

SPRY2 Is an Inhibitor of the Ras/Extracellular Signal-Regulated Kinase Pathway in Melanocytes and Melanoma Cells with Wild-Type *BRAF* but Not with the V599E Mutant

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Abstract

***BRAF* mutations result in constitutively active *BRAF* kinase activity and increased extracellular signal-regulated kinase (ERK) signaling and cell proliferation. Initial studies have shown that *BRAF* mutations occur at a high frequency in melanocytic nevi and metastatic lesions, but recent data have revealed much lower incidence of these mutations in early-stage melanoma, implying that other factors may contribute to melanoma pathogenesis in a wild-type (WT) *BRAF* context. To identify such contributing factors, we used microarray gene expression profiling to screen for differences in gene expression between a panel of melanocytic and melanoma cell lines with WT *BRAF* and a group of melanoma cell lines with the V599E *BRAF* mutation. We found that SPRY2, an inhibitor homologous to SPRY4, which was previously shown to suppress Ras/ERK signaling via direct binding to Raf-1, had reduced expression in WT *BRAF* cells. Using small interfering RNA-mediated SPRY2 knockdown, we showed that SPRY2 acts as an inhibitor of ERK signaling in melanocytes and WT *BRAF* melanoma cells, but not in cell lines with the V599E mutation. We also show that SPRY2 and SPRY4 directly bind WT *BRAF* but not the V599E and other exon 15 *BRAF* mutants. These data suggest that SPRY2, an inhibitor of ERK signaling, may be bypassed in melanoma cells either by down-regulation of its expression in WT *BRAF* cells, or by the presence of the *BRAF* mutation.**

Introduction

BRAF mutations may play an important role in initiation and/or progression of melanoma (1–3) via sustained *BRAF*–mitogen-activated protein kinase kinase–extracellular signal-regulated kinase (*BRAF*–MEK–ERK) activation (4). As such, they may be important, long searched for therapeutic targets. In melanoma, the majority of *BRAF* mutations (>90%) involve a T1796A transversion in exon 15, resulting in a V599E missense mutation (2, 5). Rarely in this cancer, exon 11 mutations, such as K438Q, are also described (5). Exon 15 mutations cluster to the kinase domain of *BRAF* and lock the molecule in its active conformation (6). In general, *BRAF* mutants lead to increased ERK activation via sustained MEK phosphorylation or interaction with Raf-1 (6), but their exact role with respect to the course and stage of melanoma remains to be determined. The high incidence (up to 80%) in nevi and metastatic lesions (2, 7) but low frequency (10%) in early-stage melanomas suggest that *BRAF* mutations may correlate with progression rather than initiation of melanoma (3, 8). Other factors may therefore be responsible for tumor

initiation in the absence of a *BRAF* mutation. In the present study, we aimed to identify such factors that may act in a wild-type (WT) *BRAF* context. Using high-throughput microarray screening, we identified the inhibitor SPRY2 being under-expressed in WT *BRAF* melanoma cells.

Materials and Methods

Cell Lines and Cell Culture. Normal melanocytes and primary melanoma cell lines were obtained from M. Herlyn (Wistar Institute, Philadelphia, PA). *BRAF* mutation status and tumor stage were determined previously (4).³ All cell lines have WT *NRAS* (4).³ Human melanocytes and melanoma cells were maintained in media as described previously (4). Fetal bovine serum was omitted according to the experimental setting. HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Sigma, St. Louis, MO) at 37°C and 10% CO₂.

Microarray-Based Gene Expression Analysis. Total RNA was isolated from cell cultures at 70% confluency by use of TRIzol reagent (Invitrogen, Carlsbad, CA), as suggested by the manufacturer. We used 5 µg of total RNA to synthesize double-stranded cDNA (Superscript Choice System for cDNA Synthesis kit; Invitrogen) and after cleanup (GeneChip Sample Cleanup Module; Qiagen/Affymetrix, Santa Clara, CA), to produce fragmented biotin-labeled cRNA (Enzo RNA Transcript Labeling Kit; Enzo, Farmingdale, NY), using the manufacturers' protocols. Human HG-U133A chips (Affymetrix, Inc.) were hybridized with 15 µg of fragmented labeled cRNA overnight at 45°C, washed (Genechip Fluidics Station 400; Affymetrix), and scanned (GeneArray Scanner; Affymetrix) according to Affymetrix protocols. Scanned images were analyzed with the MAS 5.0 software (Affymetrix), and intensities were scaled to a value of 500. Cluster3.0 was used for visualization purposes (Fig. 1A).⁴ The files of the analyzed data and the list of the 108 genes of the Ras/ERK pathway screened for the analysis can be found on the Internet.⁵

