance (Supplementary Fig. 12). A significant enrichment for mutations in axonal guidance-associated genes has also recently been observed in pancreatic cancer⁶⁷. The netrin-1 receptor *Dcc* was mutated in 24% of SB|Braf melanomas but was not detected by CIS analysis, possibly because it is a large gene. Frequent mutations in *DCC* have also been identified in human melanomas¹⁴, raising the possibility that pharmacological inhibition of downstream effectors of netrin signaling could be of clinical benefit for patients with melanoma.

Human melanomas frequently contain mutually exclusive mutations in Rho family members¹³. We identified five Rho family members (*Rac1*, *Rhoa*, *Rhot1*, *Cdc42bpb* and *Cdc42se2*) that were mutated in a mutually exclusive manner in 23% (16/70) of the SB|Braf melanomas (Fig. 4e), providing additional support for the hypothesis that corresponding

mutations are important in human melanoma. This result is also consistent with observations of a mutually exclusive relationship between Rac and Rho signaling in the control of movement in a three-dimensional culture model of melanoma metastasis⁶⁸. Extension of this analysis to other protein-coding families also identified mutually exclusive mutations among members of the SWI/SNF chromatin-remodeling complex and NFAT family members (Supplementary Fig. 13a,b and Supplementary Note). Together, our data demonstrate a systems network of deregulated pathways that contribute to melanoma progression.

Next, we assessed the clinical relevance of the SB|Braf CIS genes using microarray data for which corresponding patient survival data were available (Gene Expression Omnibus (GEO), GSE19234)⁶⁹. We considered a gene to have been validated if there was at least one probe on the array that showed a significant association with patient survival using Cox proportional hazards (CPH) regression. We found 322 CIS genes significantly associated with patient survival ($P < 0.05$; Supplementary Table 2). However, after corrections for multiple testing, only 46 genes remained significant (FDR-adjusted $P < 0.01$; Supplementary Table 22). At this significance cutoff, the number of prognosis-associated genes (46/1,139) was more than would be expected by chance from random lists of a similar size (Fisher's exact test, $P = 0.04$). Representative survival plots and log-rank survival data for these genes are shown in Figure 5a–c, Supplementary Figure 14 and Supplementary Table 2. CIS interaction network analysis showed that 12 of these genes were highly connected (Fig. 5d). To our knowledge, this is the first report identifying *ANKRD40*, *SDK1*, *THSD7B*, *DOCK5*, *GRIA3*, *ITCH*, *KDM4C*, *LPP*, *MAGI2*, *MYO10*, *NCOA3* and *PRUNE* as candidate tumor suppressors and GART as a candidate oncogene in predicting human melanoma survival outcomes.

CEP350 has a role in human melanoma progression

Among the three highest ranked CIS genes, *Cep350* is the only one not causally implicated in human melanoma. *Cep350* is a predicted TSG on the basis of the transposon insertion pattern and the finding that *Cep350* is one of only four genes identified by whole-exome sequencing to be mutated in more than one melanoma (MBM0001 and MBM0014; Supplementary Fig. 8a,c). Thirty-seven percent of SB|Braf melanomas had >1 SB insertion in *Cep350* (33 SB insertions in 19 genomes). *Cep350* may therefore represent a classical TSG. Alternatively, multiple *Cep350* insertions in the same tumor could reflect tumor heterogeneity and convergent evolution. *CEP350* was mutated in 7.2% of human melanoma genomes ($n = 25$), and these mutations included multiple stop-gain and missense mutations that were predicted to have damaging effects on CEP350 expression—features consistent with a putative role for *CEP350* as a human melanoma TSG (Fig. 6a).

To determine whether *CEP350* is a human melanoma TSG, we used stable short hairpin RNA (shRNA)-mediated knockdown of *CEP350* in two well-characterized human melanoma cell lines, A375 and COLO829 (refs. 11,16), which each harbor a *BRAF*^{V600E} mutation. We found robust *CEP350* expression in unmodified A375 and COLO829 cells and observed no significant difference in *CEP350* expression after lentiviral transduction with control shRNA. Lentiviral transduction of A375 and COLO829 cells (multiplicity of infection (MOI) of 6) with either pooled or individual non-overlapping *CEP350* shRNAs

reduced *CEP350* expression levels by ~50% (Supplementary Fig. 15a,b). For both cell lines, subcutaneous injection of cells into immunodeficient mice resulted in enhanced *in vivo* tumor formation with *CEP350* depletion (Fig. 6a–e and Supplementary Figs. 16 and 17), providing functional evidence that *CEP350* is a human melanoma TSG.

DISCUSSION

In the studies presented here, we describe a transposon-based screen in mice aimed at identifying genes that cooperate with *BRAF*^{V600E} in melanoma induction. Transposons, by their nature, are well suited to capture the full complement of genetic complexity present in tumor cells because they can identify genes that are somatically mutated in human cancer in addition to genes that are deregulated by transcriptional or epigenetic means. We also examined the entire spectrum of somatic mutations in SB-induced tumors by whole-exome sequencing. To our knowledge, this is the first time that SB-induced tumors have been characterized by whole-exome sequencing. Reassuringly, we found that the cumulative burden from SB mutagenesis was significantly higher and disproportionate to the number of non-SB mutagenic events, strongly suggesting that SB mutagenesis largely drives melanomagenesis in this model.

The vast majority of the genes identified in our screen were putative haploinsufficient TSGs. Over-representation of TSGs is an emerging, latent feature that is shared by all SB screens reported thus far for solid tumor types. Cumulative haploinsufficiencies in hundreds of genes have also been shown using human cancer cell lines³⁹ and may thus be a general mechanism through which cancer evolves. CIS genes also had more functional connections to one another than predicted by chance. In addition, there was a significant enrichment for transcription factors among CIS genes, and DNA binding sites for these transcription factors were significantly enriched in the promoters of CIS genes. Collectively, these results suggest that genes identified by SB function in large signaling networks that can be deregulated at multiple levels.

A surprising result from our study was that *BRAF*^{V600E}-mutated and *BRAF*-wild type human melanomas were similarly enriched for mutations in SB|Braf CIS genes. Examination of human melanoma genomes sequenced by The Cancer Genome Atlas (TCGA)¹³ shows that 70% of all melanomas without mutated *BRAF* have an oncogenic point mutation in *NRAS*, highlighting the importance of MAPK pathway activation in melanomagenesis. Thus, 87% (105/121) of TCGA melanoma genomes contain *BRAF* or *NRAS* mutations¹³. A likely explanation for our findings is that SB screening identifies genes that cooperate with MAPK pathway activation rather than mutant *BRAF*^{V600E} per se, with this activation occurring at a high frequency in both *BRAF*^{V600E}-mutated and *BRAF*-wild type human melanomas. Thus, a notable finding of our study is that the CIS genes identified by SB mutagenesis are operative in both *BRAF*^{V600E}-mutated and *BRAF*-wild type human melanomas.

Forty-six SB|Braf CIS genes were also identified as having potential value in predicting human patient survival outcomes. Interaction network analysis showed that 12 of these genes have a large number of connections to other genes identified in our forward genetic

screen. This suggests that pathway deregulation via alterations in networks of genes may drive tumor progression and contribute more to melanomagenesis than any single or few genetic or epigenetic alterations. Support for this hypothesis is observed in the rapidly acquired resistance to $BRAF^{V600E}$ -targeted therapies by patients carrying $BRAF^{V600E}$ mutations and the nearly 20% of patients with $BRAF^{V600E}$ mutations who are non-responders.

