Oncogenic B-RafV600E Induces Spindle Abnormalities, Supernumerary Centrosomes, and Aneuploidy in Human Melanocytic Cells

Yongping Cui1, Meghan K. Borysova1,3, Joseph O. Johnson2, and Thomas M. Guadagno1

Abstract

Activating B-Raf mutations arise in 60% to 70% of human melanomas and are thought to play a vital role in tumorigenesis, although how this occurs remains poorly understood. Wild-type B-Raf is critical for normal mitosis of human somatic cells, suggesting that mutational activation of B-Raf might compromise mitosis. We examined this hypothesis by introducing oncogenic mutant B-RafV600E into established human melanoma cells, assessing the effects on mitosis and their possible relationship to extracellular signal–regulated kinase (ERK) pathway activation. Exogenous expression of this activated B-Raf mutant led to a high incidence of aberrant spindles and supernumerary centrosomes. These mitotic abnormalities were suppressed by expression of a B-RafV600E–specific siRNA or by the addition of the mitogen-activated protein/ERK kinase–specific inhibitor U0126. Mitotic abnormalities generated by B-RafV600E also caused missegregation of chromosomes leading to aneuploidy. Because activating B-Raf mutations are detected frequently in benign nevi, we extended our studies to primary human melanocytes. Remarkably, short-term expression of B-RafV600E was sufficient to induce aneuploidy in human melanocytes or in immortalized human mammary epithelial cells. Collectively, our studies identify a novel role for the B-Raf oncogene in driving aneuploidy in melanocytic cells. We propose that disruption of mitotic controls by oncogenic B-Raf has important implications for understanding melanoma tumor development. Cancer Res 70(2): 675–84. ©2010 AACR.

Introduction

Raf kinases (A-Raf, B-Raf, and C-Raf) stimulate the mitogen-activated protein kinase (MAPK) cascade, which consists of mitogen-activated protein/extracellular signal–regulated kinase (MEK) and extracellular-signal regulated kinase (ERK). Of the three Raf kinase members, B-Raf is the most potent MEK activator (1), with prominent roles in cell growth, cell cycle progression, and survival (2, 3). In addition, mitotic roles for B-Raf in regulating spindle formation and activation of the spindle assembly checkpoint (SAC) have been shown recently in human somatic cells (4), which is consistent with mitotic functions of ERK (5–9). Mitotic activation of B-Raf requires Cdc2/cyclin B activity (10, 11), suggesting a regulatory mechanism that is distinct from Ras-mediated activation of Raffs during the G1 phase of the cell cycle. Therefore, B-Raf can promote cell proliferation by a variety of mechanisms throughout the cell cycle that extend to key roles at mitosis.

Activating B-Raf mutations arise somatically in 7% of human cancers, with a particularly high frequency (60–70%) in cutaneous melanomas (12–14). The V600E mutation accounts for at least 90% of all B-Raf mutations detected to date, which renders B-Raf constitutively active (12, 15). As such, B-RafV600E sustains 10-fold higher levels of ERK activity (16), which likely contributes to its oncogenic transforming activity shown in immortalized fibroblasts and mouse melanocytes in culture (12, 17, 18). In melanoma cells, B-RafV600E subverts adhesion-dependent controls (19, 20) and suppresses anoikis (21, 22). Targeting oncogenic B-Raf inhibits melanoma cell proliferation and survival in vitro and melanoma tumor growth and vascular development in vivo (17, 23, 24). Together, these findings underscore crucial roles for oncogenic B-Raf in tumorigenesis, yet the mechanism through which oncogenic B-Raf exerts its transforming properties remains poorly understood.

Most human tumors exhibit abnormal chromosomes numbers, known as aneuploidy, which is associated with tumor progression and poor prognosis (25, 26). Aneuploidy results from chromosome segregation errors that often arise due to faulty spindle checkpoint controls and centrosome amplification (see reviews in refs. 27, 28). Because B-Raf serves normal roles at mitosis in mediating proper spindle formation and activation of the SAC (4), we investigated whether expression of the constitutively active B-RafV600E mutant, present in most melanomas, might have detrimental effects.
effects at mitosis. Here, we showed that introducing the B-RafV600E mutant into wild-type B-Raf melanoma cells resulted in supernumerary centrosomes, aberrant spindles, and aneuploidy. Similarly, aneuploidy was rapidly induced by B-RafV600E in primary human melanocytes and hTERT-immortalized epithelial cells. From these results, we propose that the B-Raf oncogene may be a key contributor to aneuploidy in melanoma.