Plasmids. WT and V599E pEF *MYC-hBRAF* were gifts from R. Marais (Institute of Cancer Research, London, United Kingdom). Point mutations in *hBRAF* were introduced with Quikchange (Stratagene, La Jolla, CA), according to the manufacturer's instructions, and verified by capillary sequencing. pCDNA3 FLAG-mSPRY2 and pCDNA3 FLAG-mSPRY4 were supplied by J. Licht and have been described previously (10).

Cell Extraction and Immunoblotting. Whole-cell extracts were prepared in ELB+ [250 mM NaCl, 50 mM HEPES (pH 7.0), 5 mM EDTA, 10 mM β-glycerol phosphate, 10 mM NaF, 10 mM sodium vanadate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.2% Triton X-100], diluted with Laemmli buffer, and separated by SDS-PAGE. Samples were transferred to nitrocellulose membranes (*trans*-Blot; BIO-RAD, Hercules, CA), blocked in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) milk powder, and then probed with murine M2 anti-FLAG (Sigma-Aldrich; St. Louis, MO) and murine 9E10 anti-MYC antibodies (Institute of Cancer Research, London, United Kingdom) in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) milk powder. Primary antibodies were detected with an Alexa Fluor680-conjugated goat antimouse secondary antibody (Molecular Probes, Eugene, OR) and an Ody-

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³ B. L. Weber and M. Herlyn, unpublished data.

⁴ <http://bonsai.ims.u-tokyo.ac.jp>.

⁵ <http://acgh.afcri.upenn.edu/mel/MelanomaData.htm>.

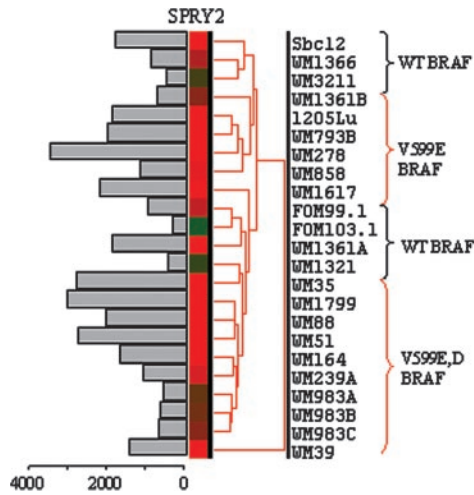


Fig. 1. Microarray-based expression analysis of *SPRY2* in melanocytes and melanoma cell lines. Fold changes are color-coded; *FOM103.1* is the reference sample for color-coding. Green, no change/down-regulation; red, up-regulation. The bar graph shows microarray signal intensities for *SPRY2*. WT, wild-type.

sey IR imaging system (Li-Cor Biosciences, Lincoln, NE) according to the manufacturers' guidelines. For detection of endogenous *SPRY2* and *BRAF*, membranes were probed with rabbit anti-*SPRY2* (Upstate, Chicago, IL) and rabbit anti-*BRAF* (Upstate) antibodies, respectively. The primary antibodies were detected with goat antirabbit horseradish peroxidase-conjugated antibody (Bio-Rad) incubated with horseradish peroxidase substrate (Bio-Rad) and detected by chemiluminescence using X-OMAT film (Kodak, Rochester, NY).