A major finding of our screen was the identification of *CEP350* as a new candidate human melanoma TSG. CEP350 is a CAP-Gly-containing protein predicted to participate in organizing, binding and anchoring microtubules at centrosomes by binding to SASS6 and CENPJ in a complex⁷⁰. CEP350 may also have a non-centrosomal role in regulating nuclear hormone receptor signaling^{71–84}. A role for *CEP350* in human melanoma was also suggested by a recent report of a familial melanoma susceptibility locus³ that mapped near *CEP350*. We demonstrate that shRNA-mediated knockdown of *CEP350* in the human melanoma cell lines A375 and COLO829 significantly accelerates tumor growth in xenograft assays. Of note, both cell lines harbor $BRAF^{V600E}$ mutations in addition to mutations in *CDKN2A* and *PTEN*. Interestingly, *Cep350* and *Cdkn2a* were identified as CIS genes only in SB|Braf^{V600E} melanoma (M.B.M., J.Y.N., N.G.C. and N.A.J., unpublished data). This suggests that, whatever tumor-suppressive role *Cep350* and *Cdkn2a* might have in tumorigenesis, it is likely to be dependent on cooperation with oncogenic *Braf*.

Our integrated studies suggest that melanoma is a systems biology problem. As predicted by the systems nature of these tumors, we found that SB|Braf CIS genes are more highly connected to one another through functional molecular links than would be predicted by chance. This observation supports the hypothesis that cancer therapies should be directed against signaling pathways themselves rather than individually mutated genes. However, exactly where to intervene in the genetic network is not a trivial problem, considering the likely presence of complex combinations of regulatory feedback loops, as has been observed from the contrasting therapeutic effects of vemurafenib on *BRAF*-mutated melanoma and colorectal cancer⁸⁵. With only a limited amount of preexisting information, the simplest method would be to disrupt genetic network connectivity by attacking its major hubs⁸⁶, as these proteins are most essential for cellular function. Removing a hub from the network would be predicted to drastically change the network signaling properties and connectedness to other parts of the network. The melanoma CIS genes identified here, including those with high connectivity to other genes in the network, provide a rich new resource of candidate genes for melanoma progression and drug targeting, in addition to a list of biological pathways and cellular processes with potential clinical importance in the treatment of human melanoma.

URLs

TgTn(sb-T2/Onc2)6070Njen mice, http://mouse.ncifcrf.gov/available_details.asp?ID=01XBG; TgTn(sb-T2/Onc2)6113Njen mice, http://mouse.ncifcrf.gov/available_details.asp?ID=01XBF; TgTn(sb-T2/Onc3)12740Njen mice, http://mouse.ncifcrf.gov/available_details.asp?ID=01XGB; Gt(ROSA)26Sortm2(sb11)Njen mice, http://mouse.ncifcrf.gov/available_details.asp?ID=01XGC; Tg(Tyr-cre/ERT2)14Bos mice,

<http://jaxmice.jax.org/strain/012328.html>; Bra^{tm1Mmcm} mice, <http://jaxmice.jax.org/strain/017837.html>; Crl:Nu(NCr)-Foxn1nu mice, <http://www.criver.com/products-services/basic-research/find-a-model/athymic-nude-mouse/>; NSG mice, <http://jaxmice.jax.org/strain/005557.html>; A375 (CRL-1619) cells, <http://www.atcc.org/products/all/CRL-1619.aspx>; COLO-829 (CRL-1974) cells, <http://www.atcc.org/products/all/CRL-1974.aspx>; Cancer Gene Census, <http://cancer.sanger.ac.uk/census/>; Circos, <http://circos.ca/>; Catalogue of Somatic Mutations in Cancer (COSMIC), <http://cancer.sanger.ac.uk/cosmic/>; Cytoscape, <http://www.cytoscape.org/>; DNACopy package, <http://www.bioconductor.org/packages/release/bioc/html/DNACopy.html>; GENCODE database, <http://www.gencodegenes.org/data.html>; GeneSetDB, <http://genesetdb.auckland.ac.nz/>; Ingenuity Pathway Analysis (IPA), <http://www.ingenuity.com/products/ipa/>; Kyoto Encyclopedia of Genes and Genomes (KEGG), <http://www.genome.jp/kegg/>; MetaCore, <http://thomsonreuters.com/metacore/>; Mouse Genome Informatics (MGI) database, <ftp://ftp.informatics.jax.org/pub/reports/index.html>; PrimerBank, <http://pga.mgh.harvard.edu/primerbank/>; The Cancer Genome Atlas (TCGA), <http://cancergenome.nih.gov/>; Database for Annotation, Visualization and Integrated Discovery (DAVID), <http://david.abcc.ncifcrf.gov/>; TRANSFAC, <http://www.biobase-international.com/product/transcription-factor-binding-sites/>.

METHODS

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

ONLINE METHODS

Mice

The following alleles were used to construct the SB|Braf mouse model of melanoma: *Tyr-cre^{ERT2}* (Tg(Tyr-cre/ER^{T2})14Bos; ref. 27); *Braf^{CA}*, (*Braf^{tm1Mmcm}*; ref. 26); T2/Onc2(TG.6070) (TgTn(sb-T2/Onc2)6070Njen; ref. 28); T2/Onc2(TG.6113) (TgTn(sb-T2/Onc2)6113Njen; ref. 28); T2/Onc3(TG.12740) (TgTn(sb-T2/Onc3)12740Njen; ref. 29); and Rosa26-LSL SBase (Gt(ROSA)26Sortm2(sb11)Njen; ref. 30). The resulting cohorts of mice were on mixed genetic backgrounds consisting of C57BL/6J, 129, C3H and FVB. Genotyping used PCR assays with primers specific to the alleles. The melanocyte-expressed tyrosinase enhancer/promoter was used to drive expression of a tamoxifen-inducible form of Cre recombinase, which permits spatial and temporal control of Cre activity in postnatal melanocytes. For Cre induction, we administered daily applications of 4-OHT (25 mg/ml in DMSO) topically on dorsal skin surfaces, tail and right ear on postnatal days (P) 2–4. An optimized 4-OHT treatment schedule was used to eliminate developmental lethality and to take advantage of a time in mouse development when melanocytes reside close to the skin surface before migrating into the hair bulb. Sibling mice were topically treated with 4-OHT and allowed to age to evaluate tumor penetrance and latency. The median latency, at which time skin masses grow to the maximal allowable size and are sent for necropsy and tissue processing, was ~36 weeks. All mouse work was completed in the AAALAC-accredited A*STAR Biological Resource Centre in accordance with approved procedures directed by the Institutional Animal Care and Use Committee (IACUC). Necropsies documented collection of all masses for subsequent analysis, and masses were split into three separate

portions: one-third snap frozen, one-third fixed in 10% neutral-buffered formalin overnight and one-third placed in fresh culture medium to attempt the creation of a melanoma cell line. Both sexes were used for experiments (Supplementary Table 1).

Histological analysis

Routine hematoxylin and eosin staining for the histological analysis of skin masses was performed on 5- μ m sections of formalin-fixed, paraffin-embedded (FFPE) specimens. Melanoma diagnosis used additional immunohistochemical staining on FFPE tissues after antigen retrieval (pH 6.0), endogenous peroxidase inhibition and overnight incubation with antibody for the neural crest marker S-100 (rabbit antibody to S-100, Z0311, Dako; 1:2,000 dilution) and a panel of antisera raised against normal melanocytic proteins (rabbit antibody to Tyrp1 (PEP1; 1:500 dilution) and rabbit antibody to Tyrp2 (PEP8; 1:500 dilution), both gifts from V. Hearing). The presence of nuclear SB transposase (SBase) was confirmed by immunohistochemistry on FFPE tissues after antigen retrieval (pH 9.0) and endogenous peroxidase inhibition followed by overnight incubation with mouse antibody to SBase (R&D Systems, AF2798; 1:200 dilution). After incubation with primary antibody, chromogen detection (with HRP polymer, anti-rabbit or anti-mouse, with Envision System from Dako) and hematoxylin counterstaining were performed. Melanin was also observed by Fontana-Masson silver staining.