Materials and Methods

Retroviral constructs and viral production. pBabe-puro and pBabe-puro-B-RafV600E retroviral vectors were a generous gift from Dr. Daniel Peeper (The Netherlands Cancer Institute, Amsterdam, the Netherlands). pSUPER-retro-B-Raf shRNA Mut-A was kindly provided by Dr. David Tuveson (Cambridge Research Institute/Cancer Research UK, Cambridge, United Kingdom) and used to selectively knock down the V600E mutant form of B-Raf as previously described (17). Retroviral vectors were transfected into HEK 293T replication-defective packaging cells for retrovirus production.

Cell lines and primary melanocytes. Human melanoma cell lines SK-MEL-5, SK-MEL-28, and A375 were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (FBS). SK-MEL-5 cells used in this study are wild type for B-Raf (12, 29), which was also confirmed by DNA sequencing analysis for exon 15, performed by the Moffitt Molecular Biology core facility (Supplementary Fig. S1). SK-MEL-28 and A375 cells carry B-RafV600E mutations (12). SBcl2 melanoma cells, originally derived from early radial growth phase primary melanomas, were obtained previously from M. Herlyn (Wistar Institute, Philadelphia, PA). SBcl2 cells are wild type for B-Raf (16, 30) and were grown in 2% tumor medium (4:1 mix of MCDB153/L15 medium, 2% FBS, 5 μg/mL insulin, 1 mmol/L CaCl2). To further assess functional B-RafV600E mutations in melanoma cell lines, phospho-ERK levels were determined by immunoblot analysis after switching to low serum for 24 h. SK-MEL-5 and SBcl2 melanoma cells switched to 0.25% FBS exhibited minimal ERK activity, whereas SK-MEL-28 and A375 (both containing B-RafV600E mutations) cells showed robust levels of phospho-ERK (data not shown). Primary human epidermal melanocytes were obtained from ScienCell Research Laboratories and cultured in their Melanocyte Medium.

Human mammary epithelial cells immortalized with human telomerase (hTERT-HME) were generously provided to us by the laboratory of Dr. Huntington Potter at the Johnnie B. Byrd Alzheimer’s Center and Research Institute (University of South Florida, Tampa, FL). hTERT-HME cells were cultured in mammary epithelial cell growth medium from Lonza.

Retrovirus infections and DNA plasmid transfections. SBcl2 cells were infected with pBabe-puro or pBabe-B-RafV600E retroviruses as described (31) and selected in puromycin (0.8 μg/mL) for 8 to 10 d. Puromycin-resistant colonies were pooled and checked for ectopic B-RafV600E expression by immunoblot analysis. Transient gene expression in SK-MEL-5 cells was done by transfection of pEBG plasmids containing an NH2-terminal glutathione S-transferase (GST) tag fused to full-length human B-RafWT or V600E, as previously described (30); transfection efficiency was routinely in the range of 80% to 90% based on immunostaining with a GST antibody. Human melanocytes or hTERT-HME cells were transfected using the Nucleofector system from Amaxa. Transfection efficiencies were estimated to be ~90% based on cotransfection with green fluorescent protein.

Immunofluorescence staining and microscopy. To assess mitotic spindles and centrosome numbers, melanoma cells were subjected to immunostaining with α-tubulin (Sigma) and γ-tubulin (Sigma) antibodies, respectively, as previously described (30). Immunostained cells were mounted with Prolong Gold containing 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) to detect chromosomes. Microscope images of mitotic cells were viewed with a fully automated, upright Zeiss Axio-Imager.Z1 microscope (Carl Zeiss MicroImaging, Inc.) using 63×/1.40 numerical aperture (NA) and 100×/1.3 NA oil-immersion objectives and DAPI, FITC, and Texas red filter cubes. High-resolution images were captured using the AxioCam MRm charge-coupled device (CCD) camera and deconvoluted using Axiovision version 4.5 software or a Leica DMi6000/TCS SP5 confocal microscope equipped with a 100×/1.40 NA Plan Apochromat oil-immersion objective, as previously described (30). All images were transferred into Photoshop v8.0 (Adobe) and saved as Tiff files.

