Tumor and Stem Cell Biology

Brick1 Is an Essential Regulator of Actin Cytoskeleton Required for Embryonic Development and Cell Transformation

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Abstract

Brick1 (Brk1) is the least-studied component of the Wave/Scar pathway involved in the branched nucleation of actin fibers. The clinical relevance of Brk1 is emphasized by correlative data showing that Von Hippel-Lindau (VHL) patients that also lose the BRK1 gene are protected against the development of tumors. This contrasts with recent evidence suggesting that the Wave complex may function as an invasion suppressor in epithelial cancers. Here, we show that the downregulation of Brk1 results in abnormal actin stress fiber formation and vinculin distribution and loss of Arp2/3 and Wave proteins at the cellular protrusions. Brk1 is required for cell proliferation and cell transformation by oncogenes. In addition, Brk1 downregulation results in defective directional migration and invasive growth in renal cell carcinoma cells as well as in other tumor cell types. Finally, genetic ablation of Brk1 results in dramatic defects in embryo compaction and development, suggesting an essential role for this protein in actin dynamics. Thus, genetic loss or inhibition of BRK1 is likely to be protective against tumor development due to proliferation and motility defects in affected cells. Cancer Res; 70(22); OF1–11. ©2010 AACR.

Introduction

Actin nucleation is tightly regulated by the Wiskott-Aldrich syndrome protein (WASP) family Verprolin-homologous protein (Wave)/Scar (suppressor of cyclic AMP receptor) nucleation-promoting factor (1, 2). The Wave/Scar proteins are components of the namesake multiprotein complex, which recruits actin monomers and triggers conformational changes in Arp2/3 complex, bringing its actin-related protein subunits (Arp2 and Arp3) into closer register, possibly to mimic an actin dimer. The Wave/Scar complex consists of five proteins: Sra1 (specifically Rac1-associated protein 1; also known as CYFIP1 or Pir121), Nap1 (Nck-associated protein; also known as Nap125, Nckap1, Kette or Hem2), Abi (Abl interactor), Wave (Scar), and Brick1 (abbreviated as Brk1 and also known as HSPC300, hematopoietic stem progenitor cell 300; ref. 3).

Whereas most members of the Wave/Scar complex have been widely studied in recent years, the relevance of the small protein Brk1 is mostly unknown. Brk1 was originally described as a recessive mutation in the maize orthologue that results in several morphologic defects in leaf epithelia (4). Arabidopsis Brk1 mutants show severe defects in trichome morphogenesis, where the participation of actin reorganization is well documented (5–7). RNA interference studies have suggested similar roles for Brk1 in Drosophila (8). The mammalian Brk1 orthologue (4) may also be a critical component of the activated Wave/Scar complex, and depletion of Brk1 in HeLa cells results in defective lamellipodia formation (9, 10).

Recent data have suggested an invasion suppressor role for the Wave complex (11). Depletion of Sra1, Nap1, or Wave2 perturbs actin dynamics, reduces epithelial adhesion, and leads to disorganization of tissue architecture. This invasion-suppressive activity contrasts with recent genetic evidence in Von Hippel-Lindau (VHL; OMIM#193300) patients. The human BRK1 locus (also known as C3orf10) maps close to the VHL gene (12, 13). VHL patients are predisposed to develop several tumors, including clear cell renal cell carcinomas (ccRCC). These patients usually inherit a normal chromosome 3 and a mutant chromosome with a specific mutation or deletion in 3p25.3, where the VHL gene is located (14). Recent studies in VHL patients have correlated the absence of specific pathologies, such as ccRCC or retinal angioma, with large 3p25.3 deletions that also affect BRK1 (12, 13, 15). In these “protected” patients, the spontaneous second hit (usually a
large deletion at least in ccRCC) results in the inactivation of not only the remaining VHL gene but also the remaining BRK1 gene. "Unprotected" VHL patients develop renal tumors in which at least one allele of BRK1 is conserved.

In this article, we analyze the cellular effects on eliminating Brk1 expression using RNA interference or genetic alleles and propose that Brk1 is required for cell proliferation, migration, and metastasis of tumor cells. These data support the notion that VHL patients with large 3p25.3 deletions affecting Brk1 may have better prognosis due to the inability of affected cells to properly support tumor development in the absence of this protein.