Transposon mobilization assays

To assess successful Cre induction and analyze whether transposition occurred in the 4-OHT-treated skin of mice in the SB and SB|Braf cohorts, we performed a modified PCR-based T2/Onc excision assay as described⁸⁷. Because melanocytes only make up 1% of skin cells, we modified the T2/Onc excision PCR cycling conditions to include a shortened elongation step to bias against the amplification of native, unmobilized donor transposons (found in keratinocytes) and increased the number of cycles (94 °C for 3 min; 94 °C for 30 s, 60 °C for 45 s and 72 °C for 60 s for 39 cycles; 72 °C for 3 min; cool to 4 °C) to detect evidence of transposon mobilization. Routine T2/Onc excision assays were performed on DNA from tail biopsies taken at weaning and used as evidence for successful Cre induction.

Mapping transposon insertion sites

Genomic DNA was isolated from flash-frozen melanomas using the PureGene Genomic DNA Purification kit (Qiagen). SB insertion reads were generated using the 454 Splink method, consisting of 454 GS Titanium sequencing (Roche) of pooled splinkerette PCR reactions with nested, barcoded primers as described³². Pre- and post-processing of raw 454 reads to assign sample DNA barcodes, filter out local hopping events from donor chromosomes, and map and orient the SB insertion sites across the entire nuclear genome of the mouse was performed³². Because of the phenomenon of local hopping known to occur with SB³³, insertions from donor chromosomes were filtered out computationally.

Identification of common insertion sites

Identification of statistically significant cooperating loci, CISs and SB insertion abundance profiles was performed using a BED-formatted file containing the genomic locations of the

filtered list of SB insertion sites from the SB|Braf melanomas (Supplementary Fig. 18 and Supplementary Tables 23 and 24). Analysis with the GKC algorithm was performed as described³² with slight modifications³⁵. Gene-centric gCIS analysis was performed as described³⁴. In an effort to highlight CISs that were likely to be of biological relevance to the initiation and progression of SB|Braf melanomas, we defined a list of 253 SB|Braf CISs with calculated genome-wide significance from GKC and gCIS by employing a more stringent genome-wide multiple-testing correction³² (Supplementary Table 2).

Exome sequencing

Genomic DNA was isolated from flash-frozen necropsy specimens from each tumor and matched spleen (normal sample, no tumor cells present as determined by routine staining with hematoxylin and eosin) using the Qiagen Gentra PureGene DNA Isolation kit protocol for tissue. DNA (4 µg per sample) was used for library preparation. Adaptor-ligated templates were purified on Agencourt AMPure SPRI beads, and fragments with an insert size of ~200 bp were excised, purified and amplified by ligation-mediated PCR (LM-PCR). Exome capture was performed using the SureSelect Mouse All Exon kit (Agilent Technologies). Captured LM-PCR fragments were run on a Bioanalyzer 2100 instrument (Agilent Technologies) to estimate enrichment, and the captured DNA libraries were sequenced using the HiSeq 2000 (Illumina) platform. High-throughput sequencing of captured libraries was carried out independently to ensure that each sample produced 10 Gb of clean read data and met the desired 100× average exome coverage. Raw image files were processed using Illumina Consensus Assessment of Sequence and Variation software 1.7 for base calling with default parameters, and sequences were generated as 90- or 100-bp paired-end reads. DNA isolation, library preparation and sequencing were performed by BGI-Americas. Subsequent bioinformatic processing using supplied fastq.gz files was performed at the Houston Methodist Research Institute using a custom-built mouse exome analysis pipeline.

Bioinformatic analysis

Detection of SB insertions in whole-exome sequencing data was accomplished using a two-step local alignment process, using the 'mem' option in bwa mapping software (version 0.7.5a-r405)⁸⁸. Reads from each sample were aligned to a 'reference' genome consisting of only transposon sequence to identify reads containing a transposon component and then aligned to the mm9 mouse reference genome, with the alignment CIGAR strings used to define 'split reads' that contained both transposon and mouse sequences and identified a unique TA in the mouse genome where an insertion occurred. Reads were required to have at least five bases mapping to the transposon, with the remaining bases mapping to the mm9 reference for use in identifying an insertion site. Additional verifications to ensure that SB insertions mapped to unique TA sites in the mm9 genome were performed, with filtering to remove SB insertions at *En2*, *Foxp2* and *Serinc3* (as a matching sequence is contained within the SB transposons).

Genomic variants (SNVs, indels and CNVs) from whole-exome sequencing data were identified by a standard processing approach, beginning with bwa (0.7.5a-r405)⁸⁸ to align sequence reads from each sample to a custom reference genome, mm9-pT2onc, comprising

the mm9 mouse genome plus the pT2/Onc2 sequence added as a new chromosome. After alignment, for each tumor-normal pair, mpileup files were generated using SAMtools (version 0.1.19-44428cd)⁸⁹ and used as the input to VarScan2 (version 2.3.6)⁹⁰ to generate SNV, indel and copy number log ratio (tumor/normal) output files. Regions of DNA gain and loss were identified using the DNACopy package (version 1.36.0) for R (version 3.0.2)⁹¹. ANNOVAR (version available on 23 August 2013)⁹² was used to identify genomic regions in which variants occurred, including genes involved, and potential functional consequence (nonsynonymous, stop gain, etc.). A detailed description of the genomic landscape of these (and other) SB mouse tumors is currently being prepared for publication (M.B.M., M.A.B., N.G.C. and N.A.J., unpublished data).

Driver gene analysis

Uniquely mappable TA sites occupied by an SB insertion with 10 reads per tumor (1,730 insertion events) were used to perform gCIS-0 kb (no promoter) analysis. When genes from a melanoma genome were found to contain more than one SB insertion in the same RefSeq gene, only the SB insertion with the highest sequence read count was used for gCIS calculations. Genes identified from 3 tumors and having corrected $P < 0.05$ were considered to be putative drivers.

Molecular interactions among CIS genes

A virtual database of 65,552 human molecular interactions was used. This database contains directional (for example, transcription factor → DNA target, kinase → protein substrate) and non-directional (for example, protein-protein binding) molecular links. Data on molecular links were sourced from GeneSetDB⁶⁶ supplemented by molecular links manually identified from the literature and accessed from the online databases MetaCore (Thomson Reuters), IPA (Qiagen) and TRANSFAC⁹³. Pairs of CIS genes found in the virtual database were visualized using the Cytoscape environment⁹⁴. Permutation analysis was performed to estimate the number of molecular links in the virtual database that would be expected to occur between human orthologs of SB integration genes due to chance alone using the R statistical environment⁹¹.

Gene expression and patient outcome association analysis

Enrichment analysis was performed using Fisher's exact test to identify associations between CCG list membership and presence in a list of genes previously associated with melanoma (for example, CONEXIC, JISTIC, etc.). Significant associations ($P < 0.05$) were considered indicative of over-representation of genes from that particular collection; more genes from the list of interest appeared in the list of CISs than would be expected by chance.

