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Perturbation and Modulation of Microtubule Cytoskeletal Elements in Response to the Potentially Oncogenic Molecules, Survivin and P53, and Cytokinesis: A Dissertation

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“REGULATION OF MICROTUBULE DYNAMICS BY SURVIVIN INDEPENDENTLY OF AURORA B”

A Dissertation Presented

By

Jack Rosa

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“Great, but I work on the centroSOME.”

All said and done, Steve runs a great lab, fueling it with his own boundless supply of passion for research and by fostering an environment of creativity.

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Perturbation and Modulation of Microtubule Cytoskeletal Elements in Response to the Potentially Oncogenic Molecules, Survivin and P53, and Cytokinesis

A Dissertation Presented

By

JACK ROSA
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Abstract

A complex network of protein filaments collectively known as the cytoskeleton carries out several crucial cellular processes. These functions include, but are not limited to, motility, cell shape, mitosis and organelle trafficking. The cytoskeleton is also highly responsive, allowing the cell to alter its shape in response to its immediate needs and environment. One of the major components of the cytoskeleton is the microtubule network. To refer to the array of microtubules in the cell as a skeleton is a misnomer. Microtubules, by virtue of their structure and nature, are highly dynamic, continuously growing and shrinking. They also bind a variety of accessory molecules that aid in regulating and directing their dynamic activity. In this way they provide a structural basis for integral cell functions that require rapid assembly and disassembly. In some cases, perturbations of the microtubule network results in structural anomalies that lead to undesirable outcomes for the cell, namely chromosomal missegregation events and instability. The accumulation of these events may induce aneuploidy, which has been a fundamental component of tumorigenesis. This dissertation examines the role of the microtubule cytoskeleton within three distinct contexts. The first chapter investigates the association of the anti-apoptotic protein survivin with the microtubule network and its potential impact upon the cell from interphase to cytokinesis. The second chapter of this dissertation explores a little-studied, microtubule-dense organelle, referred to as the midbody, and the highly orchestrated events that take place within it during cytokinesis. The third and final chapter describes a unique experimental condition that may further our understanding of the interaction between the tumor suppressor p53 and the centrosome in cell cycle regulation and tumorigenesis.
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Introduction

The cytoskeleton is a complex network of protein filaments that play critical roles in a variety of cellular functions. In higher eukaryotic cells these functions include cell growth and division, organelle trafficking, cell division, motility and the ability of the cell to adopt a variety of shapes and interact with its environment. The diversity of functions is the result of the presence of three primary types of polymer that make up the cytoskeleton: (1) intermediate filaments provide mechanical strength and resistance to physical stressors such as shear force; (2) actin filaments can associate with the cell cortex to provide structure to the cell surface, are needed for cell locomotion and polarity, and are required for cell division during cytokinesis; (3) microtubule polymers are hollow, cylindrical structures, much more rigid than the other cytoskeletal elements (actin and intermediate filaments), and are required for the intracellular trafficking of vesicles and organelles, the segregation of DNA at mitosis and act as a structural skeleton for the cell giving it shape. The three cytoskeletal networks rely heavily upon one another and a growing list of proteins (known as plakins) that bridge these networks to integrate their functions and carry out complex cellular processes and responses (Figure 1).

Microtubules, by their very nature, are highly dynamic structures that undergo continuous assembly and disassembly in the cell. Consequently, the microtubule network is perfectly equipped to transmit signals throughout the cell and to rapidly respond to signals itself. The microtubule array, emanating from a centralized region, fills the cytoplasm, interacting with various signaling proteins and organelles. In this way, the
microtubule network provides a customizable platform that is used to orchestrate activities such as cell cycle regulation, cell division and the transmission of stress-response pathways; all of which are critical to the integrity of the cell and its progeny.

The Centrosome

The centrosome is a small (~2μM) non-membranous organelle that is most often found centrally located in the cell, near the outer-membrane of the nucleus. The primary role of the centrosome is to act as the microtubule organizing center (MTOC) in animal cells. It consists of a pair of orthogonally-oriented centrioles surrounded by an amorphous collection of proteins referred to as pericentriolar material (PCM) (Paintrand et al., 1992). The PCM itself contains the microtubule nucleating activity of the centrosome (Hannak et al., 2002) and maintains a large collection of coiled-coil, scaffold molecules (Figure 2). The centrioles within the centrosome are structurally distinct from each other and have been termed the “mother” centriole, having a set of appendages at the distal end, and the “daughter” centriole, lacking the appendages. The appendages themselves appear to be involved in microtubule anchoring (Mogensen et al., 2000), ((Piel et al., 2000). The daughter centriole eventually acquires a set of appendages by late G2 phase of the cell cycle (Figure 3).

Each mammalian somatic cell typically has a single centrosome, which duplicates in coordination with the cell cycle. At interphase, the centrosome organizes the microtubule network, reaching throughout the cell. Once in mitosis, the centrosome, already
duplicated by this time, now directs the formation of mitotic spindles by being the core component of the spindle pole. Each spindle pole is inherited by its respective daughter cell, which then becomes the single centrosome of that post-mitotic cell.

Duplication of the centrosome begins with duplication of the centriole pair (consisting of mother and daughter) in late G1/early S phase. This involves separation of the individual centrioles and the growing of a new daughter centriole, orthogonally from each. The end result is the creation of two new, semi-conservative centriole pairs. The initiation of the duplication event requires the activity of CDK2/cyclin E/A (Hinchcliffe et al., 1999), (Lacey et al., 1999), (Matsumoto et al., 1999), (Meraldi et al., 1999). The new centrioles continue to elongate through G2 by undergoing a process of maturation where they accumulate components of the PCM. The mature centrosomes subsequently become the spindle poles in mitosis (Figure 3).

The PCM represents a dynamic region where many components can move to and from the centrosome, either on microtubules or via diffusion (Zimmerman and Doxsey, 2000), (Young et al., 1999), (Kubo and Tsukita, 2003). The PCM also has a highly organized and convoluted architecture; current estimations suggest that the centrosome may harbor hundreds of proteins, including coiled-coil scaffold proteins that could provide a docking platform for organizing a host of regulatory molecules and activities (Andersen et al., 2003; Dictenberg et al., 1998; Moritz et al., 1995; Young et al., 1999). This model has developed into a new understanding of the centrosome as a regulatory signal command center rather than just a microtubule-organizing center. A current model suggests that G1
arrests in the cell cycle can be triggered by disruption of the centrosome or its components. The assumption is that the centrosome’s structure is both highly organized and specific and disruption of this interconnected structure could result in perturbing the action of anchored regulatory molecules or directly induce a stress response by sensing structural and/or molecular changes (Doxsey et al., 2005a; Doxsey et al., 2005b).

Further evidence of the centrosome’s participation in regulatory pathways is derived from investigations of the tumor suppressor protein p53, which has been linked physically and functionally to the centrosome (Ciciarello et al., 2001; Morris et al., 2000; Tarapore et al., 2001; Tritarelli et al., 2004). Under normal conditions p53 is thought to play a part in the centrosome duplication cycle, linking it with the DNA replication cycle, which is, in part, initiated by late G1 activation of CDK2/cyclin E. The active CDK2/ Cyclin E complex is kept in check (especially in the event of a cellular insult) by p53 via its downstream targets, which include the potent G1CDK inhibitor p21Waf1/Cip1 (Harper, 1997; Mussman et al., 2000; Tarapore et al., 2001). In this context, it is not very surprising to see that centrosome anomalies and aneuploidy have become signature features of cancers in both human and animal models (Nigg, 2002; Pihan et al., 2003). Normal tissue with functioning p53 maintains a normal cycling-diploid population with cell cycle regulated centrosome duplication. Should a centrosomal defect arise, the cell triggers a p53-dependent, G1 arrest. In the case of p53 deficiency, as is common in cancer, centrosome defects persist in cycling cells, develop abnormal spindles and, consequently, chromosomal missegregations and genetic instability (Pihan and Doxsey, 1999; Pihan et al., 1998; Pihan et al., 2001). Alternatively, a cytokinesis defect in the absence of
functional p53 could lead to polyploid populations with supernumerary centrosomes (Gromley et al., 2003; Keryer et al., 2003). The dogma that the centrosome is a passive organelle upon which the cell cycle exerts control is being challenged. Instead, a dynamic interplay between the machinery of the cell cycle and the structure/function of the centrosome is coming to the forefront. A definitive mechanism will most likely lead to an expansion of the current list of centrosome-associated molecules but will benefit even more from a better understanding of the organization and interactions of those molecules at the centrosome.