Materials and Methods

Cell culture and short hairpin RNA expression

All cell lines were obtained from the American Type Culture Collection (ATCC) and were characterized at this cell bank by morphologic and cytogenetic studies. The VHL gene was scored for mutations in renal cells as reported previously (13) and by direct sequencing to confirm known mutations. All cells were used immediately for this work after receipt from ATCC without further authentication. Proliferation and transformation assays were done as reported previously (16, 17). Silencing experiments were done by using short hairpin RNAs (shRNA; ref. 13) or small interfering RNAs (siRNAs; Dharmacon) against BRK1 transcripts. Cell movement was scored using videomicroscopy (Leica DM1600) and Metamorph software (Molecular Devices). Wound-healing assays were quantified by evaluating the wound area recovered after 16 hours using ImageJ software. Velocity and migratory directionality were determined by tracking the positions of cell nuclei (MetaMorph). Directionality results from the division of the direct distance from start point to end point between the total track distance. For migration and invasion experiments, cells were seeded into Transwell chambers covered with an 8-μm pore membrane filter (BD Biosciences). Twenty-four hours later, cells were fixed with 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI). Cell migration and/or invasion was analyzed by detection of DAPI at the top (green nuclei) and bottom (orange) compartments of the chamber using a Leica SP5-MP confocal microscope and the IMARIS software.

In vivo metastasis assays

We made tail vein injection of 5 × 10⁵ luciferase-expressing B16F10 cells in 10-week-old female CB17/1cr severe combined immunodeficient (SCID) mice (Charles River Laboratories). Whole-body optical imaging was done using β-luciferin (Promega) and the IVIS imaging system (Xenogen).

Reverse transcription-PCR and protein immunodetection

BRK1 transcripts (468 bp) were amplified using the following oligonucleotides: Brk1_F: 5′-CGTTCGATATGTCTTGT-CGT-3′ and Brk1_R, 5′-ACTTTAAGGAAAAGTTTGAGA-3′. For immunodetection, specific antibodies against the following proteins were used: Ki67 (Master Diagnostica), active caspase-3 (RyD Systems), α-tubulin (YLI/12), Vinculin (Sigma V-9131), Brk1 (a gift from Theresia Stradal, Helmholtz Zentrum fur Infektionsforschung, Munster, Germany; and Alexis Gautreau, Laboratoire d’Enzymologie et de Biochimie Structurales, Git sur Yvette, France), anti-Wave1 (BD 612276), anti-Wave2 (Santa Cruz sc-10394), anti-Arp2 (Santa Cruz sc-15389), anti-Abi1 (a kind gift of Giorgio Scita, IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy), and anti-Actin (Sigma A4700). Filamentous actin was visualized using Alexa488 conjugated phalloidin (Invitrogen).

Generation and characterization of Brk1 mutant mice

Mouse ES cells carrying a β-galactosidase gene in Brk1 intron 1 (clone DC0002; Sanger Institute) were used to generate Brk1-deficient mice. The identification of the insertion site was done by PCR amplification using several oligonucleotides in the intron 1 of Brk1 and in the neo3 gene (Supplementary Fig. S1). Fertilized embryos were collected by flushing the uteri of pregnant females from crosses between Brk1(+/−) mice, with HEPES-buffered Medium 2 (M2; Sigma) at E1.5-E2.5. Embryos were individually cultured in vitro in potassium simplex optimized medium (KSOM; Chemicon International Inc.). To allow embryo “hatching,” blastocysts were then transferred to gelatinized 96-well plates and cultured without leukemia inhibitory factor in DMEM supplemented with 15% fetal bovine serum. All animal procedures were done according to a protocol approved by the corresponding Animal Care Committee (CBBA/42; no. 139/07).

Statistical analysis

All statistical analyses were done using GraphPad Prism 4.0 software, and means ± SEM are indicated in the corresponding figures unless otherwise indicated.