Empirical false discovery rate calculations in clinical data sets

Gene expression data were downloaded from the GEO database (GSE19234)⁶⁹, and a collection of unique microarray samples was normalized using the RMA algorithm⁹⁵, without background correction. The data set consisted of 38 patients (24 males and 14 females) with metastatic melanoma. CPH regression was used to identify associations between probe sets and metastasis-free survival in these patients, using all probe sets for the

genes in the CCG list. Where a gene was represented by multiple probe sets, the minimum P value was used for that gene. To properly correct for multiple testing (as a standard correction would not account for use of the minimum P value across multiple probes for a particular gene), a resampling approach was used to estimate the empirical FDR associated with a range of possible P -value thresholds. Briefly, this involved permuting the samples (to reflect the null hypothesis) and generating a random sample of 1,137 genes (to reflect the number of genes from the CISs represented on the microarrays). Random sampling was restricted to genes with lengths ≥ 10 kb to better reflect the distribution of gene lengths in the CISs. For each resampled gene list, the CPH analysis was applied (including selection of the minimum P value across multiple probes, where necessary), allowing P values to be generated for each gene in the resampled list. This process was repeated 100 times. The proportion of false discoveries was then calculated over a range of significance thresholds, with any of the resampled genes that achieved significance considered to be false positives, allowing a rate to be calculated relative to the non-resampled data at each possible threshold. The empirical FDR was then calculated as the mean proportion of false discoveries across all iterations of resampling. A significance threshold was chosen such that the empirical FDR was below 0.1. CISs with P values below this threshold were considered to exhibit significant association with metastasis-free survival. To supplement the CPH analysis of the gene expression data, Kaplan-Meier plots were also generated using a 'high versus low' expression approach across a range of thresholds. This involved stratifying patients into groups with 'low' or 'high' expression for each gene, on the basis of whether the expression level was above or below the threshold. This was completed in 10% increments (10–90%) for each gene, with significance at each split assessed via the log-rank test⁹⁶.

CIS analysis using GeneSetDB

We downloaded 4,076 gene sets from GeneSetDB⁶⁶ with the membership of each gene set indicated by Entrez Gene number. Entrez Gene identifiers were matched to CCGs using the Mouse Genome Informatics (MGI) database (accessed 21 September 2012). To perform enrichment analysis for each gene set, an 'occurrence matrix' (genes $\times$ tumors) of 1's and 0's was generated, identifying which CISs contained SB insertion site(s) per tumor. This matrix was used to perform enrichment analysis for each gene set by generating a 2×2 contingency table and then using Fisher's exact test for association between the occurrence of SB insertion and gene set membership. FDR-adjusted P values of <0.05 were considered to represent significant enrichment of the gene set within the CCG list, across all tumor samples. For each gene set found to be significantly enriched, waterfall plots and heat maps were produced to allow visualization of the frequency of SB insertions in members of the gene set. Waterfall plots were generated by plotting the genes in decreasing frequency of SB insertion and then ordering the samples on the basis of their insertion profile across genes. Hierarchical clustering was used to generate heat maps on the basis of the similarity of insertion patterns across both samples and tumors. In addition to the SB mouse tumor data, mutation information from sequencing studies of human melanoma was compiled into a second occurrence matrix (genes $\times$ tumors), showing the frequency at which mutations occurred in each of the CISs, across the 290 human tumors and 57 cell lines. These data were added to the data from the mouse SB occurrence matrix to allow combined (human +

mouse) waterfall plots and heat maps to be generated for each of the significantly enriched gene sets.

Cell culture

The human melanoma cell lines A375 (CRL-1619) and COLO-829 (CRL-1974) were purchased from the American Type Culture Collection (ATCC) and grown according to the manufacturer's specifications in complete medium (1× DMEM (ATCC, 30-2002) for A375 cells or 1× RPMI-1640 medium (ATCC, 30-2001) for COLO829 cells) supplemented with 10% FBS and 1× penicillin-streptomycin grown at 37 °C in 5% CO₂. All cells were free from pathogens and mycoplasma. Cells were grown for two passages (P₂) and frozen for subsequent experiments. For all experiments, P₂ cells were passaged no more than three times before use; experimental and biological replicate studies were performed using new P₂ cells each time. Lentiviral particles for five shRNA GIPZ constructs (which express TurboGFP to mark cells expressing the shRNA) targeting *CEP350* (Supplementary Table 25) or one non-targeting control (shNTC; clone V13041101) were purchased from Thermo Scientific Open Biosystems and used in pools of three shRNAs or individually. To achieve adequate knockdown of *CEP350* in human melanoma cell lines, the maximum tolerated MOI was used for all lentiviral transduction experiments. Briefly, A375 or COLO829 cells were plated at a density of 5×10^5 cells per well of a 24-well plate the night before lentiviral transduction. The next day, cells were infected for 6 h in serum-free medium mixed with lentiviral particles at an MOI of 6, consisting of individual shNTC or individual *CEP350* shRNAs or pools of three *CEP350* shRNAs. After 6 h, complete medium was added. At 48 h after infection, antibiotic selection medium, consisting of complete medium supplemented with puromycin (at 1 µg/ml for A375 cells and 0.1 µg/ml for COLO829 cells) was added every 3 d until >95% of the cells strongly expressed GFP fluorescence.

Xenograft studies

One million A375 or 2 million COLO829 cells were prepared for injection into the left flank of either female athymic nude (CrI: Nu(NCr)-Foxn1nu; strain 490; 5–8 weeks old) or male and female immunodeficient NSG (NOD.Cg-Prkdcscid;Il2rgtm1Wjl/SzJ; JAX, 005557; 6–12 weeks old) mice. Allocation to study groups was blinded. Data are expressed as the means ± s.e.m. from at least five mice per group. Experimental group sizes (n) consisted of at least five mice per group, which permitted the detection of twofold differences in survival with a power ($1 - \beta$) of 0.90, assuming a two-sided test with significance threshold $\alpha = 0.05$ and a standard deviation of less than 50% of the mean. Appropriate statistical tests were applied as indicated. Standard deviation was used to calculate variation and included in the graphs as error bars. The GFP fluorescence of subcutaneous shRNA-transduced A375 or COLO829 cells was visualized with goggles and a light source from Biological Laboratory Equipment Maintenance and Service). Within 2 h of injection, GFP-negative mice were removed from the study. Real-time quantitative reverse-transcription PCR (qRT-PCR) for human *CEP350* and *RPL13A* was performed on cDNA from a flash-frozen aliquot of 1 million injected cells to determine the levels of knockdown using the following primers obtained from PrimerBank⁹⁷: CEP350-F (PrimerBank, 171184450c2), CEP350-R (PrimerBank, 171184450c2), RPL13A-F (PrimerBank, 6912634a1) and RPL13A-R (PrimerBank, 6912634a1). All qRT-PCR data are reported as C_t values. Xenograft