**Microtubule Dynamics**

The microtubule polymer is often described as a hollow, cylindrical structure having an outer diameter of 25nm with the wall thickness being approximately 5nm. The polymer (or tube) is comprised of 13 protofilaments, each comprised of heterodimeric tubulin subunits formed from tightly bound α-tubulin and β-tubulin monomer. Microtubules exhibit a distinct polarity, having a plus (+) and a minus (-) end caused by the repetitive and parallel orientation of the tubulin subunits, where the α-tubulin subunit is oriented towards the minus end and the β-tubulin is oriented towards the plus end. The plus end of the microtubule is much more dynamic than the minus end and is capable of rapid polymerization of tubulin, inducing growth, or rapid depolymerization, resulting in shrinkage. The minus end is also capable of some shrinkage and growth; however, the dynamics are such that the net growth usually occurs at the plus end, while net shrinking occurs at the minus end (Cassimeris et al., 1987). The radial microtubule array seen in
most interphase cells is structured so that the plus ends are oriented outwards in the cytoplasm while the less dynamic minus ends are anchored at the centrosome (Bailly and Bornens, 1992). The intrinsic polymerization/depolymerization dynamics of microtubules is vital to their biological function. The dynamic nature of microtubules are what allows them to adopt different spatial arrangements that can also rapidly and drastically respond to cellular environments or perform essential functions.

Polymerization of dimeric tubulin subunits into microtubules occurs through nucleation-elongation pathways. In purified tubulin solutions, the time consuming step of nucleation requires the aggregation of 6 to 12 tubulin dimers into longitudinal oligomers. The oligomers rapidly assemble into “sheets” by lateral interactions with other tubulin dimers before forming a tube and rapidly giving way to microtubule formation. (Caudron et al., 2000; Flyvbjerg et al., 1996; Voter and Erickson, 1984). Fortunately, the cell maintains protein complexes that overcome the slow start of nucleation. As mentioned earlier, the microtubule nucleating activity of the centrosome is associated with the PCM, and one of its major components is another tubulin isoform, γ-tubulin (Stearns and Kirschner, 1994). In the PCM of the centrosome γ-tubulin is assembled into protein complexes known as γ-tubulin ring complexes (γ-TuRCs) (Moritz et al., 1995). In vitro studies have shown that the γ-TuRC can act as an effective nucleator of microtubule growth (Zheng et al., 1995). The prevailing model proposes that γ-TuRCs do not actually 'nucleate' microtubule assembly in the normal biochemical sense as in the purified tubulin solution. Rather, the γ-TuRC acts as a site onto which microtubules assemble (Keating and Borisy, 2000; Moritz et al., 2000). As a result, nucleation is significantly faster and microtubule growth
on the “seed” is essentially an elongation of the seed (Figure 4). Consistent with this model, the γ-TuRC is described as a ring or lock washer-shaped structure, 25 nm in diameter, that resembles a microtubule cross-section, hence its name (Zheng et al., 1995), (Figure 3). Once a short polymer-nucleus is assembled, growth of the polymer follows by the reversible, non-covalent addition of tubulin dimers.

During the elongation phase, incorporation of dimers at the growing plus end is faster than microtubule depolymerization (mostly occurring at the minus end). Eventually, a steady state is reached where the growth of polymer by α/β-dimer addition is balanced by its depolymerization back into free α/β-dimer. These activities are constantly acting at the microtubule ends resulting in a dynamic steady state marked by alternating periods of growth and shrinkage, otherwise known as dynamic instability (Figure 5). (Cassimeris et al., 1987; Desai and Mitchison, 1997; Drechsel et al., 1992; Erickson and O’Brien, 1992; Mandelkow et al., 1991; Mitchison and Kirschner, 1984; Walker et al., 1988; Walker et al., 1991). Microtubule dynamics are composed of five closely related parameters: the growth and shrinkage rate, the frequency of catastrophe and rescue and pauses. Catastrophe is defined as a sudden change from growth to rapid shrinkage. Conversely, rescue is the transition from rapid shrinking to growth. Pauses are a more subtle state where the microtubule is observed to neither shrink nor grow for a relatively significant period of time (Desai and Mitchison, 1997). Logic would dictate that the cell would constantly be growing new microtubules to replace those that have depolymerized completely, but this rationale would be far too simplistic.
Instead of being completely regulated by equilibrium states, complex dynamics are fueled by the energy of GTP hydrolysis (Figure 5). Each α-tubulin and β-tubulin monomer contains a GTP binding site. On the α-tubulin monomer the GTP-site is located at the dimer interface where it is never hydrolyzed or exchanged; β-tubulin, on the other hand, has an exposed GTP-binding site that can exchange between GTP and GDP. GTP binds the β-tubulin subunit of the free tubulin heterodimer and is hydrolyzed to GDP shortly after the dimer is incorporated into the polymer and remains bound to the tubulin. Under conditions in which the microtubule is growing rapidly, the rate of dimer addition is high and a GTP-bound tubulin dimer may be added before the GTP of the previously added dimer is hydrolyzed. This localized accumulation of GTP at the microtubule tip is dubbed the GTP-cap. (Erickson and O'Brien, 1992). Similarly, a low rate of dimer addition to the microtubule provides enough time to hydrolyze the GTP to the GDP form and the cap is lost (Figure 5). The binding, hydrolyzing of GTP to its GDP form and exchange of the nucleotide are central to the conformational flexibility of tubulin as it cycles through rounds of polymerization and depolymerization. Careful cryo-EM suggests that the GDP state of tubulin polymer exhibits two distinct kinks in its structure, the first being at the intra-dimer interface (GTP-containing α-tubulin site) and the second being a much larger kink at the inter-dimer contact (GDP-containing β-tubulin site). This exaggerated bend between GDP-dimers appears too great to accommodate the formation of lateral contacts (Wang and Nogales, 2005). This is consistent with cryo-EM studies of depolymerizing microtubules where GDP-tubulin resulted in highly curled oligomeric structures at depolymerizing microtubule ends (Muller-Reichert et al., 1998). The binding of GTP at the exchangeable site of the heterodimer is likely to reduce inter-dimer
bending by locally changing the conformation around the nucleotide at the inter-dimer interface and modifying the conformation of the α/β-subunit to strengthen lateral contacts. A GTP-cap favors microtubule growth and depolymerization follows when the cap is lost, allowing GDP-containing polymer to lose its lateral contacts (Figure 5).

Microtubule Associated Proteins

Microtubule dynamics are also regulated by extrinsic factors. Microtubules within cells grow more rapidly and undergo more catastrophes than in vitro microtubules polymerized from pure tubulin. This suggests that additional factors that promote growth or induce depolymerization are active in vivo. A wide variety of proteins (examples in Table 1) can alter the characteristic components of dynamic instability (catastrophe, rescue, growth, shrinkage and pause). It is a balance between microtubule stabilizing and destabilizing, binding along the microtubule that helps regulate the ratio of assembly to disassembly. (Andersen, 2000)

Stabilization against disassembly is induced by a large family of proteins appropriately named microtubule associated proteins (MAPs) (Mandelkow and Mandelkow, 1995). These proteins can weakly increase the polymerization rate of pure tubulin, strongly suppress catastrophes and promote rescues. The cumulative affect of these individual changes is to reduce the turnover of tubulin subunits and increase the fraction of tubulin in polymer. However, at the G2-M phase transition, microtubules undergo an extraordinary disassembly and re-organization to form a mitotic spindle. Conveniently,
MAPs are targeted by protein kinases such as p34\textsuperscript{cdk2}/CDK1 which, when in an active cyclin-B complex, are responsible for the transition into mitosis. Phosphorylation of MAPs reduces their affinity for microtubules, stripping the microtubules of any protection against disassembly the MAPs were providing (Drechsel et al., 1992; Masson and Kreis, 1995; Ookata et al., 1997).

In addition to stabilization, a growing class of proteins has been shown to induce microtubule "destabilization". Among these molecules is the mammalian mitotic centromere-associated kinesin (MCAK) (Ahmad et al., 1999; Andersen, 2000; Belmont et al., 1996; Belmont and Mitchison, 1996; Larsson et al., 1997; Marklund et al., 1994a; Marklund et al., 1994b; McNally and Vale, 1993; Walczak et al., 1996; Wordeman and Mitchison, 1995). MCAK is found throughout the cell but is concentrated at the centromeres/kinetochores and in the spindle poles. MCAK and its homologues destabilize microtubules both in cells and in vitro in an ATP dependent manner (Hunter et al., 2003; Maney et al., 2001) by binding microtubule ends and distorting the microtubule lattice, inducing the protofilaments to peel outwards. Recent studies have shown that the microtubule depolymerizing activity of MCAK is regulated by the Aurora B kinase of the chromosomal passenger complex. MCAK is negatively regulated by Aurora B phosphorylation, allowing the assembly of chromatin-induced microtubules which contribute to the formation of the mitotic spindle (Gorbsky, 2004; Sampath et al., 2004).
A more recent family of physically dynamic microtubule proteins is the end-binding protein family. The EB1 family represents a highly conserved group of proteins, from yeast to humans, which localize to the centrosome, spindle and cytoplasmic microtubules at their actively growing plus-end tips (Berrueta et al., 1998; Morrison et al., 1998). EB1 acts as an exquisite marker of dynamic microtubule growth and experimental data suggests it is involved in microtubule search and capture events (Carvalho et al., 2003b; Mimori-Kiyosue and Tsukita, 2003; Schuyler and Pellman, 2001). The association of EB1 molecules at the microtubule tip was found to be through a tread-milling effect. EB1 first associates with the MT polymerization ends and then dissociates behind the region of new growth (Mimori-Kiyosue et al., 2000; Tirnauer et al., 2002) (Figure 5). The growing list of plus-end binding proteins and the co-localization of these proteins has led to the proposal of a microtubule plus-end complex (Schroer, 2001).