Results

Brk1 downregulation induces cell morphology and proliferation defects in ccRCC cells

Human BRK1 is deleted along VHL in some VHL patients and it is thought to protect these patients from ccRCC. However, the Brk1 expression levels have not been tested in these tumor cells. We therefore quantified BRK1 mRNA levels in 15 ccRCC primary tumors. As depicted in Fig. 1A, BRK1 is expressed in all these tumors and, in fact, its expression levels are increased by 2- to 4-fold in most tumors and by more than 10-fold in two of these ccRCC. The only tumor with normal levels of expression corresponded to a mixed sarcomatoid renal tumor with a reduced (<20%) component of clear cells. About half of these primary tumors carry diverse mutations (including point mutations, small insertions, and deletions) in the VHL gene without any specific correlation with BRK1 expression levels (Fig. 1A).

BRK1 is also expressed in human ccRCC cell lines such as 786O and HTB46 (13), as well as Caki2, Sn12C, and U031 (data not shown). We therefore analyzed the consequences of limiting the expression of Brk1 in two different ccRCC cell lines using previously validated shRNAs (13). These shRNAs result in a significant downregulation (>60% in U031 and >80% in Sn12C...
Figure 1. Downregulation of Brk1 in clear cell renal carcinoma (ccRCC) cells results in proliferative and motility defects. A, BRK1 mRNA was scored by real-time reverse transcription-PCR in 15 different primary ccRCC samples and a normal kidney (N). Numbers in brackets indicate the Furhman grade. The relative levels of BRK1 mRNA are represented as a fold-increase versus the control tissue. VHL exons were sequenced in these primary tumors, and the mutations found are indicated according to the corresponding Ensembl sequence (ENSG00000134086). wt, wild-type sequence; N.T., not tested. B, reduction in BRK1 mRNA levels after expression of shBrk1 vectors as detected by real-time PCR. These data were normalized versus actin considering the endogenous expression levels in U031 cells as 1. Brk1 knockdown is also verified by immunoblot in U031 and SN12C cells stably expressing the shRNA constructs. C, stable shBrk1 SN12C clones display rounded morphology with a clear impairment in the formation of the actin stress fibers (phalloidin in red) observed in control cultures (arrows). α-Tubulin (α-tub), green; DNA (DAPI), blue. D, antiproliferative effect of shBrk1 vectors versus a scramble sequence (shCtrl) in U031 cells. DNA content was measured by propidium iodide. Representative micrographs are also shown illustrating the cell clamps formed in U031 shBrk1 cells.
of Brk1 expression (Fig. 1B). Brk1 knockdown provokes a dramatic reduction in actin stress fibers and reduced proliferation (Fig. 1C and D). These phenotypes are frequently accompanied by dramatic morphologic changes in shBrk1 cultures, which frequently display rounded clusters of cells rarely observed in control cultures (Fig. 1D and Supplementary Videos 1 and 2).

We next tested the specificity of the Brk1 downregulation phenotype in cell morphology by rescue experiments using RNAi-resistant forms of Brk1. Due to the difficulties in transfecting ccRCC cells, we first reproduced the cell morphology defects generated after Brk1 knockdown in U2OS cells. Transfection of U2OS cells with siRNAs against the coding region (siBrk1) or the 3’-untranslated region (siBrk1 3′UTR) of BRK1 transcripts results in lack of actin stress fibers and rounded cultures, similar to what was observed in ccRCC cells (Fig. 2A). Whereas control cells are scattered through the culture plate and exhibit a polarized morphology, Brk1 downregulation results in rounded cells that frequently form epithelial-like cellular groups with few or no protrusions and altered actin cytoskeleton (Fig. 2 and Supplementary Videos 3 and 4). Coexpression of a human Brk1-GFP fusion protein (sensitive to siBrk1 but resistant to siBrk1-3′UTR) results in the recovery of actin stress fibers in siBrk1-3′UTR cells but not in siBrk1 cells, indicating that the defects observed after RNA interference are specifically due to Brk1 knockdown. In Brk1-deficient cells, not only Brk1 but also other Wave complex proteins are downregulated, suggesting that Brk1 is required for the stability of other Wave complex proteins as previously reported (10, 18). Downregulation of Brk1 also results in defective concentration of Arp2/3 at actin-rich protrusions (Supplementary Fig. S1), indicating that although Arp2/3 total levels are maintained (Fig. 2B), they are not properly localized in the absence of Brk1.

shBrk1 cells also display a dramatic reorganization of focal adhesions as revealed by staining of vinculin, one of the major components of these structures (Fig. 2C). Vinculin-dependent adhesion sites become larger at the cell periphery with an average area of 18.8 ± 0.78 μm² per individual focal adhesion (n = 1224 individual focal adhesions in more than 50 cells), compared with the control cells that have an average of 12.09 ± 0.47 μm² (n = 558).