Fluorescence in situ hybridization analysis and metaphase chromosome spreads. Cells were treated with 1 μg/mL colchicine for 2 h, harvested by trypsinization, and washed with PBS. Cells were swollen in 65 mmol/L KCl for 5 min at 37°, fixed in cold acetic acid/methanol for 5 min at 4°, dropped onto slides, and dried at room temperature. For metaphase spreads, cells were then stained with DAPI and viewed with a Nikon E800 fluorescence microscope with a 60×/1.40 NA Plan Apo oil-immersion objective. Images were captured with a Roper Coolsnap HQ CCD camera and processed with Metamorph 5.0 and Adobe Photoshop 8.0 softwares. For interphase fluorescence in situ hybridization (FISH) analysis, slides were stained with CytoCell enumeration probes against chromosomes 2 and 8 (for SBcl2 cells) or chromosomes 3 and 10 (for human epidermal melanocytes and hTERT-HME cells) conjugated with FITC or Cy3.5 (Rainbow Scientific). Staining was carried out according to the manufacturer’s protocol. FISH samples were viewed with a fully automated, upright Zeiss Axio-Imager.Z1 microscope with a 20× objective and DAPI, FITC, and Rhodamine filter cubes. Images were produced using the AxioCam MRm CCD camera and Axiovision version 4.5 software suite. P values were calculated using a two-sample test for equality of proportions with continuity correction.

Western blot analysis. Adherent melanoma cells, washed with cold PBS, were scraped into cell lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/mL NaCl, 5 mmol/L EGTA, 0.5% NP40] containing protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 25 mmol/L NaF, 1 mmol/L sodium vanadate) and centrifuged at 14,000 rpm for 15 min at 4°C to pellet insoluble cell debris. Protein concentrations of supernatants were determined using the detergent-compatible
protein assay (Bio-Rad). Equal amounts of protein were resolved by 10% SDS-PAGE, electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore Corporation), and subjected to immunoblot analysis. Primary antibodies used include rabbit anti-B-Raf (H-145) and anti-phospho-p44/42 MAPK (Thr 202/Tyr204; E10) monoclonal antibodies (Cell Signaling) and mouse anti-ERK1 (BD Transduction Laboratories). Secondary antibodies, alkaline phosphatase–conjugated AffiniPure goat anti-mouse IgG or anti-rabbit IgG-alkaline phosphatase antibodies (Sigma), were incubated for 1 h at room temperature and then processed for chemiluminescence detection using the CDP-Star reagent (Roche).

Results

Oncogenic B-RafV600E induces spindle abnormalities and supernumerary centrosomes. Mitotic roles for B-Raf in human somatic cells have been previously shown by our laboratory (4). Because activating B-Raf mutations are detected in a high proportion of human cutaneous melanomas, we postulated that its constitutive activity may adversely affect mitosis. To test this possibility, we ectopically expressed the oncogenic B-RafV600E mutant in human melanoma cells (SBcl2 and SK-MEL-5) that are wild type for B-Raf. Moderate expression of recombinant B-RafV600E over endogenous wild-type B-Raf levels was confirmed by Western blot analysis (Fig. 1A) and, as predicted, resulted in elevated phospho-ERK levels in both melanoma cell lines. B-RafV600E–expressing cells subjected to immunostaining exhibited a high frequency (∼76%) of spindle abnormalities with misaligned chromosomes (see Fig. 1B). Approximately 55% of the abnormal spindles consisted of multipolar structures with amplified centrosomes as confirmed by costaining with anti-γ-tubulin (Fig. 1B; Supplementary Fig. S2A). The remaining abnormal spindles structures were associated with two centrosomes. Similar spindle abnormalities, including multipolar spindle structures, were generated in SK-MEL-5 melanoma cells transfected with a pGST-B-RafV600E construct (Fig. 1C; Supplementary Fig. S2B). In contrast, 90% of parental SBcl2 and SK-MEL-5 melanoma cells had normal metaphase spindles with proper chromosome alignment (Fig. 1B and C). Interestingly, overexpression of wild-type B-Raf in SK-MEL-5 cells had little effect on spindle formation (Fig. 1C).
Supplementary Fig. S2) and ERK activity (Fig. 1A), suggesting that the induced pleiotropic spindle abnormalities are due to the constitutively active B-RafV600E mutant rather than the higher levels of B-Raf protein being expressed.