Recent literature has proposed that molecules involved in the signaling pathways of apoptosis are also associated with microtubules and can influence their activity in the cell. Proteins of the Inhibitor of Apoptosis (IAP) gene family are expressed in most eukaryotic cells, and their mode of apoptosis inhibition is by preventing the maturation and proteolytic activity of initiator and effector caspases (Deveraux and Reed, 1999). The situation is further complicated by the implication of IAPs in the processes of chromosome segregation and cytokinesis (Fraser et al., 1999; Speliotes et al., 2000; Uren et al., 1999). Deletion or RNA interference (RNAi) of IAP genes in yeast and C. elegans did not directly affect apoptosis but caused mitotic abnormalities that included defective chromosome segregation and spindle midzone formation (Fraser et al., 1999; Speliotes et
Survivin is a unique member of the IAP family of proteins. It is over-expressed in the majority of human cancers and is associated with resistance to chemotherapies (Altieri, 2003; Blanc-Brude et al., 2003). By in vitro (Beltrami et al., 2004) and transgenic animal studies (Grossman et al., 2001; Okada et al., 2004) survivin has been implicated in both preserving cell viability and in the regulation of mitosis. As an inhibitor of apoptosis, survivin binds to caspases and the IAP-inhibitor Smac/Diablo (Du et al., 2000; Verhagen et al., 2000); yet survivin’s role in cell division is not fully known. The localization of survivin in the cell is somewhat controversial. It is believed to be a chromosomal passenger, localizing to kinetochores, translocating to the spindle midzone after anaphase and remaining at the midbody during cytokinesis (Skoufias et al., 2000; Speliotes et al., 2000; Uren et al., 1999; Uren et al., 2000; Wheatley et al., 2001). On the other hand, survivin has also been localized to centrosomes and spindle microtubules (Fortugno et al., 2002). In cells ectopically expressing survivin, pole-to-pole distance in mitotic cells appeared to shorten slightly in metaphase, suggesting that spindle microtubules were hyper-stabilized as seen in Taxol treated spindles (Waters et al., 1998; Yvon et al., 1999). Furthermore, in vitro studies have shown that survivin can bind polymerized tubulin with micromolar affinity (Li et al., 1998) and that a putative tubulin-binding domain exists at the C-terminal α-helix (Verdecia et al., 2000). However, the role of survivin in influencing the cytoskeleton and the consequence of that influence is not clear.

**Cytokinesis**
Cytokinesis is the final act of mitosis that ultimately results in the physical separation of a duplicating cell into two daughter cells. Currently, cytokinesis is viewed as a complex, highly orchestrated, multi-step process. The process begins in late anaphase, not long after chromosome segregation, when membrane ingression occurs at the site previously occupied by the metaphase plate. Signals, potentially mediated by the overlapping midzone of microtubules, initiate a radical reorganization of the cell cortex at the site where furrow ingression occurs (Burgess and Chang, 2005). Actin and myosin-II filaments assemble to form contractile bundles around the equatorial region of the cell. The sliding of anti-parallel actin bundles caused by myosin II induces the bundles to shorten. The overall effect resembles the tightening of a purse-string and causes the membrane to ingress (Fujiwara et al., 1978; Satterwhite and Pollard, 1992). The membrane continues to contract upon the central spindle midzone, which is dense with overlapping microtubule plus ends. The central spindle persists after anaphase well into late stage cytokinesis, and it is here that members of the chromosomal passenger complex, which include survivin and aurora B, translocate from the centromeres. Disruption of the complex leads to cytokinesis failure and regression of the cleavage furrow, causing binucleate and multinucleate cells (Bischoff and Plowman, 1999; Li, 1999; Mackay et al., 1998).

The spindle midzone is mainly comprised of overlapping, tightly bundled microtubules upon which a dynamic collection of proteins and protein complexes are organized (Figure 7A, Table 2). This structure is presumably inherited from the mitotic spindle after chromosome separation; orientation of these microtubules is such that the minus-ends
point in the direction of their respective poles with the plus-ends in the central overlapping region. The active bundling of microtubules in the midbody has been assigned to specific molecules and complexes, the centralspindlin complex and the coiled-coil protein PRC1. Centralspindlin represents a highly conserved complex composed of two proteins; (1) MKLP1 is a mitotic kinesin-like protein and (2) MgcRacGAP is a GTPase-activating protein. In vitro, the centralspindlin complex, not the individual components, has microtubule bundling activity. PRC1 is a microtubule-associated protein (MAP) with microtubule bundling capability. The kinesin-4 family member, KIF4, interacts with PRC1 and restricts it to the narrow region of the central spindle. A hierarchical organization of these components in the midzone has been difficult to define since depletion of any one of the proteins leads to loss of localization of the others (Mishima et al., 2002; Mollinari et al., 2002; Schuyler et al., 2003).

The spindle midzone persists throughout cytokinesis and is referred to as the midbody; which is a cytoplasmic bridge between the two daughter cells (Figure 7B). Due to the persistence of the microtubule bundle, it has been assumed that the microtubules themselves are stable and lack dynamic instability. Early FRAP (fluorescence rescue after photo-bleaching) analysis of midbody microtubules predicted that tubulin dynamics were much slower than those of metaphase or even interphase microtubules (Olmstead, 1998). However, the concept of the midbody that is currently coming into focus suggests that it must maintain both a temporally and structurally dynamic environment. The state of the midbody microtubules, among other components, warrants re-evaluation by means
of current improvements in cell imaging technology and the use of dynamic microtubule-reporters.

At the center of the midbody, a dense matrix of proteins, in the zone of overlapping microtubule plus ends, can be seen as a phase dense ring (Gromley et al., 2005) (Figure 7B). The “midbody ring” apparently represents a structure to which some of the central-spindle components translocate, as demonstrated by immunolocalization of MKLP1 and Centriolin (Gromley et al., 2005). Presumably, the ring represents a docking site to which components required for membrane abscission accumulate and act. Little, if anything, is known about the midbody at the moment of abscission or the fate of the midbody post-abscission.

The abscission event, the physical severing of the midbody that results in two daughter cells, is heralded by the delivery of secretory vesicles that originate from area of the Golgi and fuse in the midbody. Evidence for Golgi-derived membrane in cytokinesis was originally found in studies of C. elegans embryos treated with the drug BFA (Donaldson and Jackson, 2000; Skop et al., 2001). Late stage cytokinesis defects were observed; furrow ingression proceeded normally, but the drug prevented the terminal separation step and the furrow regressed. Since the drug did not inhibit furrow ingression, it appeared likely that the defect was caused by a specific requirement of Golgi-derived vesicles at the late midbody stage. Work from Low et al. has shown that two members of the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein
receptor) membrane fusion machinery, syntaxin 2 and endobrevin/VAMP 8 (vesicle-associated membrane protein 8) are localized to the midbody and disruption of these proteins leads to binucleate cells. Furthermore, other SNARES (snapin) and components of the exocyst complex (sec 15) are anchored at the “midbody ring” and carry out the fusion event upon delivery of membrane and separate the daughter cells (Gromley et al., 2005) (Figure 7B).

To complicate the issue further, an alternate model has been proposed which involves the endocytic pathway. The basis of this model is the dynamic localization of Rab-11, a small GTPase associated with recycling endosomes (RE) that essentially regulates the targeting and fusion of transport vesicles (Gonzalez and Scheller, 1999). At cytokinesis, RE vesicles traffic to the midbody and can be identified as such by the presence of Rab11-FIP3 and Rab11-FIP4 complexes (FIP: family of Rab11-interacting proteins) (Wilson et al., 2005). The recruitment of the Rab11 complexes is regulated by active Arf6 (a small GTPase that localizes to the midbody) and the ability of the FIP proteins to bind tightly to Arf6 in a GTP dependent manner (Figure 7).

