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Tumor suppressor interactions with microtubules: keeping cell polarity and cell division on track

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Tumor suppressor proteins protect cells and tissues from malignant transformation. Among their diverse actions, many of these proteins interact with the microtubule cytoskeleton. This review focuses on the interactions of several tumor suppressors with microtubules and speculates on how disruption of microtubule-dependent processes may contribute to cancer development and spread. We conclude that several tumor suppressors stabilize microtubules and organize microtubule arrays, functions that are likely to be important in preventing tumorigenesis. How tumor suppressors link microtubule stability with cell fate, and how their mutation affects the response of cancer cells to anti-microtubule chemotherapy drugs, remains unclear; these should prove fertile areas for future research.

Normal microtubule functioning is essential for cell polarity and division

Microtubules exist in all eukaryotic cells. They form by the polymerization of α/β tubulin dimers into protofilaments, which assemble into hollow tubules. These polymers have the length and tensile strength to act as scaffolds for moving intracellular cargoes across large distances and for controlling cellular architecture. They are highly dynamic, undergoing length and organization changes on time scales ranging from seconds to hours. Most microtubule length changes are accomplished by the addition and removal of tubulin dimers from the end of the tubule termed the plus end. The transitions between polymerization (lengthening) and depolymerization (shortening), referred to as dynamic instability, occur randomly, but they can be altered in response to temporal and spatial cues (Desai and Mitchison, 1997). These alterations are typically mediated by microtubule-associated proteins (MAPs). In a further layer of regulation, tubulin and MAPs are both subject to post-translational modifications (Drewes et al., 1998; Hammond et al., 2008; Howard and Hyman, 2003).

The placement of microtubule minus ends, which influences the organization of the array, is controlled by the centrosome-based microtubule-organizing center (MTOC). MTOCs are rich in the microtubule-nucleating protein γ-tubulin and are surrounded by pericentriolar material, which captures existing microtubule minus ends. The layout of microtubule arrays differs according to cell type and behavior (Fig. 1). Polarized epithelial cells – from which most cancers develop – place their centrosome just below the apical cell surface and extend a diffuse linear microtubule array towards the basal cell surface; apical and basal webs of microtubules are also present (Musch, 2004). In most epithelial cells, centrosomes also nucleate a microtubule-based cilium that protrudes upward from the apical cell surface to sense extracellular flow. Fibroblastic cells, by contrast, anchor their MTOC near the nucleus and extend a radial microtubule array outward to the cell periphery.

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Normally, cells have one MTOC that is duplicated early in mitosis. Paired MTOCs migrate to opposite sides of the nuclear envelope to form the two poles of the mitotic spindle. Once the nuclear envelope breaks down, a burst of microtubule polymerization creates the bipolar spindle. Microtubule plus ends then form an elaborate set of contacts with the chromosomes to promote their segregation.

Microtubule plus ends make connections with organelles and other intracellular structures. Proteins that recognize microtubule plus ends [plus-end tracking proteins (+TIPs)] can mediate stable or transient linkages of microtubule plus ends to vesicles, chromosomes and the cell cortex. These connections are vital for vesicle trafficking, cell polarization and migration, chromosome segregation, and spindle orientation within the cell.

Cells alter the spatial organization of their microtubule array in response to internal and external cues. Even subtle disruptions in microtubule length and organization can have profound effects on the cell and may promote cancer development (Mitchison, 1986). Several tumor suppressor proteins stabilize microtubules and control microtubule-dependent processes. Inactivation of these tumor suppressors impairs epithelial polarization and cell division through effects on microtubules, producing characteristics common to many cancers.

Changes in microtubule regulation could contribute to several tumor cell capabilities

An emerging view of cancer (Fig. 2) suggests that normal cells need only to acquire a select set of 'capabilities' to become malignant and escape from their tissue of origin. These capabilities include self-stimulation, evasion of restrictive signals, immortalization, angiogenesis and metastasis (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). Many of these abilities are acquired by genetic alterations; thus, genomic instability accelerates the acquisition of all the other capabilities (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). Changes in microtubule function could account for several of these capabilities, suggesting a common mechanism for tumor suppressors that regulate microtubules in promoting cancer development.

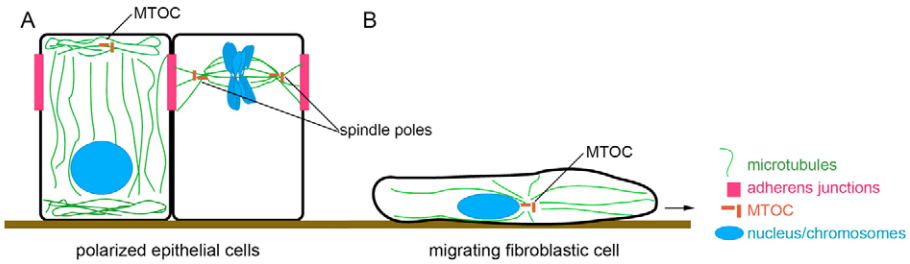

Fig. 1. Microtubule organization in epithelial cells versus fibroblastic cells. (A) Epithelial cells. The microtubule-organizing center (MTOC) is apically located during interphase. Microtubules run apico-basally and form apical and basal webs. During cell division, spindle poles extend astral microtubules to the adherens junctions to orient the spindle. (B) Migrating fibroblastic cell. The nucleus moves behind the MTOC, which extends a radial microtubule array. Microtubules extending towards the leading edge in the direction of cell migration (arrow) are selectively stabilized. Conversion from an epithelial phenotype to a more fibroblastic behavior is a component of the epithelial-to-mesenchymal transition that characterizes tumor metastasis, and misorientation of the mitotic spindle can alter the balance between stem cell replenishment and stem cell expansion.