Centrosome amplification was further quantitated in interphase cells that were immunostained with an anti-γ-tubulin antibody to assess centrosome numbers. Depending of the phase of the cell cycle, one (unduplicated) or two (duplicated) centrosomes were detected in more than 95% of the parental or vector control interphase cells (Fig. 2). In contrast, abnormal numbers of centrosomes (>2) were detected in ~30% of interphase cells for both SBcl2 and SK-MEL-5 cells transfected with GST vector (control) or GST-B-RafV600E plasmids. Representative examples of amplified centrosomes are shown in Fig. 2A and C. Thus, we conclude that oncogenic B-RafV600E induces supernumerary centrosomes in human melanoma cells.

Selective knockdown of B-RafV600E mutant reduces the incidence of mitotic abnormalities. To determine whether the mitotic abnormalities are dependent on the continued expression of the B-RafV600E mutant, a mutant-specific shRNA (pSUPER-Mut-A) was used to selectively knock down B-RafV600E levels while keeping endogenous wild-type B-Raf levels intact, as previously shown (17). SBcl2 cells selected to contain the B-RafV600E mutant were subsequently infected with pSUPER-Mut-A retrovirus, which, after 48 hours, effectively reduced B-Raf and phospho-ERK to levels comparable to that of parental SBcl2 cells (Fig. 3A). Under these conditions, a substantial reduction in amplified centrosomes (Fig. 3B) and abnormal spindles (Fig. 3C) was detected compared with B-RafV600E–expressing cells. Finally, we showed that the mitotic delay induced by the B-RafV600E mutant (30), as indicated by an increase in prometaphase cells and...
a decrease in anaphase cells, was relieved in cells treated with the B-Raf-mutant shRNA (Fig. 3D; compare B-RafV600E+/− Mut-A shRNA). Together, we conclude that persistent expression of the B-RafV600E mutant is critical to sustain centrosome amplification, mitotic delay, and spindle abnormalities in Sbc2 cells.

**Mitotic abnormalities are prevalent in melanoma cells harboring B-RafV600E mutations.** We extended our analysis of mitosis to melanoma cell lines that harbor endogenous B-RafV600E mutations (A375, SK-MEL-28, and WM1205). Interestingly, high frequencies (70–85%) of abnormal spindle morphologies and misaligned chromosomes were observed in all three B-Raf mutant melanoma cell lines but not in parental Sbc2 and SK-MEL-5 cells (Fig. 4A–C). Of the mitotic figures in the B-Raf mutant melanoma cell lines, 28% to 38% exhibited multipolar spindles associated with extra (≥2) centrosome numbers (Fig. 4C). Furthermore, knockdown of mutant B-Raf by shRNA greatly diminished the presence of supernumerary centrosomes and abnormal spindles, especially multipolar spindles, in all three melanoma cell lines (see Fig. 4C and Supplementary Fig. S3), indicating that its expression is important to sustaining these mitotic abnormalities.

To determine whether the mitotic abnormalities induced by the B-RafV600E mutant were exerted through the MEK-MAPK pathway, we treated various melanoma cells with the MEK-specific inhibitor U0126, which led to the inhibition of ERK activity (Supplementary Fig. S4). After 24 hours, mitotic cells were analyzed for spindle abnormalities and supernumerary centrosomes in the presence or absence of U0126. Treatment of B-RafV600E mutant–expressing cells with U0126 resulted in a marked reduction in supernumerary centrosomes and multipolar spindles while partially restoring normal mitotic spindles (Fig. 4D; Supplementary Fig. S4). However, irregular spindle structures (20–28% range) were only modestly reduced (∼50%) in some of the MEK-inhibited melanoma cells (A375, SK-MEL-28, and WM1205) examined, consistent with the role for MAPK activity in regulating proper spindle formation (5). Interestingly, monopolar-like spindle structures were detected in parental and B-RafV600E-expressing cells treated with U0126 (Supplementary Fig. S4A and C). Taken together, our results suggest that persistent expression of the B-RafV600E mutant and its activation of MAPK are critical for promoting and maintaining mitotic spindle abnormalities in melanoma cells.