As is usually the case, nature may already provide a plausible model for the existence of multiple membrane delivery pathways in the cell. Plant cytokinesis requires the formation of a structure called the phragmoplast that develops in the spindle midzone and is the target of vesicle fusion. With the addition of membrane, the phragmoplast expands towards the cell wall to form the cell plate and separate the daughter cells. The phragmoplast begins as the accumulation and fusion (mediated by SNARES) of Golgi-
derived transport vesicles to the midzone, and continues to grow into a tubulo-vesicular network. As the cell plate expands and matures, clathrin coated buds associate with the cell plate and clathrin coated vesicles appear around it. Presumably, endocytosis works to maintain the shape of the cell plate, removing excess membrane from the center and redistributing it to the expanding edges through a RE pathway (Camilleri et al., 2002; Hong et al., 2003; Segui-Simarro et al., 2004) (Table 2). The membranous cell plate expands eventually fusing to the parental plasma membrane, placing a cellulose-filled cell wall between the two daughter cells.
Figure 1. The Protein Filaments that Comprise the Cytoskeleton.

Each filament type (intermediate filaments, microtubules, actin filaments) is presented as an electron micrograph and as a schematic drawing. The distribution of each type of filament in interphase tissue is also shown as a schematic.
Figure 1

**INTERMEDIATE FILAMENTS**
Rope-like fibers with a diameter of 10 nm, composed of a heterogeneous family of proteins. The nuclear lamina is composed of intermediate filaments as are filaments that cross the cytoplasm, anchoring at cell junctions to provide resistance to mechanical stresses.

**ACTIN FILAMENTS**
Double-stranded helical polymers of actin protein. With a diameter of 5-9 nm and great flexibility, they form a variety of bundles, two-dimensional “sheets” and three-dimensional “gels”. Typically they concentrate at the cell cortex.

**MICROTUBULES**
Long, hollow tubes composed of α/β tubulin. Microtubules have an outer-diameter of 25 nm and are much more rigid than either intermediate or actin filaments. Typically, in interphase cells, microtubules are anchored by their “minus” end at a microtubule organizing center known as the centrosome.

Micrograph courtesy of Roger Craig

Micrograph courtesy of Richard Wade

Figure 2. The Centrosome.

A centriole pair, composed of nine triplet microtubules resulting in a typical nine-fold symmetry. Pericentriolar material (PCM) is associated with each centriole and nucleates microtubules. Only the maternal centriole has two sets of extra appendages, distal and sub-distal; the latter seems to anchor microtubules and is the site to which the centrosomal protein centriolin localizes too. A series of interconnecting fibers, different from the PCM, links the ends of the two centrioles.
Figure 2

Maternal centriole
Distal appendages
Subdistal appendages
Microtubule
PCM
Daughter centriole
Interconnecting fibers

Doxsey, 2001
Figure 3. Centrosome Duplication.

Centrioles (green), centriole appendages that mark the distal end of mature centrioles (red), and chromosomes (blue). The interphase centrosome organizes the microtubule network of the cell. As described in Figure 1, G1 centrosomes are composed of two centrioles; one centriole has sub-distal appendages that identify it as “matured” and is called the “mother” while the other centriole is dubbed the “daughter”. The two centrioles are joined by a series of interconnecting fibers. In late G1/ early S phase, duplication of the centriole pair (consisting of mother and daughter) involves separation and growing of a new daughter centriole, orthogonally, from each. The new centrioles continue to elongate through G2 undergoing a process of maturation where the centrosomes accumulate components of the PCM while the daughter centriole from G1 gains sub-distal appendages (red). Upon entering mitosis, the resulting two pairs of centrioles (or pair of centrosomes) are separated and each becomes a spindle pole in organizing the mitotic spindle. Each spindle pole is inherited by its respective daughter cell, which then becomes the single centrosome of that post-mitotic cell. Due to the nature of the centriole-duplication process, a centriole lineage can be defined, at least after one cell cycle. After a found of mitosis, one daughter cell has inherited a centrosome where one centriole can be described as a “Grandmother” (being the older centriole at the previous G1) and her own “daughter” centriole, which grew orthogonally as described above. The other daughter cell contains a centrosome composed of a “mother” centriole (having gained sub-distal appendages from the previous G2) and her own daughter centriole, which could be termed the “granddaughter”.

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Figure 3

Breakage of cohesion; centrosome maturation and separation

Centriole disengagement

Intrinsic block to reduplication

Centrosome cohesion

New centriole growth

Centriole elongation

Inhibition of de novo centrosome assembly

Tsou and Stearns, Current Opinion in Cell Biology, 2006
Figure 4. Microtubule nucleation on γ-TuRC.

The γ-TuRC, having a “lock-washer” conformation, mimics the end of a microtubule so that tubulin dimers bind to the γ-TuRC (i) and the microtubule then rapidly polymerizes (ii). The γ-tubulin would be capable of longitudinal interaction with α-tubulin (‘a’ in “i”) and laterally with both α-tubulin and β-tubulin (‘b’ and ‘c’ in “ii”).
Figure 4

Job, Valiron and Oakley, Current Opinion in Cell Biology, 2003
Figure 5. Microtubule dynamic instability.

Dynamic instability describes the coexistence of polymerizing and depolymerizing microtubule populations. GTP-tubulin is incorporated at polymerizing microtubule tips where the β-tubulin-GTP bound at the intra-dimer interface is hydrolyzed during or soon after incorporation, and Pi is subsequently released. Polymerizing microtubules infrequently shift to a depolymerization phase (catastrophe) characterized by rapid loss of GDP-tubulin subunits from the microtubule tip. Depolymerizing microtubules can also shift back to the polymerization phase (rescue). The transitions between phases are measured as frequencies (e.g. catastrophe frequency = number of catastrophes per unit time in the polymerization phase). The GTP/GDP Pi cap acts as a stabilizing structure at polymerizing tips.
Figure 5

Polymerization Phase

CATASTROPHE  

RESCUE

Depolymerization Phase

 août GTP-Tubulin  

 ø GDP-Tubulin

Figure 6. EB1 dynamics at microtubule “plus” ends.

EB1 binds laterally to microtubules and plus ends with constant dissociation, producing the observed pattern of comets or streaks transiting through the cytoplasm. Either by EB1 copolymerization with tubulin or by its recognition of a structural or chemical property of the end, EB1 accumulates in excess. Wall binding (vertical arrows) is in a steady state with dissociation, producing a faint uniform localization pattern that diminishes behind the polymerizing end. Dissociation of the incorporated EB1 shapes the tail of the comet.
Figure 7. Dynamic assembly of central-spindle/ midbody components at cytokinesis.

(A.) In post-anaphase cells, furrow ingression requires the disassembly of cortical actin and the deposition of membrane at the cleavage site. This has been observed by localization of a small GTPase involved in Recycling Endosome (RE) membrane fusion and actin remodeling, ARF6 (orange). The microtubules of the midzone, once occupied by the metaphase plate, are actively bundled together by protein complexes such as centralspindlin (a complex of two proteins: MKLP1 and MgcRacgap) and MAPs (such as PRC1 which is localized to the midbody by the kinesin KIF4) (grey, surrounding microtubule bundles of the central spindle). Another small GTPase, Rab11, regulates the targeting and fusion of transport vesicles through the endosome. In complex with FIP3, RE vesicles are trafficked to the central spindle where RE derived vesicles accumulates.

(B.) By late cytokinesis, the furrow has ingressed to meet the central spindle resulting in an intracellular bridge called the “midbody” which contains the bundle of microtubules that is the remainder of the central spindle. The central spindle appears to have poles at either end of the midbody that contain γ-tubulin (light purple). At the center of the midbody is a dense complex known as the “midbody ring” (dark purple). γ-tubulin also localizes to the ring, as do several other proteins; MKLP1, Centriolin, the secretory machinery and SNARE components. Once the membrane completes ingression ARF6 translocates to the ring and tethers RE vesicles through FIP3. In this way a concentration of RE membrane is maintained in the midbody. However, additional observations suggest that the penultimate step of cytokinesis is the asymmetric traffic of golgi-derived secretory vesicles (red) to the midbody ring. There, the fusion machinery is tethered by the centrosomal protein centriolin and abscission occurs.
Table 1. Microtubule Associated Proteins with Differing Affects on Microtubule Dynamics.

Microtubule assembly and disassembly can be regulated in vivo by trans-acting factors, microtubule-stabilizing and destabilizing proteins that bind microtubules in different ways. Stabilization can be induced by a large family of proteins known generally as MAPs. Among these proteins are MAP1, MAP2, MAP3, MAP4 and tau. MAP4 is the most abundant and ubiquitous microtubule stabilizing protein in non-neuronal cells.

Microtubule end-binding proteins represent a relatively new class of microtubule-associated protein. By either copolymerizing with tubulin subunits or associating with specific conformational changes at the microtubule “plus” end, these proteins form complexes that accumulate specifically at the polymerizing microtubule tip. These proteins may serve as attachments for dynamic microtubules to kinetochores or for interactions with the cell cortex. Microtubule-destabilizing proteins can act through a number of ways. Op18/stathmin is a small protein (19 kDa) that interacts with tubulin dimers, preventing incorporation into the polymer therefore increasing catastrophe.