Inactivation of some tumor suppressors destabilizes microtubules

As summarized in Table 1, five tumor suppressors [adenomatous polyposis coli (APC), Ras association domain family 1A (RASSF1A), von Hippel-Lindau (VHL), E-cadherin and merlin] stabilize microtubules in cell-based assays, and their inactivation destabilizes microtubules. An exception is liver kinase B1 (LKB1), which appears to destabilize microtubules in interphase cells and stabilize them in mitotic cells, although the number of studies looking at these roles is small. For most of these proteins, very few biochemical studies are available to guide our understanding of whether their microtubule interactions are direct or indirect. Regardless of the mechanism of microtubule binding, it is likely that most of these proteins act as weak microtubule stabilizers, and that loss of their function reduces microtubule stability to a degree that influences cellular processes but does not kill the cell.

symbol	capability	microtubule mechanism
	growth	sequestration/trafficking
	anti-growth	sequestration/trafficking
	death	sequestration/trafficking
	immortalization	
	vascularization	
	metastasis	epithelial polarization
	genomic instability	mitotic spindle dynamics

Fig. 2. Mechanisms by which tumor suppressor mutation-associated microtubule defects could contribute to tumor capabilities. Symbols and associated tumor cell capabilities are adapted from the Hanahan and Weinberg hallmarks wheel (with permission from Elsevier) (Hanahan and Weinberg, 2000), with the addition of a separate symbol for genomic instability. Where applicable, the roles microtubules play in preventing development of these capabilities are listed.

Microtubule destabilization may increase cell growth and survival signaling

Two major features of epithelial cell microtubules are their apico-basal polarization and their high degree of stability. Loss of these features may have implications for the trafficking of proteins that affect cell growth and survival. For example, receptor-mediated growth factor signaling may require an array of long, stable microtubules to traffic the receptor from the cell surface to the nucleus. In addition to serving as tracks for transport, microtubules also sequester some of the signaling proteins involved in pro- and anti-growth signaling (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). Examples of positive effects on growth signaling include activation of the epidermal growth factor receptor (EGFR) and the estrogen receptor (ER) upon microtubule destabilization (Manavathi et al., 2006). Alternatively, if the cell uses microtubules to sequester signaling proteins, microtubule destabilization could increase the nuclear translocation of these proteins, amplifying signaling cascades that increase cell proliferation (Massague and Weinberg, 1992).

Microtubules also sequester or scaffold some of the proteins involved in apoptosis. Microtubules bind to both pro- and anti-apoptotic regulators, further complicating the analysis of the net effect of microtubule destabilization on cell fate (Manavathi et al., 2006). The pro-survival protein survivin, which is upregulated in many cancers, is an example of an apoptotic regulator whose activity may be affected by changes in microtubule stability (Manavathi et al., 2006). The release of pro-apoptotic proteins into the cytoplasm could tip the balance between pro- and anti-apoptotic signaling, affecting cell fate decisions. Sorting out how microtubule destabilization contributes to apoptosis could be helpful in understanding tumor suppressor mechanisms.

Changes in microtubule organization and stability may contribute to loss of polarity and other epithelial-to-mesenchymal transition-like changes

Most tumors arise from epithelial cells, which form a cohesive sheet that lines the tissue in which they reside. During tumor progression,

Table 1. Familial syndromes associated with microtubule-interacting tumor suppressors

Tumor suppressor	Familial syndrome associated with germline mutation	Tumor types associated with familial syndrome	Role in microtubule stability and centrosome function	Role in polarization and cell migration	Role in mitosis
APC	Familial adenomatous polyposis (FAP)	Colorectum, retina, central nervous system	Stabilizes microtubules	Promotes cell migration	Prevents CIN and spindle misorientation
RASSF1A	–	Colorectum, diverse others	Stabilizes microtubules	Prevents cell migration	Prevents CIN
VHL	von Hippel-Lindau (VHL)	Kidney, retina, central nervous system, pheochromocytoma	Stabilizes microtubules and cilia	Prevents cell migration	Prevents CIN and spindle misorientation
E-cadherin	Familial diffuse gastric cancer (FDGC)	Breast (lobular), stomach	Stabilizes microtubule plus ends and minus ends	Prevents migration, promotes epithelial polarization	Prevents spindle misorientation
LKB1	Peutz-Jeghers syndrome (PJS)	–	Destabilizes microtubules in interphase, stabilizes them in mitosis	Promotes epithelial polarization	Prevents CIN and spindle misorientation
Neurofibromin/ merlin	Neurofibromatosis	Central nervous system, skin	Stabilizes microtubules	–	–
p53	Li-Fraumeni syndrome (LFS)	Numerous including carcinomas and sarcomas	Prevents centrosome overduplication	–	–
BRCA1	Familial breast and ovarian cancer	Breast, ovary, pancreas	Prevents centrosome overduplication	–	Promotes mitotic spindle pole focusing

This table lists the eight tumor suppressors discussed in the review. An associated familial syndrome has been documented for all tumor suppressors except RASSF1A, in which patients inherit a mutant version of one allele of the tumor suppressor gene. Tumors associated with the familial syndrome are listed; in most cases sporadic tumors occur in the same tissues. Exceptions are noted in the main text. The role in microtubule stabilization, cell polarity, chromosomal instability (CIN) and spindle orientation are listed for each tumor suppressor. References are in the main text.

these cells often lose their epithelial characteristics, including the active maintenance of cell-cell junctions, apico-basal polarization with directional protein sorting, and the capacity for sheet migration that preserves cell-cell and cell-matrix interactions. The epithelial cells detach from the extracellular matrix and from each other, acquire front-back polarity, and use fibroblastic-type migration to egress from the tissue of origin. This constellation of changes has been referred to as an epithelial-to-mesenchymal transition (EMT), or EMT-like phenomenon, after the developmental process (Etienne-Manneville, 2008; Klymkowsky and Savagner, 2009; Polyak and Weinberg, 2009). The EMT is seen in many tumor types, especially at the edges of the tumor, and it is thought to be instrumental in tumor metastasis. Recent evidence has linked the EMT to the acquisition of stem cell behavior (Mani et al., 2008). Indeed, genetic changes that promote such an EMT-like conversion have been shown to facilitate tumorigenesis (Bilder et al., 2000).