Immunoprecipitation. HEK293T cells were transfected with pEF MYC-*hBRAF* and pCDNA3 FLAG-*mSPRY* constructs, using Effectene (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. MYC-*hBRAF* complexes were immunoprecipitated from HEK293T cell extracts by mixing with rabbit A14 anti-MYC antibody (Santa Cruz Biotechnology, Santa Cruz, CA) bound to protein G-Sepharose beads for 1 h at 4°C. Samples were washed in ELB, eluted with Laemmli buffer, and subjected to SDS-PAGE. Membranes were probed for MYC-*hBRAF* and the associated FLAG-*mSPRY*, as described above. FLAG-*mSPRY* and MYC-*hBRAF* levels were determined with the Odyssey IR imaging system using Odyssey v1.0 software (Li-Cor Biosciences). Immunoprecipitations were repeated at least three times in independent experiments. The averaged results are presented as the "relative association" of FLAG-*mSPRY* with MYC-*hBRAF* (the ratio of FLAG-*mSPRY2* signal, normalized for expression levels, to MYC-*hBRAF* signal detected with the Alexa Fluor680 secondary antibody). For immunoprecipitation of endogenous *BRAF*, cell extracts were passed through protein G-Sepharose columns (Sigma) bound to anti-*SPRY2* antibody (Upstate). Samples were washed in ELB and eluted with Laemmli buffer. After SDS-PAGE and transfer, membranes were probed with anti-*BRAF* antibody (Upstate).

Small Inhibitory RNA Transfections. Small inhibitory duplex RNAs (siRNA) were prepared from DNA templates by use of the Silencer siRNA construction kit (Ambion, Austin, TX), according to the manufacturer's protocol. Sequences are available on request. To achieve transient knockout of *SPRY2*, cells were plated in 6-well plates at 30–50% confluency and transfected with 50 nM siRNA and 4 μ l of oligofectamine reagent (Invitrogen Life Technologies, Inc., Gaithersburg, MD) and OptiMEM (Invitrogen), as recommended by the manufacturer. After 4 h at 37°C, the transfection medium was removed and replaced with MCDB153. Cells were harvested at post-transfection time points as indicated.

Bromodeoxyuridine Incorporation. Incorporation assays were performed using a colorimetric bromodeoxyuridine (BrdUrd) assay (Roche) according to the manufacturer's instructions. Cells were cultured in 96-well plates at 0.5×10^4 cells/well and transfected with either control or *SPRY2*-specific siRNA, as described above. U0126 (Promega, Madison, WI) was applied at 10 μ M for 24 h at the 48-h post-transfection time point. Cells were incubated for 5 h with 10 μ M BrdUrd 48 h after siRNA transfection, fixed with FixDenat, incubated with anti-BrdUrd peroxidase antibody, washed, and incubated with 3,3',5,5'-tetramethylbenzidine solution provided by the manufacturer. Absorbance

was measured at 370 nm (reference wavelength, 492 nm). Maximum sensitivity was achieved at an absorbance range of 0.5–2.5, with sensitivity comparable to the [³H]thymidine incorporation assay. Within this range, 2-fold increases in absorbance correspond to a 5–10-fold increase in the number of proliferating cells. Wells without cells (to control for nonspecific binding to the plate) and background controls (cells incubated with anti-BrdUrd antibody) were included to control for nonspecific labeling.

Results and Discussion

We used oligonucleotide-based expression profiling (Affymetrix, Inc.) to identify gene expression changes between melanocytes and melanoma cell lines with WT *BRAF* and melanoma cells with mutant *BRAF*. We focused our initial analysis on changes in expression of components of the RAS/RAF/MEK/mitogen-activated protein kinase (MAPK) signaling pathway. Specifically, 108 transcripts belonging to this pathway were screened for changes in gene expression (see "Materials and Methods"). This analysis revealed that only *SPRY2* transcripts were down-regulated in melanocytes and WT *BRAF* melanoma cell lines compared with mutant *BRAF* cultures (Fig. 1), with a statistically significant change in average intensity on the order of 2-fold ($P < 0.05$). Quantitative reverse transcription-PCR for *SPRY2* in selected cell lines confirmed the microarray results (data not shown). *SPRY2* has been identified as inhibitor of MAPK signaling in epithelial and fibroblast cell lines (9, 10). *SPRY* was originally identified as encoding a fibroblast growth factor inhibitor that regulates tracheal branching in *Drosophila* (11). There are four human and three murine *SPRY* genes with distinct tissue expression patterns (12, 13). Present evidence suggests that *SPRY* proteins may have a role in the regulation of angiogenesis as well as kidney, lung, and limb development (12–14). *SPRY* gene expression is induced as a result of increased levels of phosphorylated (activated) ERK, creating a negative feedback loop (9, 15). The low levels of *SPRY2* in the melanocytes used in this study are consistent with the lower phospho-ERK levels that these cells show compared with melanoma cell lines (4). Melanoma cells that harbor the V599E mutation have constitutively activated ERK (4), which may account for the high levels of *SPRY2* found. Under the culture conditions used (plus serum), most of the WT *BRAF* melanoma cells used in this study exhibited phospho-ERK levels comparable to those in the mutant cells (4). In this case, low *SPRY2* levels may represent a disrupted responsiveness of *SPRY2* to phospho-ERK in these cells. This notion is further supported by the fact that a decrease in phospho-ERK after incubation with a MEK inhibitor (U0126) down-regulated *SPRY2* expression in mutant *BRAF* melanoma cells to levels similar to the ones found in WT *BRAF* cells (data not shown).