B-RafV600E induces chromosome mis-segregation and aneuploidy in human melanoma cells. To determine whether the spindle abnormalities induced by oncogenic B-Raf give rise to chromosome segregation errors, we examined later stages (anaphase/telophase) of mitosis. In contrast to vector control cells, expression of the B-RafV600E mutant in either Sbc2 or SK-MEL-5 cells resulted in a high frequency of chromosome segregation anomalies, including lagging chromosomes and chromosome bridges (Supplementary Fig. S5A–C). Similar observations were made in A375 and SK-MEL-28 melanoma cell lines, both of which carry an activating B-RafV600E mutation (Supplementary Fig. S5A, D, and E).

It stands to reason that the high incidence of chromosome mis-segregation observed in the B-RafV600E mutant expressing cells would result in aneuploidy. To test for this directly, chromosome counts on metaphase spreads were done from vector control or B-RafV600E–expressing Sbc2 cells. A mode of 46 chromosomes was observed for vector control Sbc2 cells (Fig. 5A, top graph), indicating that most of the cells in culture are diploid. In contrast, ectopic expression of B-RafV600E in Sbc2 cells, confirmed by Western blot analysis (Fig. 5A), resulted in the absence of a chromosome mode and a wider distribution of chromosome counts (15–87 chromosomes) compared with vector control cells (Fig. 5B, bottom graph). A representative chromosome spread from vector control (46 chromosomes) or B-RafV600E mutant (40 chromosomes)
SBcl2 cells is shown alongside of the graphs (Fig. 5B). As a second approach to assess for aneuploidy, interphase FISH analysis was done on nuclei of SBcl2 cells using probes specific to chromosome 2 or 8 (Fig. 5C). A low percent of nuclei (5.5% for chromosome 2 and 9.5% for chromosome 8 probes) in vector control SBcl2 cells scored positive for aneuploidy, which is expected for an established cell line. However, the incidence of aneuploidy was markedly increased for each chromosome probe in the B-Raf V600E mutant–expressing SBcl2 cells (Fig. 5C). Based on low P values from data obtained with either chromosome probe, the increase in aneuploidy for B-Raf V600E–expressing cells compared with the background for controls was determined to be statistically highly significant (see Fig. 5 legend). Taken together, we conclude that B-Raf V600E induces aneuploidy in SBcl2 cells.

**B-Raf V600E drives aneuploidy in human melanocytes and mammary epithelial cells.** To determine whether B-Raf V600E is sufficient to induce aneuploidy in a nontransformed

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**Figure 4.** Spindle abnormalities are observed at a high frequency in human melanoma cells carrying the B-Raf V600E mutation. A, a panel of human melanoma cells during mitosis containing wild-type (WT) or mutant (V600E) B-Raf. Microtubule spindles and DNA were detected by staining with an α-tubulin antibody and DAPI, respectively. Magnification, ×63. B, percent normal versus abnormal spindles in human melanoma cells at mitosis (n = 200). C, silencing B-Raf V600E reduces the frequency of spindle abnormalities and amplified centrosomes in A375, SK-MEL-28, and WM1205lu cells (n = 200). D, MEK inhibition with 10 μmol/L U0126 reduces multipolar spindles and amplified centrosomes in melanoma cells expressing the B-Raf V600E mutant (n = 200). Representative data of three independent experiments.
background, we extended these studies to primary human melanocytes and hTERT-immortalized human mammary epithelial 1 (HME1s) cells. B-Raf\textsuperscript{V600E} was introduced into hTERT-HME1s or human melanocytes and, 96 hours later (the equivalent of two population doublings), subjected to interphase FISH analysis. A low background (4–10\%) of aneuploidy was scored in nontransfected and vector (control) cells, with the majority of these cells showing the expected two signals for each chromosome probe. Strikingly, a high frequency of aneuploidy was scored in nuclei of either hTERT-HME1s or human melanocytes transfected with B-Raf\textsuperscript{V600E} (Fig. 6A and B). In addition, many of the nuclei of cells transfected with B-Raf\textsuperscript{V600E} exhibited abnormal morphology, consistent with aneuploidy, whereas control cells contained normal round-shaped nuclei. Hence, we conclude that oncogenic B-Raf\textsuperscript{V600E} is sufficient to induce aneuploidy in both primary human melanocytes and immortalized epithelial cells.