measurements were taken at least twice weekly using digital calipers while mice were conscious but restrained by one experimenter familiar with collecting caliper measurements of xenografts and blinded to the experimental group designations. GFP fluorescence was visualized at the time of caliper measurement; loss of GFP signal was considered to indicate failed engraftment, and corresponding mice were removed from the study (take rates were 80% for all cohorts). Ellipsoid tumor volumes were calculated as volume (mm^3) = $0.52(\text{length (mm)} \times \text{width}^2 (\text{mm}^2))$, where the two longest axes, length and width, were the major and minor diameter measurements, respectively; width² represents an assumption that the xenograft depth was equivalent to the diameter of the minor axis⁹⁸. Statistical significance of tumor volumes at defined time points was determined by either one-way *t* test for all cohorts relative to shNTC or by two-way, repeated-measures ANOVA with Bonferroni correction for multiple comparisons, as indicated. Area under the curve (AUC) calculations were used to estimate the magnitude of effect from cumulative growth curves. Kaplan-Meier survival curves plot the results of experiments where the outcome was time until death (days after injection to necropsy); the statistical significance for survival disparity was calculated using the log-rank (Mantel-Cox) test. Necropsy of xenograft-bearing mice was conducted by trained staff blinded to the experimental study design and included processing of end-stage xenograft masses and specimen archiving by cutting the xenograft masses into two halves, with one half fixed in formalin for histological analysis and the second half flash frozen in liquid nitrogen. For a subset of necropsy specimens, qRT-PCR analysis for human *CEP350* and *RPL13A* was performed to assess the levels of knockdown of individual clones contributing to *in vivo* tumor growth. All xenograft work was approved by the Institutional Animal Care and Use Committee at the Houston Methodist Research Institute.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank K. Mann for helpful discussions and for critical reading and editing of the manuscript; M. Eccles (Otago) and the Copeland and Jenkins laboratories in Singapore and Houston for helpful discussions; V. Hearing (National Cancer Institute) for PEP antibodies and L. Chin (Dana-Farber Cancer Institute) for *Tyr-cre^{ERT2}* mice; D. Adams (Sanger Institute), T. Whipp, R. Rance and the Wellcome Trust Sanger Institute sequencing and informatics teams for 454 sequencing and bioinformatics support; K. Rogers, S. Rogers and the Institute for Molecular and Cell Biology Histopathology Core; P. Cheok, N. Lim, D. Chen and C. Wee (Singapore) and H. Lee and E. Freiter (Houston) for assistance with tumor monitoring and animal husbandry; and A. Trevarton (Auckland) for assistance with molecular pathway analysis. Histology work was performed by the Advanced Molecular Pathology Laboratory, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore. This work was supported in part by the Biomedical Research Council, A*STAR, Singapore (to N.G.C. and N.A.J.), the Cancer Prevention Research Institute of Texas (to N.G.C. and N.A.J.), the Health Research Council of New Zealand, the University of Auckland and the New Zealand Maurice Wilkins Centre (to C.G.P.), the National Cancer Institute (to M.M. and M.W.B.) and the Melanoma Research Alliance (to M.M.). N.G.C. and N.A.J. are also Cancer Prevention Research Institute of Texas Scholars in Cancer Research.

References

1. Chin L, Garraway LA, Fisher DE. Malignant melanoma: genetics and therapeutics in the genomic era. *Genes Dev.* 2006; 20:2149–2182. [PubMed: 16912270]

2. Yokoyama S, et al. A novel recurrent mutation in *MITF* predisposes to familial and sporadic melanoma. *Nature*. 2011; 480:99–103. [PubMed: 22080950]
3. Robles-Espinoza CD, et al. POT1 loss-of-function variants predispose to familial melanoma. *Nat Genet*. 2014; 46:478–481. [PubMed: 24686849]
4. Trigueros-Motos L. Mutations in *POT1* predispose to familial cutaneous malignant melanoma. *Clin Genet*. 2014; 86:217–218. [PubMed: 24784786]
5. Nazarian R, et al. Melanomas acquire resistance to B-RAF^{V600E} inhibition by RTK or N-RAS upregulation. *Nature*. 2010; 468:973–977. [PubMed: 21107323]
6. Wilmott JS, et al. BRAF^{V600E} protein expression and outcome from BRAF inhibitor treatment in BRAF^{V600E} metastatic melanoma. *Br J Cancer*. 2013; 108:924–931. [PubMed: 23403819]
7. Flaherty KT, Yasothan U, Vemurafenib Kirkpatrick P. *Nat Rev Drug Discov*. 2011; 10:811–812. [PubMed: 22037033]
8. Damsky WE, et al. β -catenin signaling controls metastasis in Braf-activated Pten-deficient melanomas. *Cancer Cell*. 2011; 20:741–754. [PubMed: 22172720]
9. Dankort D, et al. Braf^{V600E} cooperates with *Pten* loss to induce metastatic melanoma. *Nat Genet*. 2009; 41:544–552. [PubMed: 19282848]
10. Tran SL, et al. Absence of distinguishing senescence traits in human melanocytic nevi. *J Invest Dermatol*. 2012; 132:2226–2234. [PubMed: 22513787]
11. Barretina J, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012; 483:603–607. [PubMed: 22460905]
12. Berger MF, et al. Melanoma genome sequencing reveals frequent *PREX2* mutations. *Nature*. 2012; 485:502–506. [PubMed: 22622578]
13. Hodis E, et al. A landscape of driver mutations in melanoma. *Cell*. 2012; 150:251–263. [PubMed: 22817889]
14. Krauthammer M, et al. Exome sequencing identifies recurrent somatic *RAC1* mutations in melanoma. *Nat Genet*. 2012; 44:1006–1014. [PubMed: 22842228]
15. Nikolaev SI, et al. Exome sequencing identifies recurrent somatic *MAP2K1* and *MAP2K2* mutations in melanoma. *Nat Genet*. 2012; 44:133–139. [PubMed: 22197931]
16. Pleasance ED, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*. 2010; 463:191–196. [PubMed: 20016485]
17. Prickett TD, et al. Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in *ERBB4*. *Nat Genet*. 2009; 41:1127–1132. [PubMed: 19718025]
18. Prickett TD, et al. Exon capture analysis of G protein-coupled receptors identifies activating mutations in *GRM3* in melanoma. *Nat Genet*. 2011; 43:1119–1126. [PubMed: 21946352]
19. Stark MS, et al. Characterization of the melanoma miRNAome by deep sequencing. *PLoS ONE*. 2010; 5:e9685. [PubMed: 20300190]
20. Stark MS, et al. Frequent somatic mutations in *MAP3K5* and *MAP3K9* in metastatic melanoma identified by exome sequencing. *Nat Genet*. 2012; 44:165–169. [PubMed: 22197930]
21. Turajlic S, et al. Whole genome sequencing of matched primary and metastatic acral melanomas. *Genome Res*. 2012; 22:196–207. [PubMed: 22183965]
22. Wei X, et al. Exome sequencing identifies *GRIN2A* as frequently mutated in melanoma. *Nat Genet*. 2011; 43:442–446. [PubMed: 21499247]
23. Howell PM Jr, et al. Epigenetics in human melanoma. *Cancer Control*. 2009; 16:200–218. [PubMed: 19556960]
24. Sigalotti L, et al. Epigenetics of human cutaneous melanoma: setting the stage for new therapeutic strategies. *J Transl Med*. 2010; 8:56. [PubMed: 20540720]
25. Yancovitz M, et al. Intra- and inter-tumor heterogeneity of BRAF^{V600E} mutations in primary and metastatic melanoma. *PLoS ONE*. 2012; 7:e29336. [PubMed: 22235286]
26. Dankort D, et al. A new mouse model to explore the initiation, progression, and therapy of BRAF^{V600E}-induced lung tumors. *Genes Dev*. 2007; 21:379–384. [PubMed: 17299132]
27. Bosenberg M, et al. Characterization of melanocyte-specific inducible Cre recombinase transgenic mice. *Genesis*. 2006; 44:262–267. [PubMed: 16676322]