Conversely, the protein katanin severs microtubules, generating new polymer-ends lacking a GTP-cap, which can lead to catastrophe. Destabilization can also be induced by molecular motors such as the mitotic, kinesin-related protein MCAK. By binding to microtubule ends, the protein can distort the microtubule lattice to promote the “peeling outward” of tubulin protofilaments resulting in catastrophe.
Table 1

<table>
<thead>
<tr>
<th>Microtubule Associated Proteins (MAPs)</th>
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</thead>
<tbody>
<tr>
<td><strong>Stabilizing MAPs</strong></td>
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<tr>
<td><strong>Microtubule end-binding proteins</strong></td>
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Table 2. Conserved Components of the Midbody Spindle in Mammals and the Phragmoplast in Plants.

In mammals, the spindle not only functions in chromosomal segregation, it also defines the division plane for cytokinesis. Non-kinetochore, overlapping microtubules reside within the intracellular bridge between dividing cells. This "midbody" maintains a dense protein matrix at the region of microtubule overlap that bundles the microtubules together (PRC1 and Kif4, MKLp1 and Mg RacGap). The density of protein also includes golgi-derived membrane and membrane-fusion proteins (syntaxin 2), suggesting that the midbody microtubules must be maintained until new membrane can be inserted at the site of abscission. Similarly in plants, cytokinesis is mediated by an array of microtubules and associated molecules, known as the phragmoplast, to form a new cell wall between daughter cells.
### Table 2

**Components of the Midbody-spindle and the Phragmoplast**

<table>
<thead>
<tr>
<th>Mammals</th>
<th>Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC1</td>
<td>MAP-65</td>
</tr>
<tr>
<td>MgcRacGAP</td>
<td>At4g24580</td>
</tr>
<tr>
<td>MKLP1/CHO1, RabK6/MKLP2</td>
<td>At1g20060, (AtT20H2.17)</td>
</tr>
<tr>
<td>AuroraB/AIM-1</td>
<td>At-Aurora 1, -2</td>
</tr>
<tr>
<td>INCENP</td>
<td>—</td>
</tr>
<tr>
<td>Survivin</td>
<td>—</td>
</tr>
<tr>
<td>Borealin/CDCa8</td>
<td>—</td>
</tr>
<tr>
<td>Cdc14a</td>
<td>—</td>
</tr>
<tr>
<td>KIF4</td>
<td>FRA1/At5g47820, At3g50170, At5g60930</td>
</tr>
<tr>
<td>BimC</td>
<td>TKRP125, DcRP120-2</td>
</tr>
<tr>
<td>PLK1-4</td>
<td>At4g24400</td>
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<tr>
<td>TACC1</td>
<td>—</td>
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<tr>
<td>ch-TOG/XMAP215</td>
<td>MOR1/GEM1</td>
</tr>
<tr>
<td>KIFC1/CHO2, KIFC2, KIFC3</td>
<td>AtK(1-4)/AtKat(A-D), AtK5, AtKCBP</td>
</tr>
<tr>
<td>EB1</td>
<td>AtEB1</td>
</tr>
<tr>
<td>TD-60</td>
<td>At1g19880</td>
</tr>
<tr>
<td>CLIP-170/Restin</td>
<td>MCLIP-170</td>
</tr>
<tr>
<td>Dynamin II</td>
<td>DRP2A, AtDRP1/Phragmoplastin, ADL1A, ADL1E</td>
</tr>
<tr>
<td>p34cdc2/CDK1</td>
<td>CDC2</td>
</tr>
<tr>
<td>Syntaxin 2</td>
<td>KNOLLE/AtSYP111</td>
</tr>
<tr>
<td>Formin/mDia</td>
<td>AtFH5</td>
</tr>
</tbody>
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Otegui, Verbrugghe and Skop, 2005
Chapter 2:

Survivin Modulates Microtubule Dynamics and Nucleation Throughout the Cell Cycle
Contributions and Acknowledgements

The data presented in Chapter 2 is published in *Molecular Biology of the Cell*: Rosa, J., Canovas, P., Islam, A., Altieri, D. C., and Doxsey, S. J. (2006). Survivin modulates microtubule dynamics and nucleation throughout the cell cycle. Mol Biol Cell 17, 1483-1493. Initial adenoviral transductions of the pAD-surivin and pAD-GFP constructs were conducted by Ashraful Islam, Ph.D.. RNAi and subsequent verification by westerns was conducted by Pedro Canovas, Ph.D.. Microinjections, live cell imaging, tissue fixation and immunofluorescence, microtubule regrowth assays, subsequent quantification and analysis of cytoskeletal elements were all conducted by Jack Rosa.
Introduction

Survivin is a member of the Inhibitor of Apoptosis (IAP) gene family (Salvesen and Duckett, 2002), which is over-expressed in nearly every human tumor and frequently associated with resistance to therapy, and unfavorable outcome (Altieri, 2003).

Experimental work carried out in vitro (Beltrami et al., 2004) and in transgenic animals (Grossman et al., 2001; Okada et al., 2004) has assigned a dual function to survivin: protection from apoptosis and regulation of cell division. While the cytoprotective function of survivin has recently come into better focus (Blanc-Brude et al., 2003; Marusawa et al., 2003) and linked to the upstream initiation of mitochondrial apoptosis (Dohi et al., 2004), the mechanism by which survivin participates in cell division is still unclear.

Although survivin-like IAP molecules in model organisms appear to participate predominantly or exclusively in cytokinesis (Speliotes et al., 2000; Uren et al., 1999), reduction or loss of survivin in mammalian cells has been associated with a panoply of cell division defects that include supernumerary centrosomes (Li et al., 1999), aberrant spindle assembly (Giodini et al., 2002), mislocalization of mitotic kinases (Wheatley et al., 2001), loss of mitotic checkpoint(s) (Lens et al., 2003), and cytokinesis failure with appearance of multinucleated cells (Li et al., 1999). Adding further complexity to its potential role in mitosis, survivin localizes to multiple sites on the mitotic apparatus, including centrosomes, microtubules of the metaphase and central spindle, kinetochores and midbodies (Fortugno et al., 2002). Previous experiments of antibody microinjection
suggested a potential role of survivin in spindle microtubule assembly, reflected in a phenotype of flattened mitotic spindles depleted of microtubules (Giodini et al., 2002). Similar observations were reported in knockout studies, and homozygous deletion of survivin resulted in nearly complete absence of mitotic spindles (Okada et al., 2004), and appearance of disorganized tubulin bundles (Uren et al., 2000). This model may fit well with the observation that survivin forms a complex with some of the chromosomal passenger proteins, notably Aurora B (Adams et al., 2001), and the more recently described Borealin/hDasa B (Gassmann et al., 2004; Sampath et al., 2004). It has been proposed that the chromosomal passenger complex is a regulator of kinetochore attachment and cytokinesis (Adams et al., 2001), and is important for bipolar spindle formation in a pathway independent of Ran-GTP involving Aurora B-dependent phosphorylation of the microtubule destabilizing Kin I kinesin, MCAK (Gassmann et al., 2004; Sampath et al., 2004). Survivin can enhance the activity of Aurora B (Bolton et al., 2002), suggesting a model whereby survivin regulates spindle formation through Aurora B.

In this study, we examined the role of survivin in microtubule dynamics and its potential dependence on the chromosomal passenger complex. Using time-lapse live imaging of two independent microtubule markers, the microtubule plus-end protein EB1 and α-tubulin, we found that survivin functions as a novel regulator of microtubule dynamics and nucleation in interphase and throughout mitosis, and that this pathway is independent of Aurora B activity.
Results

Depletion of Survivin by RNA Interference Increases Microtubule Dynamics.

Because of its reported localization to microtubules (Fortugno et al., 2002), and its ability to alter microtubule organization during mitosis (Giodini et al., 2002), we asked whether survivin modulated microtubule dynamics in living cells. We first examined microtubule dynamics in cells depleted of survivin by small interfering RNAs (siRNA). Transfection of RPE cells with a previously characterized dsRNA oligonucleotide targeting survivin (S4) (Beltrami et al., 2004) resulted in >90% reduction in survivin levels by immunoblotting 48 h later, whereas a control dsRNA oligonucleotide (VIII) had no effect on survivin levels (Figure 1A). We next examined several parameters of microtubule dynamics including microtubule growth, catastrophe (transitions from growth to shrinking), shrinking, rescue (transitions from shrinking to growth) and pause (periods between growth and shrinking) in interphase cells stably expressing GFP-labeled α-tubulin (Desai and Mitchison, 1997). We found that the average frequency of catastrophe (#/sec) and dynamicity (dimers exchanged at the tip/ lifetime of microtubule, see materials and methods, (Toso et al., 1993; Waterman-Storer et al., 2000)) were significantly higher in survivin-depleted cells (Figure 1C, E) compared with control cells (Figure 1B, D, n>25 microtubules from 5 cells, two experiments/condition); other parameters were not significantly different from controls. Each parameter of microtubule dynamics is independently presented in Table 1. Histories of growth and shrinking events of 5 microtubules per condition are shown in Figure 1B-E and in movies 1 and 2. In addition, we analyzed cultures by fluorescence microscopy with an antibody to acetylated
tubulin, a post-translationally modified tubulin found in stabilized microtubules (Bulinski et al., 1988). The acetylated tubulin signal was diminished compared with controls (Supplementary Figure 3). The decrease in acetylated tubulin staining and increased frequency of microtubule catastrophe demonstrate that survivin depletion increases microtubule dynamics.