**Brk1 is required for cell transformation and motility**

Similarly to ccRCC cells, downregulation of Brk1 in U2OS cells results in a significant inhibition of cell proliferation (Fig. 3A and Supplementary Fig. S2). Because VHL patients with germline loss of Brk1 seem to be protected against tumor formation, we decided to analyze the susceptibility of primary cells to malignant transformation by oncogenes after knocking down Brk1. We used primary Cdk4-R24C murine embryonic fibroblasts that have increased susceptibility to malignant transformation (17). As shown in Fig. 3B, Brk1 is required for full malignant transformation of primary cells by oncogenes such as Ras or a combination of Ras plus E1A, suggesting a protective effect of Brk1 loss against malignant transformation of primary cells. Taken together, the results obtained in ccRCC cells, U2OS, and primary cultures suggest that Brk1 is required for proper proliferation of a wide spectrum of normal and tumor cells.

To test the consequences of Brk1 depletion in cell motility, we first did a wound healing assay in vitro in different cell types. Wound closure is significantly impaired in U2OS and ccRCC shBrk1 cells, which only manage to cover <30% to 40% of the wound area whereas control cells spread over 50% to 65% of the wound (Fig. 3C). This is not likely a consequence of defective proliferation because the doubling time of these cultures is ~35 hours and the test is done only in 16 hours. Additionally, we have done the same assay in ccRCC cells that harbor VHL mutations (786O cell line) and we have observed similar defects in migration and wound recovery (Supplementary Fig. S3A), suggesting that the effect mediated by Brk1 downregulation is independent of the VHL status. Interestingly, time-lapse imaging of wound healing revealed differences in the motility of the cells located at the wound edges. To quantify these differences, U2OS cells were seeded at low confluence and allowed to migrate randomly during 16 hours (Fig. 3D). shBrk1 cells display a significantly reduced speed (21.04 ± 0.7 μm/h; n = 21) when compared with control cells (32.44 ± 2.1 μm/h; n = 25). In addition, whereas control cells are able to “travel” linear distances longer than 100 μm, shBrk1 cells only perform small random movements with a reduction in directionality (D/T ratio). Downregulation of Brk1 in ccRCC cells also results in similar defects in motility (see Supplementary Videos 1 and 2).

**Brk1 downregulation results in impaired migration, invasion, and metastatic potential**

Downregulation of Brk1 also results in similar defects in chemotactic responses on Transwell filters. U2OS or ccRCC shBrk1 cells were seeded in the upper compartment of Transwell chambers and serum-supplemented medium was used as a chemoattractant in the lower compartment. After 48 hours, almost half of the control U2OS cells were able to cross the porous membrane, whereas only 15% of shBrk1 cells were able to migrate (Fig. 4A). Similar defects were observed in ccRCC cells which displayed a significant reduction of 20% to 50% in the ratio of cells that migrated through the membrane. Downregulation of Brk1 also affects the performance of these tumor cells in invasion assays using collagen IV–coated Transwell chambers. As depicted in Fig. 4B, U2OS and ccRCC shBrk1 cells suffered a significant reduction in their invasion capacity. These faults are not due to defects in the secretion of matrix metalloproteinases and the degradation of the extracellular membrane because all these cultures displayed similar behavior in collagen degradation assays (Supplementary Fig. S3). Hence, Brk1 seems to be required for cell migration and invasion of tumor cells, likely as a consequence of its critical role in actin dynamics and a subsequent alteration in cell motility.