**Discussion**

Previous work from our laboratory showed normal mitotic functions for B-Raf in human somatic cells (4) as well as a causal role for oncogenic B-Raf\textsuperscript{V600E} in promoting hyperactivation of the SAC (30). In this study, we showed that constitutive B-Raf\textsuperscript{V600E} signaling abrogates mitosis in human melanoma cells. In particular, B-Raf\textsuperscript{V600E} induced pleiotropic spindle abnormalities, supernumerary centrosomes, and chromosome mis-segregation resulting in aneuploidy. The
A persistência dessas aberrações mitóticas foi encontrada dependente da expressão contínua do mutante B-RafV600E e sua ativação constitutiva do caminho MEK/MAPK. Consistente com nossos achados, células de melanoma humanas carregando mutações endógenas B-RafV600E foram também encontradas com aberrações mitóticas semelhantes, sugerindo que os resultados de expressão ectópica do mutante B-RafV600E são relevantes de um modo fisiológico.

A amplificação do centrosoma foi geralmente não observada em células parentais SBcl2 e SK-MEL-5, mas foi facilmente observada em células de melanoma carregando mutações ativando B-Raf endógenas ou ao introduzir o mutante B-RafV600E nas células SBcl2 ou SK-MEL-5 (Fig. 1, 2, e 4). Ainda que o mecanismo pelo qual o mutante B-RafV600E induz amplificação do centrosoma não seja compreendido, uma possibilidade envolvendo p53 porque a amplificação centrosomal anormal é frequentemente associada com função prejudicada do p53 na câncer humano (27). Portanto, examinamos a resposta ao DNA de danos p53 na célula SBcl2 e SK-MEL-5. Indução de p53 e seu alvo p21 após tratamento com radiação parecem ser normais (Suplemento Fig. S6), indicando que os efeitos de signaling oncolítico B-RafV600E sobre amplificação do centrosoma não são dependentes da perda ao mesmo tempo de função do supressor tumoral p53. Além disso, expressão ectópica de B-RafV600E tinha pouco ou nenhum efeito sobre a proteína p53 ou fosforilação (resíduos Ser15 e Ser20) (Suplemento Fig. S7). Isso sugere que outros fator(es) agindo em concórdia ou downstream do signaling oncolítico B-Raf são responsáveis pela indução de amplificação centrosomal anormal em células de melanoma.

A atividade do MAPK foi encontrada importante para manutenção de amplificação do centrosoma em células de melanoma (Fig. 4D; Suplemento Fig. S4). Impressionantemente, a incidência de centrosomas supernumerários em B-RafV600E-expressing melanoma foi rapidamente reduzida dentro de 24 horas após o tratamento com o inibidor MEK U0126. Yun e colaboradores (32) forneceram efeitos semelhantes de redução de centrosomas amplificados em células tratadas com o inibidor MEK PD98059. Acredita-se que uma redução rápida em centrosomas amplificados por MEK inhibição teria sido um resultado de duplação do centrosoma sendo suprimida no absent de atividade MAPK. De fato, espinhas monopôlar contendo um único centrosoma foram observadas especificamente em células de melanoma tratadas com U0126 (ver Suplemento Fig. S4), sugerindo um possível papel no MAPK atividade em duplicação do centrosoma em células de melanoma. Alternativamente, células contendo centrosomas supernumerários podem exibir um efeito mais intenso.