To independently validate results obtained with GFP-α tubulin-expressing cells, we used GFP-tagged EB1 as a marker for the plus ends of growing microtubules. Recent studies have shown that GFP-EB1 accurately reflects microtubule growth rates and the number of growing microtubules, including those nucleated from centrosomes (Piehl et al., 2004; Tirnauer et al., 2004). Stable expression of GFP-EB1 in control cells (VIII) revealed GFP-EB1 foci moving outward from the centrosome as previously described (movie 3) (Piehl et al., 2004). The number of GFP-EB1 foci in survivin-depleted cells was increased compared with control cells (Figure 2A-C). When EB1 foci were collectively displayed as a single projected image in control cells, long tracks representing extended periods of microtubule growth were observed (Figure 2D, F and H, movie 3). Survivin-depleted cells expressing similar levels of GFP-EB1 (see below) had shorter EB1 tracks (Figure 2E, G, and H, movie 4). These results are consistent with an increase in the number of growing microtubules and a higher rate of catastrophe (Gliksman et al., 1993). The changes in microtubule parameters observed in survivin-depleted cells occurred in the absence of changes in total cellular tubulin levels (Supplementary Figure 4). These results confirm data from GFP-α-tubulin-expressing cells and verify that GFP-EB1 is a reliable marker for microtubule dynamics.
The increase in the number of EB1 foci demonstrated that more microtubules were present in survivin depleted cells and suggested an increase in the number of microtubules nucleated from centrosomes. To test this directly, cells were treated with nocodazole to depolymerize microtubules then washed to remove the drug and to allow regrowth of centrosomal microtubules. By counting the number of EB1 foci stained with anti-EB1 emanating from centrosomes, an accurate determination of microtubule nucleation could be determined as described (Piehl et al., 2004; Tirnauer et al., 2004). We found a significant increase in the number of EB1 foci following siRNA-mediated depletion of survivin compared to cells treated with a control siRNA (Figure 3A), demonstrating an increase in the number of centrosome-nucleated microtubules.

**Expression of Survivin Suppresses Microtubule Dynamics and Nucleation at Multiple Cell Cycle Phases.**

Based on the increase in microtubule dynamics and nucleation observed in survivin-depleted cells, we reasoned that elevated survivin levels would suppress these parameters. To test this prediction, we first examined GFP-EB1 movements in living COS-7 cells microinjected with a plasmid encoding GFP-EB1 together with a plasmid encoding HA-surivin or a control protein, β-galactosidase. As expected, multiple GFP-EB1 foci emanated from the centrosome in control β-galactosidase-expressing cells (Figure 4A, B, see movie 5) and long GFP-EB1 tracks marking EB1 movements over time were observed (Figure 4B, D). When the same cell was subsequently treated with Taxol to suppress microtubule dynamics, GFP-EB1 movements were abolished and no
GFP-EB1 foci or tracks of EB1 movements were detected (Figure 4C, see movie 6). Survivin-expressing cells revealed a phenotype similar to that of Taxol-treated cells. In many cells (~80%), no detectable GFP-EB1 foci were observed and GFP-EB1 track projections yielded little to no linear dimension (Figure 4E, see movie 7). GFP-EB1 levels achieved in these experiments were roughly similar in all cells examined (+/-11%, see below) and never approached levels known to induce microtubule changes (Ligon et al., 2003). Consistent with the decrease in the number of GFP-EB1 foci, the number of centrosomal EB1 foci and hence the number of microtubules nucleated from centrosomes, was decreased (Figure 3B).

We next examined the effect of elevated survivin levels in mitotic cells, where microtubules are more dynamic than interphase cells (Rusan et al., 2001). We used living COS-7 cells over-expressing either survivin or β-galactosidase (control), and expressing similar levels of GFP-EB1. We found that survivin-expressing cells had significantly less spindle-associated GFP-EB1 and more cytoplasmic GFP-EB1, which was reversed in control cells (supplementary Figure 1). Moreover, survivin-expressing cells showed little to no detectable microtubule growth, as most spindles (85%, n=20) contained a negligible number of organized GFP-EB1 foci (Figure 4F, G, see movie 11). While EB1 foci were present in some spindles (~15%, n = 20), their number never exceeded ~10% of control levels (Supplementary Figure 5). As expected, control cells showed multiple GFP-EB1 foci moving away from both spindle poles (Figure 4F, H, see movie 10). Results from living cells were also confirmed in fixed cells. In control experiments, fixed β-galactosidase-expressing cells contained organized bipolar spindles with numerous EB1
foci (Figure 4I-K). Conversely, fixed cells expressing survivin had little to no spindle-associated GFP EB1 foci (Figure 4L-N) and revealed small or disorganized mitotic spindles as previously reported (Giodini et al., 2002).

We next examined the effect of survivin on microtubule dynamics during cytokinesis. In control cells expressing β-galactosidase (Figure 5A-C), GFP-EB1 foci in midbodies were numerous (Figure 5A). They moved away from the center of the midbody (the zone that does not stain for microtubules at asterisk, Figure 5D, arrow, see Gromley et al., 2005) as well as toward the midbody center (Figure 5E, arrow), showing that microtubules were growing in both directions at this site (better visualized in movies 8 and 9). These data suggest that midbody microtubules are highly dynamic, of dual polarity and undergo bidirectional growth both toward and away from the midbody center. In comparison, GFP-EB1 foci in survivin-expressing cells (Fig. 5F-H) were either undetectable or diminished in number. The expressed survivin (Figure 5G) primarily accumulated with the endogenous protein at the midbody (Li et al., 1999), suggesting that this was the site of action of the ectopically expressed protein. Consistent with previous observations (Speliotes et al., 2000; Uren et al., 1999), survivin-expressing cells with reduced microtubule dynamics often failed cytokinesis and generated multinucleated cells (Supplementary Figure 6).
Expression of Survivin Stabilizes Microtubules in Interphase and Mitosis.

Because of its role in modulating microtubule dynamics, we next asked whether survivin influenced microtubule stability in fixed cell preparations. We expressed GFP tagged survivin in COS-7 cells using a replication-deficient adenovirus (GFP-survivin) (Mesri et al., 2001), and analyzed cultures by fluorescence microscopy for acetylated tubulin (see above). Interphase cells expressing GFP-survivin showed an increase in the amount of acetylated tubulin compared with GFP-expressing control cells (Figure 6A-D). In addition, cells in cytokinesis showed an increase in the amount of acetylated tubulin at midbodies when survivin levels were increased (Figure 6EH). Moreover, survivin-expressing interphase cells treated with the microtubule-depolymerizing agent nocodazole, showed increased resistance to microtubule depolymerization compared with controls (Figure 6K). Twenty min after nocodazole treatment most microtubules were depolymerized in control cells (Figure 6I, GFP), while microtubules persisted in survivin expressing cells at this time (Figure 6J) and for an additional 40 min.

Depletion or Pharmacologic Inhibition of Aurora-B Kinase Does Not Affect Microtubule Dynamics or Nucleation.

To investigate the mechanism by which survivin modulates microtubule dynamics and nucleation, we first tested whether Aurora-B perturbed microtubule dynamics when depleted by RNAi. Aurora-B depletion was achieved using siRNAs previously employed in studies to deplete Aurora-B (Hauf et al., 2003). These effectively reduced Aurora B
levels in RPE cells by 60-80 percent (Figure 7A). Reduction of Aurora B expression by siRNA was associated with formation of binucleated cells presumably due to cytokinesis failure (Figure 7B, C), in agreement with published results. However, analysis of microtubule stability using acetylated tubulin antibodies under these experimental conditions revealed no significant differences between control and Aurora B siRNA-treated cultures (Figure 7D-F). To formally test whether Aurora B suppression by siRNA affected microtubule dynamics, we used time-lapse imaging of stably transfected cells expressing GFP-labeled α-tubulin. In these experiments, the frequency of microtubule rescue and catastrophe, the duration of microtubule pause, growth and shrinking and the rate of growth and shrinking were indistinguishable from cultures treated with control (VIII) or Aurora B-directed siRNA (Figure 8A, B, Table 1). To independently validate these results, we used time-lapse imaging of microtubule growth in living cells expressing GFP-EB1. In these experiments, Aurora B suppression by siRNA did not significantly alter microtubule growth distances and the number of GFP-EB1 foci, as compared with control (VIII)-transfected cells (Figure 8C, D). We also showed that immunoprecipitation of survivin from logarithmically growing or mitotic HeLa cells did not pull down detectable Aurora B, although the survivin-binding protein Hsp 90 effectively co-immunoprecipitated with survivin (Figure 8E, synchronized, mitotic cells immunoprecipitated with survivin did not pull down detectable levels of Aurora B; Supplementary Figure 7). These biochemical experiments suggest that at least some survivin was not bound to Aurora B kinase in HeLa cells under these conditions, a result different from studies done in Xenopus extracts or cells ectopically expressing the
proteins (see Discussion) (Beardmore et al., 2004; Bolton et al., 2002; Temme et al., 2005).