28. Dupuy AJ, Akagi K, Largaespada DA, Copeland NG, Jenkins NA. Mammalian mutagenesis using a highly mobile somatic *Sleeping Beauty* transposon system. *Nature*. 2005; 436:221–226. [PubMed: 16015321]
29. Dupuy AJ, et al. A modified *Sleeping Beauty* transposon system that can be used to model a wide variety of human cancers in mice. *Cancer Res*. 2009; 69:8150–8156. [PubMed: 19808965]
30. Starr TK, et al. A transposon-based genetic screen in mice identifies genes altered in colorectal cancer. *Science*. 2009; 323:1747–1750. [PubMed: 19251594]
31. Copeland NG, Jenkins NA. Harnessing transposons for cancer gene discovery. *Nat Rev Cancer*. 2010; 10:696–706. [PubMed: 20844553]
32. March HN, et al. Insertional mutagenesis identifies multiple networks of cooperating genes driving intestinal tumorigenesis. *Nat Genet*. 2011; 43:1202–1209. [PubMed: 22057237]
33. Uren AG, et al. Large-scale mutagenesis in p19^{ARF}- and p53-deficient mice identifies cancer genes and their collaborative networks. *Cell*. 2008; 133:727–741. [PubMed: 18485879]
34. Brett BT, et al. Novel molecular and computational methods improve the accuracy of insertion site analysis in *Sleeping Beauty*-induced tumors. *PLoS ONE*. 2011; 6:e24668. [PubMed: 21931803]
35. Mann KM, et al. *Sleeping Beauty* mutagenesis reveals cooperating mutations and pathways in pancreatic adenocarcinoma. *Proc Natl Acad Sci USA*. 2012; 109:5934–5941. [PubMed: 22421440]
36. Karreth FA, et al. In vivo identification of tumor-suppressive *PTEN* ceRNAs in an oncogenic *BRAF*-induced mouse model of melanoma. *Cell*. 2011; 147:382–395. [PubMed: 22000016]
37. Ni TK, Landrette SF, Bjornson RD, Bosenberg MW, Xu T. Low-copy *piggyBac* transposon mutagenesis in mice identifies genes driving melanoma. *Proc Natl Acad Sci USA*. 2013; 110:E3640–E3649. [PubMed: 24003131]
38. Davoli T, et al. Cumulative haploinsufficiency and triplosensitivity drive aneuploidy patterns and shape the cancer genome. *Cell*. 2013; 155:948–962. [PubMed: 24183448]
39. Solimini NL, et al. Recurrent hemizygous deletions in cancers may optimize proliferative potential. *Science*. 2012; 337:104–109. [PubMed: 22628553]
40. McFadden DG, et al. Genetic and clonal dissection of murine small cell lung carcinoma progression by genome sequencing. *Cell*. 2014; 156:1298–1311. [PubMed: 24630729]
41. Liu G, Aronovich EL, Cui Z, Whitley CB, Hackett PB. Excision of *Sleeping Beauty* transposons: parameters and applications to gene therapy. *J Gene Med*. 2004; 6:574–583. [PubMed: 15133768]
42. Futreal PA, et al. A census of human cancer genes. *Nat Rev Cancer*. 2004; 4:177–183. [PubMed: 14993899]
43. Swanton C. Intratumor heterogeneity: evolution through space and time. *Cancer Res*. 2012; 72:4875–4882. [PubMed: 23002210]
44. Shitashige M, et al. Traf2- and Nck-interacting kinase is essential for Wnt signaling and colorectal cancer growth. *Cancer Res*. 2010; 70:5024–5033. [PubMed: 20530691]
45. Lelièvre H, Chevrier V, Tassin AM, Birnbaum D. Myeloproliferative disorder FOP-FGFR1 fusion kinase recruits phosphoinositide-3 kinase and phospholipase C γ at the centrosome. *Mol Cancer*. 2008; 7:30. [PubMed: 18412956]
46. Nan H, et al. Genome-wide association study identifies nidogen 1 (*NID1*) as a susceptibility locus to cutaneous nevi and melanoma risk. *Hum Mol Genet*. 2011; 20:2673–2679. [PubMed: 21478494]
47. Amos CI, et al. Genome-wide association study identifies novel loci predisposing to cutaneous melanoma. *Hum Mol Genet*. 2011; 20:5012–5023. [PubMed: 21926416]
48. Barrett JH, et al. Genome-wide association study identifies three new melanoma susceptibility loci. *Nat Genet*. 2011; 43:1108–1113. [PubMed: 21983787]
49. Bishop DT, et al. Genome-wide association study identifies three loci associated with melanoma risk. *Nat Genet*. 2009; 41:920–925. [PubMed: 19578364]
50. Brown KM, et al. Common sequence variants on 20q11.22 confer melanoma susceptibility. *Nat Genet*. 2008; 40:838–840. [PubMed: 18488026]
51. Falchi M, et al. Genome-wide association study identifies variants at 9p21 and 22q13 associated with development of cutaneous nevi. *Nat Genet*. 2009; 41:915–919. [PubMed: 19578365]

52. Macgregor S, et al. Genome-wide association study identifies a new melanoma susceptibility locus at 1q21.3. *Nat Genet.* 2011; 43:1114–1118. [PubMed: 21983785]
53. Teerlink C, et al. A unique genome-wide association analysis in extended Utah high-risk pedigrees identifies a novel melanoma risk variant on chromosome arm 10q. *Hum Genet.* 2012; 131:77–85. [PubMed: 21706340]
54. Sabatino M, et al. Conservation of genetic alterations in recurrent melanoma supports the melanoma stem cell hypothesis. *Cancer Res.* 2008; 68:122–131. [PubMed: 18172304]
55. Brunner AL, et al. Transcriptional profiling of lncRNAs and novel transcribed regions across a diverse panel of archived human cancers. *Genome Biol.* 2012; 13:R75. [PubMed: 22929540]
56. Khaitan D, et al. The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. *Cancer Res.* 2011; 71:3852–3862. [PubMed: 21558391]
57. Derrien T, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res.* 2012; 22:1775–1789. [PubMed: 22955988]
58. Harrow J, et al. GENCODE: the reference human genome annotation for The ENCODE Project. *Genome Res.* 2012; 22:1760–1774. [PubMed: 22955987]
59. Harrow J, et al. GENCODE: producing a reference annotation for ENCODE. *Genome Biol.* 2006; 7(suppl):S4.1–S4.9. [PubMed: 16925838]
60. Akavia UD, et al. An integrated approach to uncover drivers of cancer. *Cell.* 2010; 143:1005–1017. [PubMed: 21129771]
61. Lin WM, et al. Modeling genomic diversity and tumor dependency in malignant melanoma. *Cancer Res.* 2008; 68:664–673. [PubMed: 18245465]
62. Xing F, et al. Concurrent loss of the PTEN and RB1 tumor suppressors attenuates RAF dependence in melanomas harboring ^{V600E}BRAF. *Oncogene.* 2012; 31:446–457. [PubMed: 21725359]
63. Sanchez-Garcia F, Akavia UD, Mozes E, Pe'er D. JISTIC: identification of significant targets in cancer. *BMC Bioinformatics.* 2010; 11:189. [PubMed: 20398270]
64. Huang DW, et al. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Res.* 2007; 35:W169–W175. [PubMed: 17576678]
65. Kanehisa M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 2000; 28:27–30. [PubMed: 10592173]
66. Araki H, Knapp C, Tsai P, Print CP. GeneSetDB: a comprehensive meta-database, statistical and visualisation framework for gene set analysis. *FEBS Open Bio.* 2012; 2:76–82.
67. Biankin AV, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature.* 2012; 491:399–405. [PubMed: 23103869]
68. Sanz-Moreno V, et al. Rac activation and inactivation control plasticity of tumor cell movement. *Cell.* 2008; 135:510–523. [PubMed: 18984162]
69. Bogunovic D, et al. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci USA.* 2009; 106:20429–20434. [PubMed: 19915147]
70. Le Clech M. Role of CAP350 in centriolar tubule stability and centriole assembly. *PLoS ONE.* 2008; 3:e3855. [PubMed: 19052644]
71. Patel H, Truant R, Rachubinski RA, Capone JP. Activity and subcellular compartmentalization of peroxisome proliferator-activated receptor α are altered by the centrosome-associated protein CAP350. *J Cell Sci.* 2005; 118:175–186. [PubMed: 15615782]
72. Lim J, et al. A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. *Cell.* 2006; 125:801–814. [PubMed: 16713569]
73. Vandamme J, Völkel P, Rosnoblet C, Le Faou P, Angrand PO. Interaction proteomics analysis of Polycomb proteins defines distinct PRC1 complexes in mammalian cells. *Mol Cell Proteomics.* 2011; 10:M110.002642. [PubMed: 21282530]
74. Danielsen JM, et al. Mass spectrometric analysis of lysine ubiquitylation reveals promiscuity at site level. *Mol Cell Proteomics.* 2010; 10:M110.003590. [PubMed: 21139048]
75. Hutchins JR, et al. Systematic analysis of human protein complexes identifies chromosome segregation proteins. *Science.* 2010; 328:593–599. [PubMed: 20360068]