Figura 6. B-RafV600E induz aneuploidia em hTERT-HMIE1s e melanócitos humanos primários. B-RafV600E ou plasmids vectoriais vazios foram transferidos em células hTERT-HMIE1 em passagem precoce (hTERT-HMIE1) ou melanócitos humanos primários com uma eficiência de transmissão de ~90% (ver Os Materiais e Métodos). Análise FISH interphase foi realizada usando sondas para cromossomos 3 (vermelho) e cromossomos 10 (verde). A. percentagem aneuploidia detectada para cromossomos individuais nos nucleos não transfetados (não TF), somente vector (vazio), e B-RafV600E (V600E) hTERT-HMIE1. Nota que ~40% dos núcleos foram detectados para aneuploidia com qualquer sonda. B. percentagem aneuploidia detectada para cromossomos individuais nos nucleos não transfetados, somente vector–transfeitado, e B-RafV600E–transfeitado human melanocytes. Note que 44% de núcleos foram positivos para aneuploidia com qualquer sonda. Representação de imagens de FISH para análise de hTERT-HMIE1 e human melanocytes são mostrados abaixo de cada gráfico. FISH analysis was calculated from at least 200 nuclei. All results are representative of three independent experiments.
sensitivity to MEK inhibition and succumb to cell death due to the mitotic stresses imposed by multipolar spindles.

Consistent with overexpression of B-RafV600E causing aneuploidy in SBCcl2 cells, melanoma cells with endogenous activating B-Raf mutations (A375 and SK-MEL-28 cells) also scored positive for aneuploidy (Supplementary Fig. S8). Thus, our studies suggest a novel link between oncogenic B-RafV600E and aneuploidy in melanoma. Although activating B-Raf mutations are found in 60% to 70% of cutaneous melanomas, alterations in chromosomal numbers are typically detected in 95% of primary and metastatic melanomas (33–35), suggesting that other genetic mutations (independent of B-Raf mutations) can contribute to aneuploidy in melanoma. For instance, activating N-Ras mutations are detected in 18% to 22% of human melanomas (36, 37) and may also have a causal role in promoting aneuploidy. Indeed, it has been known for some time that activated Ras is associated with chromosome instability in human cancer (38, 39). Interestingly, acute expression of H-RasV12 in thyroid PCCL3 cells was shown to promote centrosome amplification and defects in spindle checkpoint regulation (40, 41). However, in contrast to the spindle checkpoint effects mediated by oncogenic B-Raf in melanoma cells (30), H-RasV12 was shown to accelerate mitotic progression by overriding the SAC in a MAPK-independent manner (40). Finally, it has been shown that gene copies of cyclin-dependent kinase 4 or cyclin D (CCND1), downstream effectors of the MAPK pathway, are amplified in melanomas (42, 43) and may contribute to cell cycle defects that feed into mitotic dysfunction.

A large proportion (~82%) of benign nevi harbor activating B-Raf mutations (44), lending to the idea that B-Raf activation is an early and critical step in the development of melanocytic neoplasia. Whereas genetic evidence supports an early role for B-RafV600E in nevus formation (45), sustained B-RafV600E activity is also associated with oncogene-induced senescence (46, 47), explaining why most nevi never develop into invasive melanomas and remain dormant over long periods of time. Then, how do melanocytes harboring activated B-RafV600E mutations progress to neoplasia? The results from our study show that oncogenic B-RafV600E is sufficient to rapidly induce aneuploidy in primary human melanocytes (Fig. 6A) and raise the possibility that random mitotic errors may occur during early nevus formation. Although mitotic errors in most cells would lead to mitotic catastrophe or cell death, we speculate that induction of aneuploidy in proliferating melanocytes could allow for additional genetic changes to occur, which, if tolerated, might contribute to melanoma initiation. This would be in line with several reports showing that additional mutations in melanoma susceptibility genes (i.e., p16INK4a, ARF, and PTEN) cooperate with oncogenic B-Raf (or Ras) to allow for melanoma initiation (see review in ref. 46).

In summary, our work shows for the first time a link between the B-Raf oncogene and chromosome instability in melanoma that is explained, in part, by the ability of B-RafV600E to induce spindle abnormalities and supernumerary centrosomes. These findings have important implications for understanding the mechanisms that drive the development and progression of melanoma and may aid in developing effective therapeutic interventions that target Raf signaling and mitosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Yongping Cui, Meghan K. Borysova, Joseph O. Johnson, et al.

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