EMT-like processes are associated with dramatic reorganizations of the epithelial microtubule array. The linear, highly stable apico-basal array converts to a fibroblastic pattern, collecting minus ends at an MTOC near the nucleus and increasing plus-end dynamic instability. This facilitates fibroblast-type migration, with extension of a leading edge and retraction of the rear of the cell (Wen et al., 2004). Microtubules are required for both maintaining epithelial polarity and generating some of the complex EMT-associated phenotypes (Dugina et al., 1995; Ivanov et al., 2006; Ligon and Holzbaur, 2007; Meng et al., 2008; Shaw et al., 2007; Waterman-Storer et al., 2000; Yap et al., 1995; Yu et al., 2003). Thus, it is possible that changes in microtubule stability and organization that are induced by tumor suppressor mutations could promote EMT-like phenomena that are advantageous to tumor formation or metastasis.

Another way in which microtubule destabilization may contribute to an EMT is by weakening cell-cell junctions that are needed for maintaining epithelial polarity. Interestingly, several microtubule-interacting tumor suppressors localize to cell-cell junctions. E-cadherin forms a crucial homotypic adhesion molecule for these junctions (van Roy and Berx, 2008); APC, LKB1 and merlin localize to junctions; and RASSF1A and VHL have both been proposed to play a role in junction formation, the latter through controlling levels of E-cadherin and tight junction components (Calzada et al., 2006; Dallol et al., 2005; Evans et al., 2007; Harten et al., 2009). Microtubule minus and plus ends connect to cell-cell junctions to deliver junctional components and to transmit polarity information to the cell; loss of these connections has been implicated in junction disassembly (Ligon and Holzbaur, 2007; Shaw et al., 2007; Waterman-Storer et al., 2000; Yap et al., 1995). Microtubule-interacting tumor suppressors are all potential candidates to link microtubules to these junctions, and their inactivation could disrupt epithelial polarity, reduce epithelial barrier function, impair ciliogenesis and alter spindle orientation (Amin et al., 2009; Flaiz et al., 2008; Lallemand et al., 2003; Shibata et al., 1994; Yu et al., 1999).

Microtubule destabilization could also contribute to an EMT-like process by disconnecting microtubules from actin. All of the microtubule-interacting tumor suppressors discussed in this review also bind to actin or an actin regulatory protein (Dallol et al., 2005; Kamada et al., 2001; McClatchey and Fehon, 2009; Nathke, 2005; Perez-Moreno et al., 2003; Tsukita et al., 1992; Zhang et al., 2008). The role of microtubule-actin linkages in cancer protection is

unknown, but interactions between the two polymer systems are known to facilitate epithelial polarization and cell migration, as well as mitotic spindle orientation.

Small changes in microtubule regulation may impair chromosome segregation and induce aneuploidy

Probably the most obvious consequence of microtubule destabilization is its effect on mitotic spindle function. Formation of the spindle requires near-complete depolymerization of the microtubule array, followed by a burst of new polymerization. An elaborate set of microtubule plus-end interactions with the chromosomes then occurs. Mitotic spindle defects lead to chromosomal instability (CIN) and aneuploidy, which is associated with many cancer types (Pellman, 2007). Mitotic abnormalities include spindle multipolarity, chromosome misattachments and cytokinesis defects (Fig. 3).

Multipolar spindles form by centrosome overduplication or inheritance of additional centrosomes from a previous cell cycle. Some cells correct spindle multipolarity by clustering centrosomes into a single spindle pole or by expelling them from the cell; some cells with multipolar spindles fail to complete mitosis (Acilan and Saunders, 2008). For cells that do not correct spindle multipolarity, progression through mitosis produces aneuploid daughter cells (Zyss and Gergely, 2009). Multipolar spindles have been found in many tumor types as well as in early carcinoma in situ lesions, suggesting a possible contributory role to the development of invasive cancer (Lingle et al., 1998; Pihan et al., 2003).

The failure of chromosomes to attach to the spindle also causes aneuploidy. These failures arise when kinetochore microtubules cannot form stable connections to chromosomes, or when they bind to more than one chromosome (Cimini and Degrassi, 2005). Some attachment defects are sensed by the spindle assembly checkpoint, but a tumor suppressor mutation may inactivate normal checkpoint signaling and allow mitosis to continue despite these mistakes. Chromosome misattachments are seen in many tumor types and are thought by many investigators to facilitate tumorigenesis.

A final source of aneuploidy is the failure of cytokinesis following proper or improper chromosome segregation. Cytokinesis requires microtubules for both specifying the cytokinesis plane and delivering membrane components to the correct cortical site (Barr and Gruneberg, 2007). Failure to complete cytokinesis produces a single, tetraploid daughter cell. Tetraploidy, in turn, accelerates other genetic changes (Ganem et al., 2007).

The possible cellular outcomes following chromosome segregation errors include cell death, survival as an aneuploid cell and evolution with further genomic instability. Because many tumor suppressors also promote apoptosis signaling, their inactivation can lead to the dangerous combination of aneuploidy without appropriate apoptosis. The tumor suppressors discussed in this review have all been associated with one or more of these mitotic errors. Thus, their inactivation contributes to the overarching role of genomic instability in promoting cancer development.

In addition, since mitotic fidelity is so sensitive to microtubule destabilization, it may be impaired by heterozygosity for a microtubule-interacting tumor suppressor, a state that is not usually thought to promote cancer development. Such a role has been proposed for APC and may apply to other tumor suppressors as well (Nowak et al., 2002).

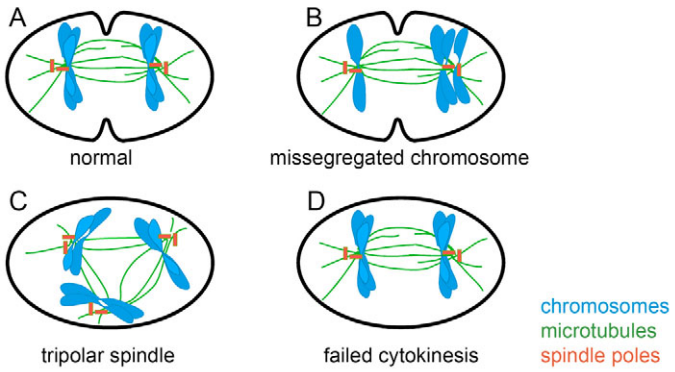

Fig. 3. Mitotic defects leading to whole-chromosome aneuploidy. (A) An anaphase cell that has undergone proper chromosome segregation and begun anaphase. (B-D) Tumor suppressor-associated mitotic spindle defects. (B) Individual chromosomes can segregate incorrectly owing to failed attachments of kinetochore microtubules. (C) Multiple chromosomes can segregate to the wrong daughter cell owing to tripolar spindle formation. (D) Cytokinesis can fail, resulting in a single, tetraploid daughter cell.