Because recent data suggests that depletion of Aurora B can affect the cellular levels of survivin and vice versa (Honda et al., 2003), we used the pharmacologic agent Hesperadin to inhibit the activity of the kinase as done previously (Hauf et al., 2003). Treatment with Hesperadin dramatically reduced phosphorylation of the Aurora B target protein histone H3 compared with cells exposed to an inactive Hesperadin analog (Figure 9A). Hesperadin induced defects in spindle organization and chromosome alignment (17/17 spindles and 0/10 controls, Figure 9B-D). Spindles were usually more narrow and sometimes longer than controls and the two halves of the spindles were misoriented in that they were not aligned 180 degrees from one another but crescent-shaped. Chromosomes were often positioned outside the area occupied by spindle microtubules (Figure 9C, D). The spindle defects in Hesperadin-treated cells were distinct from those observed in survivin expressing cells, where spindles were often shortened in the pole-to-pole dimension but had normally aligned chromosomes (Figure 4G) (Giodini et al., 2002). The different spindle disruption phenotypes suggested that Aurora B and survivin affected spindles by different mechanisms, providing additional support for the idea that these proteins functioned independently and not as members of a common protein complex. Cells treated with either Hesperadin or the inactive analog showed no differences in the rates of microtubule growth or shrinking, the duration of microtubule growth, shrinking or pause, the frequency of microtubule catastrophe or rescue as collectively measured by time lapse and fixed cell imaging of GFP-α tubulin or GFP-
Moreover, Hesperadin treatment of survivin-depleted cells showed no effect on microtubule nucleation suggesting that there was no contribution of Aurora B in the context of reduced survivin (Figure 3C). This was consistent with our data showing that survivin depletion did not significantly affect Aurora B levels and vice versa (Figure 7A, see discussion). Taken together with data from the Aurora B depletion experiments, these results show that inhibition of Aurora B activity or levels has no effect on microtubule dynamics despite induction of dramatic defects in mitosis under both conditions. The microtubule dynamics and nucleation changes seen in cells with altered survivin levels appear to be independent of Aurora B and could be induced by a fraction of survivin that is not associated with the chromosomal passenger complex or by survivin within the complex.

Discussion

A Unifying Model for the Multiplicity of Survivin Phenotypes?

The work in this chapter provides new mechanistic insights into the complex functions of survivin at multiple cell cycle stages. We propose a model in which survivin modulation of microtubule dynamics and nucleation contributes to microtubule-based functions at multiple cell cycle stages and at all cellular sites to which the protein is localized. In this model, survivin could modulate the organization and/or function of mitotic spindles (Giodini et al., 2002; Li et al., 1999; Okada et al., 2004), the spindle checkpoint (Carvalho et al., 2003a; Lens et al., 2003), midbody activities (Adams et al., 2001),...
centrosome-mediated microtubule nucleation and organization (Li et al., 1999) and interphase microtubule-based processes.

Two Pathways to Regulate Microtubule Dynamics.

The mitotic function of survivin is thought to be related to its localization to kinetochores, and in particular its association with at least some “chromosomal passenger proteins” (Wheatley et al., 2001). In addition to its potential involvement in proper kinetochore attachment, central spindle formation and cytokinesis, the chromosomal passenger complex has been more recently implicated in a Ran-GTP independent pathway of bipolar spindle assembly via Aurora B inhibitory phosphorylation of the microtubule depolymerizing activity of the Kin I kinesin, MCAK. It remains possible that survivin indirectly regulates MCAK activity and therefore microtubule stability, at the kinetochore. However, our data argue for the existence of a separate, survivin selective/specific, pathway for modulating microtubule dynamics. For example, the complex could be selectively involved in modulating microtubule stability at kinetochores, while survivin could participate in more global mechanisms of spindle assembly/function, midbody function and interphase microtubule nucleation/organization. The differences in the ability to detect an interaction of Aurora B and survivin observed by different investigators will require further investigation. It is possible that these differences reflect an interaction that is labile or transient. In any case, we believe that the ability of survivin to modulate microtubule nucleation and dynamics
in an Aurora B-independent manner could be achieved whether the protein is alone or in a complex with Aurora B and other members of the chromosomal passenger complex.

Survivin-dependent Changes in Microtubule Dynamics in Interphase

An important conclusion of this study is that the effect of survivin depletion or overexpression on microtubule dynamics was not restricted to mitosis, but occurred throughout multiple cell cycle phases, including interphase. There is already convincing evidence that survivin expression may be induced outside mitosis, and this has been experimentally validated for cytokine-stimulated hematopoietic progenitors, angiogenic endothelial cells, and tumor cells where survivin is abundantly over-expressed at all cell cycle phases (Altieri, 2003). This may reflect distinct transcriptional mechanisms of survivin gene expression at different cell cycle phases, as exemplified by the recently reported role of E2F family proteins at inducing survivin expression at the G1/S transition. Accordingly, the ability of survivin to control microtubule dynamics at multiple cell cycle phases may have dramatic repercussions for cancer cells, promoting aneuploidy at cell division but also potentially altering cell polarity through disruption of microtubule dynamics and nucleation in interphase. Moreover, the interphase effects of survivin on microtubules provides additional evidence for an Aurora B-independent activity as the kinase is thought to be mitosis-specific. In summary, our data demonstrate that survivin functions in a continuum throughout multiple cell cycle phases and its role is centered on the regulation of microtubule dynamics and nucleation. The independence of this pathway from Aurora B kinase expression and activity suggests that it may
provide a novel mechanism of microtubule regulation in both mitotic and interphase
cells, and a potential critical point of intervention for molecular antagonists of survivin as
rational anti-cancer agents (Altieri, 2003). The over-expression of survivin in nearly
every human tumor underlines the importance of this endeavor.

To clarify the use of different cell lines, the initial experiments were based on survivin
overexpression in mitotic cells using EB1 as a marker for live analysis. COS-7 cells were
selected because of their response to thymidine arrest and tolerance to nuclear
microinjection of the survivin and control plasmids. Hela cell lines were used in
collaboration with the Altieri lab for transductions with adenoviral constructs and blot
analysis. Finally, the reason RPE cells were used in quantifying various microtubule
parameters was due to the access of stable cell lines that express GFP-EB1 or GFP-α-
tubulin that allowed us to do live analysis. Unfortunately, the RPE cell lines are sensitive
to physical stress and are particularly ill-suited for microinjection and, in some cases,
chemical synchronization. Finally, for the purposes of live-microscopy at mitosis, RPE
cells are particularly challenging in that they round-up considerably and are potentially
even more sensitive to fluorescent light.

It is understandable that there are caveats in comparing data obtained from RPE cells
with the EB1 dynamics observed in COS-7 cells. However, these were independent
experimental approaches that were internally controlled and provided complimentary
results. Survivin is a highly conserved IAP and the diversity and multiplicity of
experimental approaches used lend support to the conclusion that survivin may play a role in microtubule dynamics.

Materials and Methods

Cell cultures. Cervical carcinoma HeLa cells and monkey COS-7 cells were obtained from the American Type Culture Collection (ATCC) and were maintained in culture according to the manufacturer's specifications. COS-7 were routinely used in microinjection experiments due to their tolerance for the procedure while the Altieri lab has characterized and routinely conducted adenoviral transductions, RNAi and subsequent blots in Hela. Diploid, telomerase-immortalized human RPE-1 cells (hTERT-RPE-1) were obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA) (Morales et al., 1999) and stably transfected with either GFP-EB1 or GFP-α-tubulin for the purposes of RNA followed by live analysis.