SPRY proteins may act as competitive inhibitors of MAPK signaling by at least two potential mechanisms. The COOH termini of *SPRY2* and *SPRY4* can bind to RAF1, inhibiting kinase activity (16). In addition, *SPRY* proteins uncouple receptor tyrosine kinase signaling from the activation of RAS, possibly by sequestration of the GRB2, an adaptor protein required for RAS activation (9, 10). As the inhibitory function of *SPRY2* in MAPK signaling is thought to be cell-context-dependent (9, 17), we asked whether *SPRY2* has an inhibitory effect in melanocytes and/or melanoma cell lines. We therefore designed siRNAs complementary to *SPRY2* and tested their ability to reduce *SPRY2* transcript and protein levels, regulate ERK phosphorylation, and alter cell growth characteristics. To this end, *SPRY2*-specific siRNA (or scrambled-sequence siRNA for control) was transfected into normal melanocytes and melanoma cell lines by use of Oligofectamine (Invitrogen). Whole-cell lysates were prepared at 24-h intervals (0–96 h). Maximum reduction in *SPRY2* protein levels was achieved at the 48- and 72-h time points (data not shown). At 48 h post-transfection, normal melanocytes assumed a morphology previously described as characteristic of increased levels of phospho-

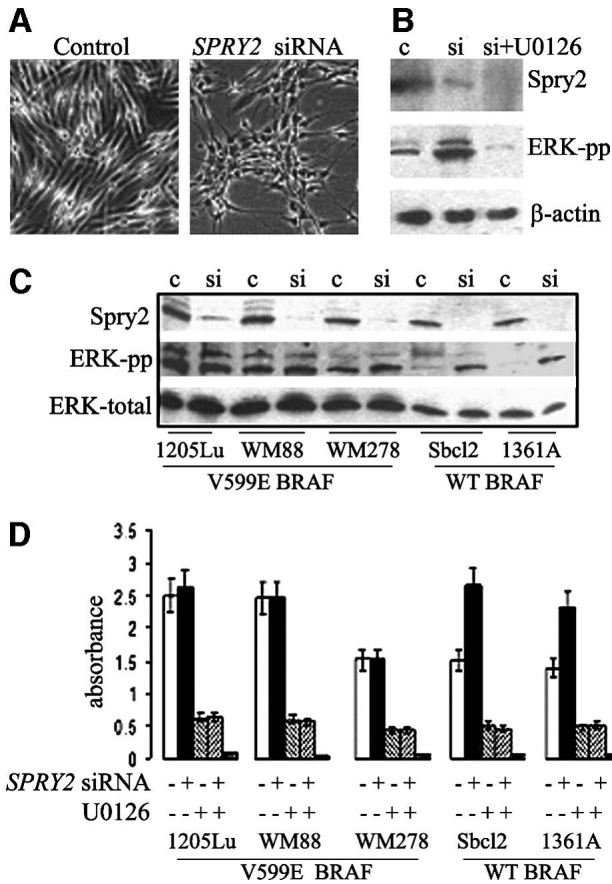


Fig. 2. SPRY2 effect on extracellular signal-regulated kinase (ERK) activation and cell proliferation in wild-type (WT) and mutant *BRAF* melanoma cell lines. **A**, phenotype of normal melanocytes (FOM74) after transfection with control small inhibitory RNA (siRNA) or with *SPRY2* siRNA. **B** and **C**, phospho-ERK levels after *SPRY2* siRNA (or control siRNA) transfection of normal melanocytes (**B**) and primary melanoma cell lines (**C**). *Third lane in B* corresponds to *SPRY2* siRNA sample incubated with the MEK inhibitor U0126. *Lanes c*, control; *Lanes si*, *SPRY2* siRNA transfection; *Sbc12*, 1361A-WT *BRAF*; *WM278* and *WM88*, 1205Lu-*BRAF* V599E. **D**, bromodeoxyuridine incorporation in the same melanoma cell lines as in **C** after control siRNA or *SPRY2* siRNA transfection, with or without incubation with the mitogen-activated kinase inhibitor U0126. The *fifth column* in each group represents background values. *Bars*, SE.

ERK (Ref. 18; Fig. 2A), whereas the phenotype of the melanoma cell lines did not change (data not shown).