Spindle misorientation may increase the pool of tumor stem cells

A final mitotic defect caused by impaired astral microtubule function is spindle misorientation within the cell. The spindle orientation axis sets the cell division plane, which determines daughter cell inheritance and positioning. Asymmetric partitioning is usually seen in stem cell compartments and is considered to be a means of replenishing the stem cell pool. During development and injury/regeneration, a shift to symmetric division allows the stem cell compartment to expand (Morrison and Kimble, 2006). A similar switch in a tumor might also expand the stem cell population, impacting chemotherapy resistance and metastatic potential. Loss of astral microtubule attachments to the cell cortex could revert asymmetric spindle orientation to a symmetric pattern or could randomize spindle orientation, thereby increasing the cancer stem cell pool (Morrison and Kimble, 2006).

In summary, microtubule destabilization has the potential to alter some fundamental cellular processes that are known to be abnormal in cancer. These include altering cell growth and death signaling pathways, reducing cell polarity, increasing EMT-like migration, causing chromosome segregation errors, and producing spindle misorientation with expansion of the stem cell pool. Mutation of a microtubule interacting tumor suppressor would not need to cause all of these defects simultaneously; even one or two of these effects could send cell polarity or mitosis off track.

Tumor suppressors that regulate microtubule function

Despite their diverse structures and other cellular activities, several tumor suppressors with known associations to human cancer also stabilize microtubules. Reduced microtubule stability and its consequences, caused by inactivation of these proteins, may contribute to the evolution of a large percentage of human cancers.

APC

APC was initially identified as the disease gene for familial adenomatous polyposis (FAP), an inherited syndrome of early-onset colorectal polyposis (Nishisho et al., 1991). These patients develop hundreds of colonic polyps, some of which inevitably progress to carcinoma (Fodde and Khan, 1995). Families with germline APC mutations also exhibit extra-intestinal manifestations, including malignant gastrointestinal and nervous system tumors, benign congenital hypertrophy of the retinal pigment epithelium (CHRPE), osteomas, and dental and skin abnormalities. Somatic mutation of APC was subsequently shown to account for over 80% of sporadic colorectal cancers (Bodmer et al., 1989). Mutation of APC thus accounts for tens of thousands of colorectal tumors annually.

The *APC* gene encodes a ~300-kDa protein that is responsible for cell growth, cell survival, DNA repair and RNA trafficking, as described in many comprehensive reviews (Aoki and Taketo, 2007; Fearnhead et al., 2001; Nathke, 2000; Nathke, 2004; Rusan and Peifer, 2008; Sieber et al., 2001). There is a second APC homolog, APC2, which has been suggested also to have a role in tumor suppression (Jarrett et al., 2001; van Es et al., 1999). Most of the APC mutations identified from FAP families and sporadic cases are clustered in central repeat regions of APC that bind to β-catenin. APC downregulates β-catenin and prevents uncontrolled growth by reducing the transcription of c-Myc and cyclin D (He et al., 1998; Rubinfeld et al., 1996; Tetsu and McCormick, 1999; Wong and Pignatelli, 2002).

APC-truncating mutations also remove carboxy-terminal APC regions that bind to cytoskeletal components (Hanson and Miller, 2005; Nathke, 2004; Nathke, 2005). APC-microtubule interactions include the direct binding of APC to microtubules by a basic region of APC, binding of APC to the microtubule plus-end tracking protein EB1, binding of APC to the microtubule-destabilizing protein mitotic centromere-associated kinase (MCAK), and binding of APC to microtubule-based kinesin motors (Banks and Heald, 2004; Deka et al., 1998; Jimbo et al., 2002; Munemitsu et al., 1994; Smith et al., 1994; Su et al., 1995). Phosphorylation of APC by glycogen synthase kinase 3β (GSK3β) reduces its affinity for microtubules, just as phosphorylation of traditional MAPs reduces their levels of microtubule binding (Zumbrunn et al., 2001). APC is a potent microtubule stabilizer, and the addition of the C-terminal regions that are lost upon tumor-associated mutation was found to dramatically increase microtubule polymerization and stability in an in vitro assay (Deka et al., 1998; Munemitsu et al., 1994; Nakamura et al., 2001; Zumbrunn et al., 2001). APC also localizes to centrosomes, although its function there is less well studied (Louie et al., 2004).

Because most tumorigenic APC mutations simultaneously abrogate APC–β-catenin interactions and APC-microtubule binding, it has been difficult to separate these roles experimentally. A mouse harboring a truncating APC mutation distal to the β-catenin binding regions (*Apc*1638T) showed developmental abnormalities but did not spontaneously form tumors (Smits et al., 1999). By contrast, human patients with distal APC mutations that preserve β-catenin interactions but eliminate binding to cytoskeletal components develop attenuated polyposis (Resta et al., 2001). The existence of these kindreds suggests that the loss of APC-cytoskeletal interactions may be sufficient for tumorigenesis.

Intestinal epithelial cells use a form of sheet migration, maintaining apico-basal polarization as they move upward from the crypt base. Microtubules deliver APC to the leading edges of migrating cells (Mimori-Kiyosue et al., 2000; Nathke et al., 1996). In a fibroblast scratch wound assay, APC stabilized a population of microtubule ends facing the leading edges of migrating cells; in addition, cells that were transfected with tumor-associated APC mutants showed increased migration through Transwell filters (Wen et al., 2004). Forced expression of full-length APC in mouse intestines caused changes in crypt morphology that were attributed to excessive cell migration (Munemitsu et al., 1994; Wong et al., 1996). Cell migration was reduced in intestinal crypts in which APC was inactivated and in cultured cells in which APC was eliminated (Kroboth et al., 2007; Munemitsu et al., 1994; Sansom et al., 2004). These results are all consistent with a role for APC in enhancing cell migration. Cell migration depends heavily on actin polymerization at the leading edge and APC interacts with several actin-associated proteins (Iizuka-Kogo et al., 2005; Mimori-Kiyosue et al., 2007; Watanabe et al., 2004; Wen et al., 2004). Thus, APC may mediate an important microtubule-actin association during the migration process.