Finally, we did in vivo metastasis assays in mice. We generated several stable shBrk1 clones using the highly metastatic B16F10 mouse melanoma cell line, carrying a constitutive expression of the luciferase reporter gene. First, we tested these clones in vitro using wound-healing assays to confirm that Brk1 repression affects B16F10 cells in a similar manner to
Figure 2. Brk1 regulates the actin cytoskeleton and cell adhesion. A, cellular defects in U2OS cells expressing siRNAs against the coding region (siBrk1) or the 3' UTR (siBrk1-3UTR) of human Brk1. These reagents provoke changes in morphology and loss of actin stress fibers (arrows). A Brk1-GFP fusion transcript (devoid of the 3' UTR) rescues the defects observed in siBrk1-3UTR but not siBrk1 siRNAs, which also target the transcript of the fusion protein, resulting in decreased levels of the GFP signal. B, downregulation of Brk1 in U2OS cells stably expressing Brk1-specific shRNAs as verified by reverse transcription-PCR and Western blot (WB). C, shBrk1 U2OS cultures form cellular clusters with rounded morphology and cortical distribution of actin and vinculin (red; arrowheads). Details of vinculin (white insets) staining in control and shBrk1 cells suggest enlarged focal adhesions in the absence of Brk1. The area of each focal adhesion located at the cell periphery was quantified in more than 50 cells per assay. Focal adhesion sites (n = 1224) range up to an area of 250 µm² in siBrk1 cells, whereas control cells (n = 558) have a maximum area of 100 µm² (P < 0.0001; unpaired t test). Bars represent 10 μm.
ccRCC or U2OS cells and verify Brk1 downregulation by reverse transcription-PCR. Additionally, we could observe that shBrk1 B16F10 cells display as well aberrant actin stress fiber distribution (Supplementary Fig. S4). Subsequently, these cells were injected in the tail vein of CB17/Icr SCID mice, and the animals were examined weekly for luciferase signal. After 9 or 16 days, control animals showed a remarkable luciferase bioluminescent signal at the thorax, whereas mice boosted with shBrk1 cells barely showed a residual luciferase signal (Fig. 4C). In addition, the Kaplan-Meier survival curve of these mice indicates that shBrk1 cells are less aggressive in inducing the lethal pulmonary metastasis (Fig. 4D). Macroscopic (Fig. 4C) and histologic (Supplementary Fig. S4) analysis of lungs from treated animals reveal a dramatic reduction in the number of tumor masses upon Brk1 downregulation.

Taken together, these observations suggest that Brk1 is required for proper proliferation and migration of tumor cells, and suppression of Brk1 expression impairs the formation of aggressive metastatic nodules in vivo.

Brk1 is a ubiquitous protein essential for mouse development

Because large genomic deletions in VHL patients may affect the BRK1 gene, we next tested the cellular defects caused by complete genetic ablation of Brk1 using a Brk1-null allele in the mouse. Brk1(+/−) mice were generated by microinjecting Brk1(+/−) ES cells into developing blastocysts. Brk1(+/−) mice are viable and fertile and do not develop evident abnormalities during the first 18 months of life. Because the Brk1(−) mutant allele expresses...
the β-geo gene under the endogenous Brk1 transcriptional regulatory sequences (Supplementary Fig. S5), we analyzed the pattern of expression of mouse Brk1 transcripts using β-galactosidase as a reporter. Brk1 is ubiquitously expressed in the developing embryo as detected by whole-mount β-galactosidase activity at embryonic day (E)6.5 or during midgestation (Supplementary Fig. S6). At this stage, β-galactosidase is clearly detected in neuroepithelium, spinal cord, root ganglia, ependyma, vascular endothelia, fibroblasts, and heart. Similar results have been observed using in situ hybridization with specific antisense oligonucleotides (Supplementary Fig. S6). Brk1 is also expressed