76. Varjosalo M, et al. The protein interaction landscape of the human CMGC kinase group. *Cell Rep*. 2013; 3:1306–1320. [PubMed: 23602568]
77. Emanuele MJ, et al. Global identification of modular cullin-RING ligase substrates. *Cell*. 2011; 147:459–474. [PubMed: 21963094]
78. Udeshi ND, et al. Methods for quantification of *in vivo* changes in protein ubiquitination following proteasome and deubiquitinase inhibition. *Mol Cell Proteomics*. 2012; 11:148–159. [PubMed: 22505724]
79. Woods NT, et al. Charting the landscape of tandem BRCT domain-mediated protein interactions. *Sci Signal*. 2012; 5:rs6. [PubMed: 22990118]
80. Bandyopadhyay S, et al. A human MAP kinase interactome. *Nat Methods*. 2010; 7:801–805. [PubMed: 20936779]
81. Wagner SA, et al. A proteome-wide, quantitative survey of *in vivo* ubiquitylation sites reveals widespread regulatory roles. *Mol Cell Proteomics*. 2011; 10:M111.013284.
82. Glatter T, Wepf A, Aebersold R, Gstaiger M. An integrated workflow for charting the human interaction proteome: insights into the PP2A system. *Mol Syst Biol*. 2009; 5:237. [PubMed: 19156129]
83. Kim W, et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell*. 2011; 44:325–340. [PubMed: 21906983]
84. Yan X, Habedanck R, Nigg EA. A complex of two centrosomal proteins, CAP350 and FOP, cooperates with EB1 in microtubule anchoring. *Mol Biol Cell*. 2006; 17:634–644. [PubMed: 16314388]
85. Stites EC. The response of cancers to BRAF inhibition underscores the importance of cancer systems biology. *Sci Signal*. 2012; 5:pe46. [PubMed: 23074264]
86. Azmi AS, Wang Z, Philip PA, Mohammad RM, Sarkar FH. Proof of concept: network and systems biology approaches aid in the discovery of potent anticancer drug combinations. *Mol Cancer Ther*. 2010; 9:3137–3144. [PubMed: 21041384]
87. Collier LS, Carlson CM, Ravimohan S, Dupuy AJ, Largaespada DA. Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature*. 2005; 436:272–276. [PubMed: 16015333]
88. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009; 25:1754–1760. [PubMed: 19451168]
89. Li H, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009; 25:2078–2079. [PubMed: 19505943]
90. Koboldt DC, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res*. 2012; 22:568–576. [PubMed: 22300766]
91. R Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; 2013. 2008
92. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*. 2010; 38:e164. [PubMed: 20601685]
93. Knüppel R, Dietze P, Lehnberg W, Frech K, Wingender E. TRANSFAC retrieval program: a network model database of eukaryotic transcription regulating sequences and proteins. *J Comput Biol*. 1994; 1:191–198. [PubMed: 8790464]
94. Shannon P, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003; 13:2498–2504. [PubMed: 14597658]
95. Irizarry RA, et al. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 2003; 31:e15. [PubMed: 12582260]
96. Harrington DP, Fleming TR. A class of rank test procedures for censored survival data. *Biometrika*. 1982; 69:553–566.
97. Spandidos A, Wang X, Wang H, Seed B. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res*. 2010; 38:D792–D799. [PubMed: 19906719]

98. Henare K, et al. Dissection of stromal and cancer cell–derived signals in melanoma xenografts before and after treatment with DMXAA. *Br J Cancer*. 2012; 106:1134–1147. [PubMed: 22415295]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

SB-mediated mutagenesis promotes melanoma formation in *BraF*^{V600E} mutant mice. (a) Pigmentation changes in SB|BraF mice appear uniform at 25 weeks of age with all 4-OHT-painted surfaces appearing almost completely black; this was most obvious on tail skin in comparing sibling mice. Green, yellow and white asterisks denote BraF, SB|BraF and SB sibling mice, respectively. (b) Image of the underside of a dorsal skin specimen after it was removed during necropsy of an SB|BraF mouse. In wild-type mice, the dorsal skin was uniformly non-pigmented, but many individual black clones of *BraF*^{V600E}-positive nevi that covered 4-OHT-painted surfaces were found in SB|BraF mice. Scale bar, 5 mm. (c) Kaplan-Meier survival curves comparing experimental SB|BraF and control sibling SB and BraF mice (log-rank test, $P < 0.0001$). (d-i) Histology and tumor classification from sections of skin masses stained with hematoxylin and eosin (d-f) and undergoing immunohistochemistry (IHC) analysis (g-i). (d) Melanoma showing both a loose cell pattern and a more typical melanoma pattern with nerve-like structures (400×). (e) Melanoma displaying focal schwannomatous features containing pigment granules (400×). (f) Melanoma differentiation focus with tumor cells containing pigment granules (1,000×). (g) S-100 expression in the nucleus of melanoma cells from an unpigmented melanoma (100×); inset, low magnification depicting robust S-100 expression throughout the tumor and adjacent normal skin lacking S-100 expression. (h) Cytoplasmic staining for the melanocyte-specific protein Tyrp1 using antiserum to Tyrp1 (PEP1) (400×). (i) Robust staining of nuclear SB transposase in SB|BraF melanoma cells (400×). Scale bars, 100 μm (d-i); inset, 3 mm (g).

Figure 2.

Whole-exome sequencing of SB|Braf genomes. **(a)** Statistically significant SNV and indel mutations from six melanomas, including SB|Braf CIs (red) and six genes with known roles in melanoma biology (*Dusp6*, *Irak1*, *Map4*, *Mlana*, *Ttn* and *Xirp2*). **(b)** The protein-altering mutations found in each of the six genomes. **(c)** SNV mutational signature. Details regarding SNV and indel mutations appear in Supplementary Tables 16–20.

Figure 3. Landscape of candidate driver genes mutated in SB/Braf melanoma. **(a)** Driver genes for early progression consisting of 21 CIS genes with mean and median SB insertions containing 10 or more 454 sequence reads per tumor. All genes contained SB insertions in three or more melanomas and had corrected $P < 0.05$ by gCIS-0 kb analysis. Genes with red data points were mutated in at least 30% of SB/Braf melanomas. **(b)** Heat map showing the landscape of SB insertions in 17 candidate driver genes for early progression across SB/Braf melanoma genomes (one genome per column). Right, CIS gene symbols and the percentage of melanomas ($n = 70$) where each gene is altered by SB insertion. Four genes, *Cdkn2a*, *Cep350*, *Phlpp1* and *R3hdm1*, occur on donor chromosome 1 or 4, and an adjustment for censored genomes was therefore made to the calculated percentage of altered melanomas by only considering non-donor chromosome genomes: *Cdkn2a* (20/38), *Cep350* (15/50), *Phlpp1* (7/50) and *R3hdm1* (4/50). Bottom, 70% (49/70) of SB/Braf melanoma genomes had one or more SB insertions in 17 candidate driver genes for early progression. The genomes selected for whole-exome sequencing are highlighted in yellow. A quantitative catalog of SB insertions on an individual tumor basis can be found in Supplementary Figure 18. WES, whole-exome sequencing.