APC also plays a major role in chromosome segregation. Studies correlating mutations in APC with aneuploidy and CIN in tumors and cancer cell lines have shown various degrees of association (Kearney et al., 1993; Tighe et al., 2001). A role for APC mutations in impairing chromosome segregation was shown in embryonic stem cells expressing truncated APC as the sole form of the protein in the cell (Fodde et al., 2001; Kaplan et al., 2001). Based on cell culture studies, various models for the mechanics of chromosome segregation defects have been proposed, including interference with the spindle assembly checkpoint, impaired chromosome attachments to kinetochore microtubules, and a failure to correct chromosome defects (Caldwell et al., 2007; Dikovskaya et al., 2007; Draviam et al., 2006; Green et al., 2005; Zhang et al., 2007; Zhang et al., 2009). These ideas are thoughtfully discussed in a recent review (Rusan and Peifer, 2008).

APC also plays a role in orienting the spindle within the cell (Caldwell et al., 2007; Fleming et al., 2009). In fly stem cells, APC was needed for asymmetric spindle orientation and was proposed to limit the size of the stem cell compartment. In cultured mammalian tumor cells, APC RNA interference (RNAi) induced astral microtubule depolymerization, causing spindle misorientation and cytokinesis failure (Caldwell et al., 2007). In mouse intestines, the presence of mutant APC led to spindle misorientation without reducing astral microtubule length, suggesting a role in astral microtubule attachment to the cell cortex (Fleming et al., 2009). In summary, APC stabilizes microtubules and links the microtubule and actin polymer systems. Its inactivation disrupts cell migration, chromosome segregation and mitotic spindle orientation.

RASSF1A

Although no familial syndromes involving mutation of the *RASSF1* gene have been identified, epigenetic inactivation of the A isoform of *RASSF1* is one of the most common molecular events in human cancer (Donninger et al., 2007). Both promoter hypermethylation and somatic mutations have been described; hypermethylation of the *RASSF1A* promoter is well documented in small cell and non-

small cell lung cancer and other solid tumors (Donninger et al., 2007). Strong evidence for the causative role of this epigenetic inactivation in cancer was provided by the finding that targeted deletion of *Rassf1a* in mice increases the incidence of many types of spontaneous, radiation- and carcinogen-induced tumors (Tommasi et al., 2005; van der Weyden et al., 2005).

RASSF1A is one of seven isoforms of the *RASSF1* gene (*RASSF1A-G*), which itself belongs to a family of 10 genes (*RASSF1-RASSF10*). The *RASSF1A* isoform encodes a 39-kDa protein with a C-terminal Ras association domain (Donninger et al., 2007). It acts as a Ras effector, which may require heterodimerization with the related NORE1 (later designated as RASSF5) protein (Ortiz-Vega et al., 2002; Richter et al., 2009). Another major function associated with RASSF1A is regulation of the G1-S cell cycle transition through interactions with the c-Jun N-terminal kinase (JNK) pathway, the transcription factor $p120^{E4F}$ or cyclin D1 (Donninger et al., 2007). RASSF1A also acts as a pro-apoptotic factor, interacting with several apoptotic pathway components (Richter et al., 2009).

RASSF1A stabilizes microtubules. Green fluorescent protein (GFP)-RASSF1A colocalized and co-sedimented with microtubules from cell extracts, and some cancer-associated mutations of RASSF1A abolished its microtubule localization (Dallol et al., 2004; Liu et al., 2003; Rong et al., 2004). A microtubule-binding region of the RASSF1A protein has been mapped, and interaction with the microtubule-stabilizing protein MAP1B has been reported (Dallol et al., 2004; Liu et al., 2003). Overexpression of GFP-RASSF1A increased microtubule stability and prevented microtubule depolymerization by drug or cold treatment, and *RASSF1A* null fibroblasts were more sensitive than controls to anti-microtubule drugs (Liu et al., 2003; Rong et al., 2004; Vos et al., 2004).

Interestingly, the addition of RASSF1A to *RASSF1A* null cells reduced migration in a wound-healing assay, whereas RASSF1A RNAi increased cellular migration (Dallol et al., 2005). Both of these results are consistent with a role for the protein in preventing cell migration, which is the opposite effect from that seen for APC.

In mitotic cells, enhanced GFP (EGFP)-RASSF1A localized to spindle microtubules and it was retained at the spindle poles in the presence of the microtubule-depolymerizing agent nocodazole (Liu et al., 2003). Antibody staining showed the spindle pole but not the spindle microtubule association, suggesting that lower affinity microtubule binding may require overexpression of RASSF1A (Guo et al., 2007; Liu et al., 2008). RASSF1A overexpression caused an increase in monopolar spindles and cytokinesis failure (Guo et al., 2007; Liu et al., 2003), whereas RASSF1A RNAi caused a premature exit from mitosis, with multipolar spindles and misaligned and lagging chromosomes (Liu et al., 2003; Song et al., 2004). As a possible mechanism, RASSF1A is both an activator of, and a substrate for, the mitotic kinase Aurora A, which controls centrosome separation and is itself frequently upregulated in tumors (Rong et al., 2007). In support of this, a *RASSF1A* mutation that mimicked Aurora phosphorylation bypassed the mitotic delay caused by Aurora A RNAi (Song et al., 2009).

In summary, RASSF1A, like APC, stabilizes microtubules. Unlike APC, RASSF1A appears to be an inhibitor of cell migration. Its loss causes pleiotropic effects on the mitotic spindle, which could be mediated by either microtubule destabilization or effects on Aurora kinases, or both.