Figure 4. Brk1 is required for tumor cell spreading. A, migration (Transwell) and (B) invasion (collagen-coated Transwell) assays showing significant deficiencies in U2OS, SN12C, and U031 cells after downregulation of Brk1 (shBrk1). Cells that crossed the Transwell membrane are in orange, whereas the green signal indicates cells that remain at the upper compartment. C, bioluminescent imaging of living SCID mice i.v. injected with B16F10 cells expressing a luciferase gene and a control vector or shBrk1 vectors. Quantification of the luciferase signal into living animals 9 and 16 d postinjection (Turkey’s multiple comparison test \(P < 0.001\)). Macroscopic view of the lungs taken from mice number 1, 4, and 5 at 2 wk postinjection. D, Kaplan-Meier survival curve. Median survival is 18 d in control mice and 24 d in shBrk1 mice (survival log-rank test analysis \(P < 0.005\)).
in most adult tissues with a particular overrepresentation in brain or kidney as observed by Northern blot analysis or β-galactosidase activity (Supplementary Fig. S7). A ubiquitous distribution of Brk1 transcripts is also detected, both in human and mouse, after meta-analysis of available microarray or expressed sequence tag data (Supplementary Fig. S8).

Complete genetic ablation of Brk1 induces embryonic lethality as no Brk1(−/−) mice can be detected at birth.

Figure 5. Early embryonic lethality in the absence of Brk1. A, representative images of E4.5 and E6.5 embryos in culture. Most Brk1(+/+) and Brk1(+/−) embryos hatch out of the zona pellucida producing trophoblasts that attach to the plate (open arrow), liberating the inner cell mass (asterisk). B, immunofluorescence of wild-type and Brk1-deficient embryos after 4 d in culture. Brk1(+/+) embryos display a localization of actin filaments (green) at the cellular cortex. Brk1(−/−) embryos display aberrant structures within cells that are not compacted and actin is not properly concentrated. C, an unusual E8.5 Brk1(−/−) embryo (out of 27 E8.5 embryos analyzed) was identified by PCR analysis showing abnormal size and structure. D, E7.5 Brk1(−/−) in decidua embryos (dashed yellow lines) are composed of a small and disordered mass of cells with abundant apoptotic figures (filled arrowheads). H&E, hematoxylin and eosin staining. Bars indicate 200 μm (top and left) or 50 μm (bottom right). Fluorescence analysis (right) indicates that actin (as detected with a red-tagged phalloidin) is polarized in wild-type embryos (white arrowheads) but generates a diffuse signal in Brk1-deficient embryos (bottom left and right inset). The few remaining mutant cells display positive signal (yellow arrowheads) for active caspase-3 (green; bottom right). Asterisks indicate trophoblast cells highly loaded with actin. DNA (DAPI), blue signal. Bars indicate 20 μm (upper and bottom left), 10 μm (top inset) or 50 μm (bottom right inset).
Because Brk1 is highly expressed in mouse at the blastocyst stage (Supplementary Fig. S8), we then analyzed fertilized embryos in vitro. E1.5 embryos were isolated and cultured during 6 days in vitro. By E4.5, some embryos display an abnormal phenotype with undeveloped blastocysts showing abnormal morphologies (Fig. 5A). One day later, both Brk1(+/-) and Brk1(+-) blastocysts hatched, producing a compacted inner cell mass surrounded by tropheoblasts, whereas the abnormal Brk1(-/-) blastocysts remain as a degenerated blastocyst. Interestingly, all Brk1(-/-) embryos show an aberrant actin network (Fig. 5B). Whereas in wild-type compacted blastomeres, actin is precisely localized at the cell cortex, Brk1(-/-) blastocysts display an irregular actin staining indicating the absence of proper actin fibers at the apical and basolateral boundary. Lack of the proper actin structure is likely to prevent the proper compaction of the embryos and the establishment of intercellular junctions required at the eight-cell stage as previously proposed in E-cadherin-deficient mice (19–21). Despite these severe defects in vitro, a few Brk1(-/-) embryos get implanted, although they are dramatically underdeveloped (Fig. 5C). Whereas wild-type cells are well organized into the different embryonic layers with polarized actin, Brk1(-/-) cells are disorganized and display a diffuse distribution of actin. These mutant embryos are reduced to a mass of abnormal cells with evident signs of apoptosis depicted by cleavage caspase-3 staining (Fig. 6D). All together, these results show the essential roles of Brk1 in the reorganization of the actin cytoskeleton and cell survival, at least during embryonic development.