To determine whether the siRNA-mediated *SPRY2* knockout had an effect on ERK activation, we prepared whole-cell extracts from *SPRY2* siRNA-transfected and control sequence siRNA-transfected melanocytes 48 h post-transfection. These experiments demonstrated that the observed morphological changes were correlated with ERK phosphorylation (Fig. 2B). Both the phenotypic change (data not shown) and the increase in ERK phosphorylation (Fig. 2B) seen after transfection with *SPRY2* siRNA were reversible after exposure to the MEK inhibitor U0126, indicating the dependence of the phenotype on ERK activation. These data demonstrate that normal melanocytes respond to *SPRY2* down-regulation by increasing ERK phosphorylation and that *SPRY2* functions as an inhibitor of MAPK signaling in normal melanocytes.

We also measured phospho-ERK levels in melanoma cell lines, with and without a *BRAF* mutation (V599E), transfected with *SPRY2* siRNA (Fig. 2C). These experiments demonstrated that knock-down of *SPRY2* protein is associated with increased phospho-ERK in melanoma cell lines with WT *BRAF* (Fig. 2C) compared with control transfections with scrambled *SPRY2* siRNA. In contrast, phospho-ERK levels remained unchanged after *SPRY2* siRNA transfection in melanoma cell lines with mutant *BRAF* (V599E; Fig. 2C).

To determine whether *SPRY2*-induced modulation of MAPK signaling plays a role in regulation of cell growth, we used BrdUrd incorporation as a surrogate for cellular proliferation. As predicted by alterations in phospho-ERK levels, transfection with *SPRY2* siRNA was associated with significantly increased BrdUrd incorporation ($P < 0.0015$), whereas incorporation was unaffected by *SPRY2* siRNA transfection in melanoma cell lines with mutant *BRAF* (Fig. 2D). Treatment with the MEK inhibitor U0126 showed that a functional RAF/MEK/ERK signaling cascade is required for cell proliferation in these cells, as captured by BrdUrd incorporation (Fig. 2D).

Previous studies showed that *SPRY4* binding to the COOH terminus of RAF1 is required for inhibition of ERK phosphorylation after growth factor stimulation (16). In addition, *SPRY2* and *SPRY4* may act upstream of Ras, interfering with Grb2-Sos complex formation (9). Because Ras is constitutively activated in melanoma cells (4), we hypothesized that *SPRY2* may inhibit the ERK pathway downstream of Ras (at the