VHL

The *VHL* gene is mutated in the germline of patients with familial von Hippel-Lindau syndrome, which is characterized by renal cell cancer, malignancies of the cerebellum and retina, pheochromocytomas, visceral cysts and other tumors (Kaelin, 2008). Loss of heterozygosity at the *VHL* locus and *VHL* promoter methylation were subsequently shown to be responsible for a large number of sporadic kidney cancers (Nyhan et al., 2008). Thus, VHL fulfills a gatekeeper role in the renal epithelium similar to that of APC in the intestine.

VHL encodes a protein of 30 kDa (VHL_{30}), with translation from an internal initiation codon producing a 19-kDa isoform (VHL_{19}) with a truncated amino terminus. Both isoforms are often discussed together in publications, making it difficult to interpret the individual functions of the two isoforms. VHL acts as the substrate recognition component of an E3 ubiquitin ligase that promotes degradation of the transcription factor hypoxia-inducible factor 1α (HIF1α) in the presence of oxygen (Kaelin, 2007; Nyhan et al., 2008). Cells lacking VHL behave as if they were experiencing hypoxia: they upregulate HIF1, leading to expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF).

As with APC and RASSF1A, VHL stabilizes microtubules. A decade after its discovery, an immunofluorescence study using a novel antibody revealed that VHL is localized to microtubules (Hergovich et al., 2003). It is not clear why earlier studies failed to show this localization using other antibodies and GFP fusions. Potential explanations include the frequent use of immunohistochemistry (IHC, which is inadequate for imaging microtubules) and the possibility that C-terminal GFP fusions that did not localize correctly. Equally important may be a distinction between the two VHL isoforms, as only the less-abundant VHL_{30} appears to localize to microtubules (Hergovich et al., 2003). VHL_{30} was observed as being bound to microtubules in a co-pelleting assay; this binding was later shown to be mediated by the kinesin Kif3A (Hergovich et al., 2003; Lolkema et al., 2007). VHL stabilizes microtubules that are exposed to the microtubule-depolymerizing drug nocodazole, an ability that is abrogated by disease-associated VHL mutations (Hergovich et al., 2003; Hergovich et al., 2006). Fluorescence recovery after photobleaching (FRAP) experiments suggested that VHL might reduce the turnover of microtubules at the periphery of cultured cells (Lolkema et al., 2004). This ability of VHL to stabilize microtubules depends on two serine residues in VHL_{30} that are substrates for phosphorylation by GSK3β (Hergovich et al., 2006). In this way, VHL is similar to APC in interacting with GSK3β and similar to other microtubule-stabilizing proteins whose activities are reduced by GSK3β phosphorylation.

VHL was recently found to be required for the microtubule-based process of ciliogenesis, which is especially important in renal development, and to bind to the ciliary kinesin-2 complex (Kuehn et al., 2007; Lolkema et al., 2007; Schermer et al., 2006). A failure to support ciliogenesis correlates with disease mutations (Lutz and Burk, 2006; Thoma et al., 2007), and loss of VHL causes ciliary depletion, but only when GSK3β is also inhibited (Thoma et al., 2007). Loss of ciliogenesis might play a role in tumor development by disrupting cell polarity and altering tissue architecture.

An elevation of HIF1 caused by VHL inactivation also represses the expression of the crucial cell-cell junction protein E-cadherin,

which is itself a microtubule-interacting tumor suppressor. Increased levels of HIF also repress the expression of the tight junction components occludin and claudin (Calzada et al., 2006; Evans et al., 2007; Harten et al., 2009). The resulting loss of adherens and tight junctions may reduce epithelial polarity and contribute to an EMT-like process, further reducing microtubule stability.

A role for VHL in mitotic spindle dynamics was discovered recently. The depletion of VHL by RNAi caused chromosome segregation defects leading to whole-chromosome aneuploidy; this correlated with a reduction of the mitotic checkpoint protein Mad2 (Evans et al., 2007). VHL RNAi also caused mitotic spindle misorientation, which correlated with the loss of microtubule-stabilizing regions of the protein, and was associated with shortened astral microtubules (Evans et al., 2007).

In summary, VHL acts as a microtubule-stabilizing protein, both in the cytoplasm and the primary cilia. It maintains E-cadherin and tight junction protein levels and hence cell-cell attachment, contributing to epithelial polarity. It protects cells from aneuploidy and orients the mitotic spindle, thus potentially regulating genomic stability and the size of the stem cell pool.

E-cadherin

Hereditary mutation in the *CDH1* gene that encodes E-cadherin causes the rare hereditary diffuse gastric cancer (HDGC) syndrome (Dunbier and Guilford, 2001). These germline mutations account for about a third of hereditary gastric cancers and 1% of gastric cancers overall (Dunbier and Guilford, 2001). Affected patients are also predisposed to lobular breast cancer, and recently, a family with lobular breast cancer, but without gastric cancer, was found to harbor an E-cadherin mutation (Masciari et al., 2007). Highlighting its role in lobular breast cancer pathogenesis, E-cadherin mutations have been seen in the precursor lesion lobular carcinoma in situ (LCIS), and reduced E-cadherin expression is used clinically to help distinguish between ductal and lobular breast cancers (Cowin et al., 2005; Lerwill, 2004). Epigenetic inactivation of E-cadherin is common in multiple cancer types (Strathdee, 2002).

E-cadherin is the epithelially expressed member of the cadherin gene family, which includes many additional genes that encode cadherin proteins (Halbleib and Nelson, 2006). These proteins include N-cadherin, which is implicated in EMT-like processes. E-cadherin encodes a single-pass transmembrane protein of ~120 kDa that mediates calcium-dependent homotypic adhesion between epithelial cells (van Roy and Berx, 2008). Cadherins are needed for the formation of both adherens junctions and tight junctions between cells. Their intracellular domains coordinate actin polymerization through α-catenin; these domains also stimulate signaling pathways, the best studied of which is the regulation of Wnt signaling through interactions with β-catenin.