Discussion

Despite the extensive information on the different Wave/Scar complex components, the small protein Brk1 has been largely overlooked in mammals. Brk1 is the highest conserved member of the Wave/Scar pathway (10) with clear orthologues in plants and animals but not in yeast. Brk1 is not an essential component of the Wave/Scar complex because Sra1, Nap1, Wave, and Abi can still form a complex in the absence of Brk1 (22). However, partial downregulation of Brk1 has a dramatic effect on the total levels of the components of the Wave complex (Fig. 2 and refs. 10, 23) and in the localization of Wave and Arp proteins to cellular protrusions. Brk1 is also known to be present in a large pool of free protein that associates to the Wave complex during the assembly of these macromolecular structures (10, 22).

The in vivo requirements for Wave/Scar complex proteins have revealed a rich variety of phenotypes associated to actin defects. Wave2-null embryos die by E10.5-E12.5 due to defects in cell migration and cardiovascular development (23, 24). Genetic ablation of other members of the complex, such as Nap1 or Abi, suggests similar roles for these proteins in midgestation, cytoskeleton dynamics, organogenesis, and neural biology (25–27). Lack of Brk1 results in abnormal distribution of actin at the cell cortex of blastomeres. At this stage, a morphologic reorganization called compaction results in outer polarized blastomeres assembling typical epithelial intercellular junctions rich in actin filaments at the apical pole underlying and adjacent to the contact zone between blastomeres (28). Cytochalasin D, a potent inhibitor of actin polymerization, disrupts these structures and inhibits intercellular flattening (29). Lack of Brk1 is likely to impair the establishment of specialized cellular junctions required for a fully polarized epithelium. The developmental arrest caused by Brk1 deficiency occurs earlier than in the absence of any other member of the Wave complex, a difference that may be explained by the presence of diverse paralogues for the later proteins (e.g., Wave1-3, Abi1-3, Sra1-2). In addition, Brk1 may
have additional Wave-independent roles in which the large pool of free Brk1 may be involved (22). The latter possibility is further supported by the fact that Nap1-deficient embryos develop morphogenetic defects and lethality by E9 once the epiblast and mesoderm layers are already organized despite the complete absence of Wave complexes (26).

How does Brk1 function affect tumor susceptibility in VHL patients? Approximately 20% to 30% of VHL patients have small or large (0.5 to 250 kb) germline deletions affecting VHL and, in some cases, blanking genes (15). For the tumor to develop, a spontaneous second hit occurs during the patient’s lifetime that inactivates the wild-type VHL allele. In a significant number of tumors, this second hit consists of large deletions due to Alu-mediated recombination because the BRK1 and VHL genes lie in a region of high Alu density (15). Thus, the final retention of BRK1 is mostly determined by the inherited allele. Those individuals that inherit small deletions or point mutations affecting VHL but not BRK1 (Fig. 6A) are susceptible to develop tumors upon the second hit (usually a large deletion). However, individuals that inherit large deletions affecting BRK1 and VHL (Fig. 6B) will be protected because the second hit will produce Brk1-deficient cells, which are defective in proliferation, polarization, and motility and are likely to be inefficient in tumor formation. In fact, recent analysis has shown a perfect correlation between the loss of BRK1 and protection from ccRCC in a recent survey of 127 individuals with germline VHL deletions (30).

The fact that other components of the Wave complex (e.g., Sra1) may act as invasion suppressors (11) suggests that the Wave complex may have different activities depending on the cellular scenario or the protein composition. For instance, whereas downregulation of Sra1 does not alter cell proliferation, downregulation of the close paralogue Sra2 results in a dramatic reduction in cell proliferation (11). Genetic ablation of Wave2 also results in significant deficiency in proliferation (23), and original data from other Wave complex members suggest a requirement for Wave2/3 and Abi in tumor cell migration and invasion (31–33). Thus, different sets of Wave complexes may have distinct roles in favoring proliferation or invasion. Alternatively, it is also possible that Brk1 may have additional unknown, Wave-independent functions. However, the essential roles of Brk1 in actin organization and cell function reported here, along with the recent identification of the strong requirements for other actin regulators in tumor development (34), suggests the therapeutic value of inhibiting Brk1 or actin dynamics in ccRCC or other tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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