Figure 4.

Reduced netrin signaling in SB|Braf melanoma extends the phenotypic consequence of alterations in the Rho family of GTPases. **(a)** Network of the functional connections from GeneSetDB linking *RHOA* and *RAC1*. In total, 30 and 35 CISs (gray nodes) had functional links (black lines) to *RHOA* and *RAC1*, respectively. **(b,c)** Permutation analyses demonstrated that the likelihood of identifying the numbers of CIS genes functionally linked to *RHOA* **(b)** and *RAC1* **(c)** by chance alone was exceedingly low (empirical $P < 0.0001$). Histograms illustrate the results of permutation analyses: x axes show the number of genes in each of 10,000 random sets of genes that have a functional link to *RHOA* or *RAC1* signaling in the database of molecular interactions, and y axes show frequency. Blue dashed lines indicate 95% confidence intervals, and green arrows indicate the number of CISs with functional links to *RHOA* or *RAC1* signaling in the database of molecular interactions. **(d)** Schematic of the netrin signaling network, including core protein components encoded by genes mutated in SB|Braf genomes. Data for direct interactions of netrin signaling proteins

(shown by the overlap of ovals) were from GeneCards and Pathway Commons. Symbols in bold correspond with CISs; *Dcc* was not a CIS, but inactivating SB insertions were observed in 24% of SB|Braf melanomas. (e) Heat map depicting the mutually exclusive distribution of mutations among Rho family members in SB|Braf melanomas and human melanoma genomes from published results^{13,14,17,18,20–24}.

Figure 5.

Significant clinical associations between SB|Braf CIS genes, RNA abundance and patient survival. (a–c) Survival plots for patients with metastatic melanoma based on the expression of three representative SB|Braf CIS orthologs. A poor patient survival outcome is predicted by reduced expression of *ANKRD40* (a), *SDK1* (b) and *THSD7B* (c). (d) Six CIS interaction networks of functional connections between 12 human SB|Braf CIS gene orthologs that show clinical association with RNA abundance and patient survival (red nodes) and other SB|Braf CIS orthologs (gray nodes), including the known cancer drivers *FBXW7*, *FYN*, *GNAQ*, *NSD1*, *NUP98*, *PTEN* and *RAC1*.

Figure 6.

CEP350 is a melanoma tumor suppressor. **(a)** *CEP350* alterations encoded in human melanoma genomes. Amino acid positions in gray, black and red text denote synonymous, nonsynonymous and non-damaging, and nonsynonymous and damaging alterations, respectively. Single and double asterisks denote 2 genomes with 2 mutations. **(b–e)** *CEP350* depletion accelerates xenograft progression *in vivo*. Primary shRNA experiment consisting of 1 million A375 cells stably expressing control shNTC construct (MOI of 6; $n = 10$) or 3 non-overlapping, pooled shRNA constructs for *CEP350* (clones 689, 691 and 694, each at MOI of 2; $n = 10$) injected subcutaneously into immunodeficient NSG mice. **(b)** Xenograft volumes calculated over a 1-month period. **(c)** The cohorts receiving the *CEP350* shRNA pool grew significantly faster than matched cohorts receiving control shNTC. The growth curves differed significantly ($P < 0.0001$) when compared by two-way, repeated-measures ANOVA with Bonferroni correction for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, significant differences between the shNTC and *CEP350* shRNA groups. **(d)** Time to necropsy was substantially reduced in cohorts with *CEP350* shRNA. **(e)** Secondary shRNA experiment consisting of 1 million A375 cells stably expressing control shNTC construct (MOI of 6; $n = 10$) or one of five individual *CEP350* shRNA constructs (MOI of 6; $n = 10$ per group) injected subcutaneously into NSG mice. Xenograft volumes calculated 15 d after injection show that tumor volumes met or exceeded the maximal shNTC volumes for between 20–70% of mice per cohort, with statistically significant accelerated tumor volumes achieved by *CEP350* shRNA clones 481, 494 and 694. P values were calculated by one-

factor ANOVA ($P = 0.112$) with Bonferroni post-test. Error bars, mean $\pm$ s.e.m. AUC, area under the curve.

Table 1

The 104 CIS orthologs with the highest frequency of somatic mutations in 347 human melanoma genomes

Number of somatic mutations in human melanoma genomes	Number of SB Braf CIS orthologs	Human ortholog of the CIS-targeted gene(s)
186	1	<i>BRAF^a</i>
155	1	<i>LRP1B</i>
119	1	<i>CSMD3</i>
105	1	<i>ADAM28</i>
104	1	<i>USH2A</i>
103	1	<i>APOB</i>
96	1	<i>EPHA6</i>
94	1	<i>MLL3</i>
91	3	<i>PCDH15, PTPRD, SYNE1</i>
88	2	<i>ERBB4^a, HYDIN</i>
87	2	<i>GRIA3^b, PTPRT</i>
85	1	<i>CSMD1</i>
84	1	<i>PKHD1</i>
82	1	<i>SCN11A</i>
81	1	<i>COL5A1</i>
75	1	<i>GPR98</i>
73	2	<i>CNTNAP2, ODZ1</i>
72	1	<i>PLCB1</i>
70	2	<i>GRID2, MAGI1</i>
68	1	<i>FLNB</i>
67	1	<i>BAI3</i>
66	1	<i>THSD7B^b</i>
64	1	<i>SDK1^b</i>
60	2	<i>CNTN5, LRP1</i>
59	1	<i>CDH6</i>
56	2	<i>CADM2^b, CNTN4</i>
55	2	<i>MAP3K1, NRXN1</i>
54	2	<i>EGFLAM, NPR1</i>
53	5	<i>HERC2, KIAA1217, MACF1, MYO3A, NLRP4</i>
52	3	<i>DMD, GRM8, ZAN</i>
51	4	<i>DPP10, GRM7, NF1, PDE7B</i>
50	2	<i>NRXN3, ODZ3^c</i>
49	1	<i>FBN1</i>
48	4	<i>ADAMTS12, CTNNA3, FLI1, TRIO</i>
47	1	<i>EP300</i>
46	4	<i>A2BP1, C8ORF34, MCTP1, SRGAP3</i>
44	1	<i>BIRC6</i>

Number of somatic mutations in human melanoma genomes	Number of SB Braf CIS orthologs	Human ortholog of the CIS-targeted gene(s)
43	6	<i>ADAM17, AHNAK, ARID1A, CNTNAP5, NCOA3^b, ZNF536</i>
42	1	<i>TCF4</i>
41	6	<i>BCLAF1, LTBP1, MAGI2^b, MYST4, ZFH3c, ZFYVE26</i>
40	2	<i>FLT3, WDFY3</i>
39	2	<i>EP400^b, SCN7A</i>
38	5	<i>CDKN2A^{a,c}, FRMPD4, MAPKBP1, TRPM3, UBR5</i>
37	3	<i>KIAA2018, RPTOR, SPAG16</i>
36	8	<i>CHD1, DNER, GREB1, LPHN3, NCKAP5, ODZ4, PTPRZ1, TRERF1</i>
35	8	<i>FGF12, FRYL, IL7R, OCA2, PARD3, PCNX, PKP2, SIPA1L1</i>

^aKnown melanoma drivers.

^bSignificant clinical associations between SB|Braf CIS genes, RNA abundance and patient survival.

^cCandidate driver genes mutated in SB|Braf melanoma.