An appreciation of the interactions of E-cadherin with microtubules is beginning to emerge (Hall, 2009; Stehbens et al., 2009). The formation of E-cadherin-dependent cell contacts is associated with a dramatic reorganization of microtubules and changes in their dynamic properties (Bre et al., 1990; Buendia et al., 1990; Wadsworth and Bottaro, 1996). Cadherins and their associated junctional complexes can substitute for centrosomes in their ability to stabilize microtubule minus ends (Chausovsky et al., 2000). Recently, E-cadherin-dependent adherens junctions were shown to anchor microtubule minus ends and, possibly, to nucleate new microtubules at these junctions, with implications for trafficking molecules towards the junctions (Meng et al., 2008). The stabilization of minus ends converts the dominant microtubule behavior from treadmilling (continuous minus-end depolymerization coupled to plus-end polymerization) into plus-end dynamic instability (Chausovsky et al., 2000). Adherens junctions also interact independently with microtubule plus ends to enhance their stability (Waterman-Storer et al., 2000).

Loss of E-cadherin-mediated cell adhesion is thought to be a crucial step in the EMT, presumed to be a prerequisite for the development of metastasis (Schmalhofer et al., 2009). Many metastasis-associated pathways converge on the downregulation of E-cadherin expression (Cano et al., 2000; Guarino et al., 2007). Loss of E-cadherin is often most noticeable at the tumor front, where cells egress from the tumor.

A role for E-cadherin in mediating spindle orientation was described recently. A dominant negative form of E-cadherin caused spindle misorientation without disrupting epithelial junctions or polarity, but E-cadherin RNAi misoriented spindles only when another cadherin (cadherin 6) was also eliminated (den Elzen et al., 2009). Altogether, E-cadherin plays bidirectional roles in enhancing the stability of microtubule plus and minus ends, maintaining epithelial polarity, and orientating the mitotic spindle.

LKB1

LKB1, which is also called serine/threonine kinase 11 (STK11), is mutated in the familial Peutz-Jeghers syndrome (PJS). Affected individuals develop mucocutaneous pigmentation and multiple, benign hamartomatous intestinal polyps. The incidence of colorectal cancer in PJS patients is independently amplified by many-fold, and cancers of other sites including the breast, ovary, lung and testes are also increased (Hearle et al., 2006). Somatic LKB1 mutations have been identified in a subset of sporadic non-small cell lung cancers, especially in male smokers, as well as in a variety of other sporadic tumors (Giardiello et al., 2000; Ji et al., 2007; Matsumoto et al., 2007). LKB1 is thus a key tumor suppressor in several tissues.

LKB1 encodes an ~50-kDa protein with serine-threonine kinase activity (Hezel and Bardeesy, 2008). It phosphorylates downstream targets that are involved in two separate, yet linked, activities: energy utilization and cell polarity (Hezel and Bardeesy, 2008). LKB1 activates several members of the AMP-activated protein kinase (AMP kinase) family, shifting the cell from a state of ATP consumption to one of ATP production. The *C. elegans* homolog of LKB1 was identified as partitioning defective gene 4 (Par4) in a screen for mutants that failed to properly carry out asymmetric cell division in the developing embryo (Watts et al., 2000). Mammalian LKB1 is the only protein that has been shown to cause single cells to autonomously generate apico-basal polarity in the absence of cell-cell contacts (Baas et al., 2004). The AMP kinases that are downstream of LKB1 phosphorylate the mammalian target of rapamycin (mTOR), accelerating tight junction formation. Together, the overall activity of LKB1 is to increase energy production and to enhance epithelial cell polarization.

In addition to its effects on cell polarity, LKB1 was demonstrated to regulate microtubule stability through effects on proteins that control microtubule dynamics. LKB1 phosphorylates the microtubule affinity-regulating kinases (MARKs), which in turn

phosphorylate MAPs such as the stabilizing protein Tau (Kojima et al., 2007). In vitro, a cascade of phosphorylation from LKB1 to MARK2 to Tau reduced initial rates of microtubule polymerization (Kojima et al., 2007). In cultured cells, the expression of LKB1 did not alter the microtubule array, but it did suppress microtubule regrowth following nocodazole washout (Kojima et al., 2007). These results suggest that LKB1 reduces microtubule stability, which is opposite to the activity of the other tumor suppressors discussed thus far.

LKB1 also influences cell division. It colocalizes with meiotic spindles in mouse oocytes, which are tethered to the oocyte cortex (Szczepanska and Maleszewski, 2005). Mutations of the fly LKB1 homolog cause structural defects in the mitotic spindle, including polyploidy, improperly segregated chromosomes, reduced astral microtubule density, and loss of the normal asymmetry of spindle orientation – all of these phenotypes are consistent with reduced stability of spindle microtubules (Bonaccorsi et al., 2007; Lee et al., 2007). In summary, LKB1 differs from the other tumor suppressors in having more complex effects on microtubules, but it has similar roles in promoting epithelial polarization and in protecting mitotic spindle dynamics.

Neurofibromin and merlin

The neurofibromatoses are familial cancer syndromes caused by the mutation of two tumor suppressors, neurofibromatosis 1 and 2 (NF1 and NF2), which encode neurofibromin and merlin (also called schwannomin), respectively. These syndromes are associated with benign tumors of the nervous system, pigmentary lesions, malignant tumors of the peripheral nerve sheath (MPNST), gliomas and myelodysplastic syndromes of the blood (Reed and Gutmann, 2001). NF2 mutations have been found in sporadic schwannomas, meningiomas, melanomas and mesotheliomas (Reed and Gutmann, 2001; Xiao et al., 2003).

The *NF1* gene product, neurofibromin, is a 280-kDa protein that acts as a GTPase activating protein (GAP) for Ras. When neurofibromin function is reduced, Ras remains in an active state that promotes cell proliferation through growth factor pathways and MAP kinase signaling cascades (Trovo-Marqui and Tajara, 2006). The *NF2* gene product, merlin, is a 65-kDa protein with homology to the ezrin, radixin and moesin (ERM) family of proteins, which organize plasma membranes and link them to underlying cortical actin (McClatchey and Fehon, 2009; Scoles, 2008). Merlin anchors the actin cytoskeleton to the overlying membrane (Xu and Gutmann, 1998). Similar to neurofibromin, it also controls cell proliferation through a Ras mechanism. Merlin also regulates the Rho GTPase Rac, which is involved in cell-cell and cell-matrix adhesion (Xiao et al., 2003). Finally, merlin was suggested to play a role in stabilizing adherens junctions, which are needed for epithelial cell polarization (Lallemand et al., 2003). Thus, these proteins contribute to epithelial polarity.