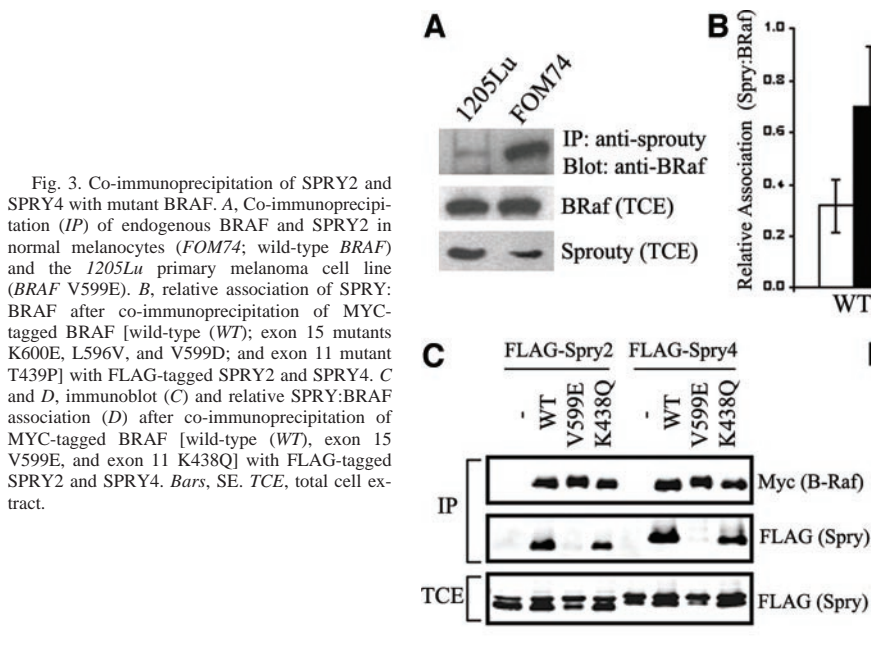


Fig. 3. Co-immunoprecipitation of *SPRY2* and *SPRY4* with mutant *BRAF*. **A**, Co-immunoprecipitation (IP) of endogenous *BRAF* and *SPRY2* in normal melanocytes (FOM74; wild-type *BRAF*) and the 1205Lu primary melanoma cell line (*BRAF* V599E). **B**, relative association of *SPRY*:*BRaf* after co-immunoprecipitation of MYC-tagged *BRAF* [wild-type (WT); exon 15 mutants K600E, L596V, and V599D; and exon 11 mutant T439P] with FLAG-tagged *SPRY2* and *SPRY4*. **C** and **D**, immunoblot (**C**) and relative *SPRY*:*BRaf* association (**D**) after co-immunoprecipitation of MYC-tagged *BRAF* [wild-type (WT), exon 15 V599E, and exon 11 K438Q] with FLAG-tagged *SPRY2* and *SPRY4*. *Bars*, SE. *TCE*, total cell extract.

level of Raf), rather than upstream. Thus, we asked whether SPRY2 and/or SPRY4 could bind to BRAF, and whether, if so, this interaction is altered by the presence of a *BRAF* mutation. Endogenous BRAF co-immunoprecipitated with SPRY2 from whole-cell lysates of FOM74 melanocytes that expressed WT BRAF, but not from lysates of 1205Lu melanoma cells that expressed mutant V599E BRAF (Fig. 3A). To determine whether various tumor-derived BRAF mutations affected binding to SPRY2 and SPRY4, we coexpressed FLAG-tagged *SPRY2* or FLAG-tagged *SPRY4* and MYC-tagged mutant or WT *BRAF* in HEK293T cells. These experiments showed that exon 15 BRAF mutants (V599E, V599D, L596V, and K600E) did not bind SPRY2 or SPRY4, whereas WT BRAF and nonactivating exon 11 mutants (K438Q, T439P) efficiently bound both SPRYs (Fig. 3, B–D).

These results suggest that in the case of melanoma cell lines with WT BRAF, SPRY2-dependent inhibition may be mediated by direct interaction of SPRY2 with BRAF. In V599E mutant melanoma cells, SPRY2 does not show any inhibitory effect. This observation can be explained either by the mutation-mediated disruption of SPRY2-BRAF association, or it can simply be a result of the overwhelming increase in kinase activity of BRAF exon 15 mutants.

In summary, we have shown that *SPRY2* is underexpressed in melanocytes and WT BRAF melanoma cell lines compared with V599E melanoma cells. Knocking down SPRY2 in these cells has a positive impact on ERK signaling only in a WT BRAF context. In addition, SPRY2 is capable of binding BRAF, but the interaction is disrupted by exon 15 mutations in BRAF. These findings suggest that loss of SPRY expression may enhance levels of active ERK, enabling the development and growth of melanoma cells of a WT BRAF profile. Further studies should provide insight into the role of the SPRY family in these processes, with the potential benefit of optimizing *BRAF* as a therapeutic target in melanoma.

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