Merlin localizes to microtubules by immunofluorescence (Gregory et al., 1993) and binds to microtubules in an in vitro assay (Muranen et al., 2007). The phosphorylation of merlin is thought to unfold it and promote its microtubule binding (Muranen et al., 2007; Scoles, 2008; Xu and Gutmann, 1998). Both neurofibromin and merlin have been co-purified from HeLa cells in large complexes with microtubule-associated kinesin-1 (Hakimi et al., 2002). Recombinant merlin increased tubulin polymerization in an in vitro assay, and merlin increased microtubule turnover as assessed by FRAP (Muranen et al., 2007). Roles for neurofibromin and merlin in the microtubule-based transport of vesicular cargoes have been proposed and remain to be tested (Hakimi et al., 2002).

Centrosome-interacting tumor suppressors: p53 and BRCA1

Two important tumor suppressor proteins, p53 and breast cancer 1 (BRCA1), bind to centrosomes and repress centrosome duplication. Their loss can promote centrosome overduplication, which can in turn lead to spindle multipolarity and aneuploidy.

Hereditary mutation of p53 causes the Li-Fraumeni cancer syndrome, and p53 mutations are among the most common cancer-associated mutations (Malkin, 1994). In addition to its well-known roles in controlling cell death and cell cycle progression, the p53 gene product also controls centrosome number. Cells lacking p53 undergo abnormal centrosome amplification by two mechanisms: cell cycle disruption and physical interaction with centrosomes (Fukasawa et al., 1996; Shinmura et al., 2007). The physical interaction prevents centrosome overduplication independently of p53 binding to DNA (Tarapore et al., 2001). Interestingly, in prolonged culture, p53 mutant cells eventually suppress this centrosome amplification, suggesting that the selective benefit of the abnormality might be transient (Chiba et al., 2000). Finally, p53 has been noted to localize to mitotic spindle poles, but defects in mitotic spindle dynamics owing to a loss of p53 have not been demonstrated (Morris et al., 2000; Tritarelli et al., 2004).

Germline mutation of the breast and ovarian cancer tumor suppressor gene *BRCA1* accounts for the familial breast and ovarian cancer syndrome. In addition to its major role in DNA repair, BRCA1 also regulates centrosome duplication. BRCA1 binds to centrosomes and mitotic spindle poles (Hsu and White, 1998); its structural partners at the centrosome include the microtubule-nucleating protein γ-tubulin and proteins that control mitotic spindle assembly (Hsu and White, 1998; Joukov et al., 2006; Sankaran et al., 2007). BRCA1 ubiquitinates γ-tubulin and prevents its over-recruitment to the centrosome (Sankaran et al., 2007; Starita et al., 2004). In its absence, cells develop supernumerary centrosomes that show an increased microtubule-nucleating capacity (Starita et al., 2004; Xu et al., 1999). This effect may be tissue specific, as it could not be produced in non-breast cell lines (Starita et al., 2004). The depletion of BRCA1 also reduced the mitotic spindle pole focusing that was induced by activated Ran in Xenopus egg extracts, a centrosome-independent form of spindle assembly (Joukov et al., 2006). These effects on spindles have the potential to promote aneuploidy.

Implications of microtubule-interacting tumor suppressor inactivation for cancer diagnosis and treatment

Ultimately, it will be useful to know whether we can exploit tumor suppressor interactions with microtubules to guide diagnostic and therapeutic practice. If changes in microtubule stability impact the evolution of a pre-neoplastic lesion to an invasive cancer, or alter the metastatic potential of a tumor, this information could inform decisions about how aggressively to treat a patient. Easy diagnostic readouts of microtubule stability will be needed, as current biochemical assays of microtubule polymerization require fresh

material, and microtubule imaging depends on sophisticated optics and special sample handling. Assaying surrogates for microtubule destabilization may be easier and more practical than assaying microtubules directly.

We also do not know whether defective microtubule regulation predicts the response of a tumor to chemotherapy. Anti-microtubule drugs are one of the largest and most effective classes of chemotherapeutic drugs. Many of the currently used anti-microtubule drugs, such as paclitaxel and vinca alkaloids, are subject to multidrug resistance efflux, further complicating the interpretation of their effects relative to microtubule regulation (Morris and Fornier, 2008). This may soon change with the introduction of the epothilones and other compounds that are not subject to drug efflux (Morris and Fornier, 2008). The ability to predict the effects of these drugs on tumor cells based on measures of existing microtubule stability in the tumor would be a major advance.

Anti-microtubule drugs are thought to work by causing mitotic arrest followed by cell death. If all anti-microtubule drugs acted by this same pathway, however, one would expect similar effects on cell death, and these are not always seen. Thus, the connections between microtubule stability and the cell death machinery need to be better understood.

Alterations in microtubule stability could either synergize with, or antagonize, anti-microtubule drugs, and mitotic spindle defects could be an asset or a liability for cell survival (Chandhok and Pellman, 2009). Recent work has begun to demonstrate that, following mitotic arrest, cell fate decisions are not only cell type and drug type specific, but also vary within a clonal cell population that has been exposed to the same drug (Brito and Rieder, 2008; Gascoigne and Taylor, 2008; Orth et al., 2008). It will thus be interesting to determine whether the response of cancer cells to anti-microtubule drugs depends on existing microtubule defects induced by tumor suppressor mutations.

Just as tumor suppressor mutations may influence the response of a cell to anti-microtubule drugs, treatment with these drugs may alter the function of a microtubule-bound tumor suppressor. Experiments to determine interactions between anti-microtubule drugs and microtubule-interacting tumor suppressors could help to narrow the gap between a fascinating biological problem and more individualized cancer therapy.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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