Antibodies. Antibodies used were α-tubulin (Sigma, #DM1α), acetylated-tubulin (Sigma, #6-11B-1), β-galactosidase (Roche Applied Science, Indianapolis, IN, #1083104), tyrosinated-tubulin (rabbit W2) (Gurland and Gundersen, 1995), survivin (Fortugno et al., 2002), anti-EB1(BD Biosciences, Franklin Lakes, NJ, cat #610534), anti γ-tubulin (polyclonal peptide antibody raised against amino acids AATR, Covance, Princeton, NJ, HM2569) and HA (Roche Applied Science, #3F10). As secondary antibodies, we used anti-mouse cy3 (Molecular Probes, Eugene, OR), anti-rabbit cy5 or FITC (Jackson ImmunoResearch, West Grove, PA) or anti-rat cy3 (Molecular Probes).
**Microinjection and live cell imaging.** COS-7 cells were synchronized by double-thymidine block (Quintyne and Schroer, 2002) and upon release from the S phase block, were microinjected into the nucleus with plasmids containing HA-surivin or β-galactosidase (200 ng/μl) together with 25 ng/μl of an EB1-GFP plasmid (a gift from L. Cassimeris, Lehigh University, PA) using an Eppendorf transjector 5246 and Micromanipulator (Brinkmann, Westbury, NY). Cells were returned to the incubator for 8-10 h then used for live imaging or fixed and stained for other antigens as indicated using methods described previously (Gromley et al., 2003).

**Adenoviral transduction.** The replication-deficient adenoviruses encoding survivin (pAd-surivin) or GFP (pAd-GFP) were described (Mesri et al., 2001). Cells (2.5x10^7) were transduced at multiplicity of infection (m.o.i.) of 50 for 24 h at 37°C, washed and replenished with fresh growth medium for further analysis.

**Microtubule quantification in fixed cells.** Hela cells plated at comparable density were transduced with pAd-GFP or pAd-surivin, treated with 10 μM of the microtubule-depolymerizing agent nocodazole (Sigma, St. Louis, MO) and harvested at increasing time intervals between 5 and 60 min (Hergovich et al., 2003). Cells were fixed in ice-cold methanol, washed three times in PBSAT, and stained with antibody to acetylated-tubulin (Gromley et al., 2003) followed by a secondary anti-IgG conjugated to cy3 and mounting on glass slides (Prolong Antifade, Molecular Probes, Eugene, OR). Microtubule fluorescence was quantified by acquiring 13 optical sections (333±50 nm) using wide
field fluorescence microscopy (Olympus, JAPAN) and a 63X objective from 6 random fields. For individual cell measurements, we obtained fluorescence values of areas outside those containing microtubules and subtracted this background from the total value (typically 5-10% of total cellular fluorescence). The fluorescence intensity (integrated optical density, IOD) (Dictenberg et al., 1998) for every optical section in every full cell profile or within an entire population was calculated using Metamorph software (above) or IP Lab software (version 3.5.4, Scanalytics, Fairfax, VA) as described previously (Gromley et al., 2003; Purohit et al., 1999). These values were then averaged to calculate the fluorescence of the total population.

**RNA interference (RNAi).** Double stranded (ds) RNA oligonucleotides targeting survivin (S4), Aurora B or a control unrelated sequence (VIII) were described (Altieri, 2003; Beltrami et al., 2004). Cells were transfected with 50 nM of the various dsRNA oligonucleotides using oligofectamine (3 µl/well) reagent (Life Technologies, Grand Island, NY) and replenished with growth medium after 4 h. After 24-48 h, in the absence of any detectable apoptosis, cells were harvested and analyzed for reduction of survivin levels by immunoblotting, or used for live analysis of EB1-GFP or GFP-α-tubulin dynamics.

**Microtubule re-grow assay (nucleation).** Cells grown on coverslips were either transfected with the indicated dsRNA oligonucleotides or transduced with the indicated adenovirus as described above. Cells were then incubated for 4 hours in 8µM nocodazole at 37°C and then for an additional 30 minutes on ice prior to washing. Coverslips were
than washed twice in 50ml ice-cold PBS and incubated at 37°C for 2 minutes to allow microtubule growth and subsequently fixed in -20°C methanol, washed three times in PBSAT, and stained with antibodies to EB1 and γ-tubulin followed by cy3 and FITC-conjugated secondaries respectively. EB1 foci were quantified by acquiring 16 optical sections (200±50 nm) using wide field fluorescence microscopy (Olympus, JAPAN) and a 100X objective from random fields. EB1 foci were individually counted from the entire z-series and final images are presented as maximum projection of all planes used. Centrosomes containing 2 γ-tubulin foci were used. Graphs represent data taken from 25-30 cells in 2 experiments.

**Live Microscopy of GFP-EB1.** Cells (COS-7 or RPE) were plated on coverslips (25 mm diameter) and were placed in a chamber (PDMI-2; Harvard Apparatus, Holliston, MA) in complete medium with CO2 exchange (0.5 liters/min) at 37°C. Cells were imaged every 3 seconds for 2 or more min using a 100x objective on an inverted microscope (Olympus IX-70). Images were captured on a CoolSnap HQ CCD camera (Roper Scientific, Trenton, NJ). Time-lapse movies of EB1 movements were obtained. In some cases, individual images were concatenated to produce linear elements representing the total distance traveled and providing a measure of the total amount of microtubule growth during the imaging period (Metamorph software 4.6, Universal Imaging Corp., Downingtown, PA). Using similar visualization techniques, we quantified the number of growing microtubules in cells. Where indicated, taxol (10 μM, Sigma) was added to live microscopy media. Resulting movies are shown at a rate of 15 frames per second.
Time-lapse analysis of cells expressing GFP-α-tubulin. RPE cells stably expressing GFP-α-tubulin were transfected with survivin-specific S4 or control VIII dsRNA oligonucleotides, and imaged by confocal laser scanning microscopy. Microtubule growth, catastrophe, shrinking and rescue, as well as growth/catastrophe transition rates for individual microtubules were calculated from images collected as time-lapse movies from several random areas of the cytoplasm that in most cases comprised the leading edge of the cell. Average transition values were obtained from 5 microtubules. Similar results were obtained from 5 individual cells in two different experiments. For targeting of Aurora B kinase, RPE GFP-α tubulin cells were first transfected with control (VIII) or Aurora B-directed dsRNA oligonucleotide for 36 h and analyzed by Western blotting. In independent experiments, cells were treated with the Aurora B kinase inhibitor, Hesperadin (100 nM for 6 h) characterized in previous studies (Hauf et al. 2003; Sessa et al., 2005). For analysis of microtubule dynamics, cells prepared as described above were observed using an inverted Zeiss microscope equipped with a 100X, N.A. 1.4 objective lens, a spinning-disk confocal scan head (Perkin–Elmer), and a MicroMAX interline transfer cooled charge-coupled device camera (Roper Scientific, Trenton, NJ). All images (16-bit) were acquired using a single-wavelength (488 nm) filter cube. Image acquisition was controlled by Ultraview RS software (Perkin-Elmer, Boston, MA). Time-lapse sequences were acquired at 3 sec intervals by using an exposure time of 0.2 sec at four optical planes per interval with a Z-step of 0.3 μm. Resulting movies are shown at a rate of 15 frames per second.
**Quantification of microtubule dynamics.** Individual microtubules were analyzed as described (Rusan et al., 2001). Briefly, time-lapse images were exported from the proprietary Ultraview software and imported into Metamorph software (Universal Imaging, Media, PA) for further analysis. A stack of four optical planes was used to make a Z-projection at each time point and a time-lapse movie was reconstructed. The position of the microtubule end was tracked using the “track points” function in Metamorph, which was linked to Excel to produce a history plot of each microtubule. Growth and shortening phases were identified based on the history plots. The frequency of catastrophe was calculated by dividing the sum of the number of transitions from growth to shortening and pause to shortening by the sum of the duration of growth and pause. The frequency of rescue was calculated by dividing the sum of the number of transitions from shortening to growth and shortening to pause by the duration of shortening. Microtubule dynamicity was calculated as the total number of tubulin dimers exchanged at the microtubule end (using 1624 dimers/µm) considering the lifetime of the microtubule (Toso et al., 1993; Waterman-Storer et al., 2000). The value from each microtubule was used to calculate an average for each experiment and was used in Table 1. The time spent in each phase (shrink, growth, and pause) was recorded and the percent time spent in each phase was calculated for each microtubule. The percent time was averaged individually and used in Table 1.

**Immunofluorescence of phosphorylated histone H3.** RPE GFP-α tubulin cells were grown on glass coverslips, treated with control or 100 nM Hesperadin for 6 h, and fixed in -20°C methanol for 30 min. Coverslips were stained with DAPI and an antibody to
phosphorylated H3 (Upstate Biotechnology, Lake Placid NY, cat# 6-570) followed by cy5 secondary reagents. Images were acquired using the Metamorph software as described above.

**Quantification of phosphorylated H3 fluorescence.** The method used was similar to the quantification EB1-GFP fluorescence (supplemental material). Briefly, 0.2 μm optical sections were taken of each mitotic cell for DAPI, GFP and cy5. Since phosphorylated H3 specifically labels the chromatin, a region of the cytoplasm was used as background to subtract from the cy5 fluorescence. The DAPI labeling for each corresponding phosphorylated H3 image was used to define a region of interest based on the “threshold image” function in Metamorph. The region was transferred to the appropriate phosphorylated H3 image and the fluorescence in the defined region (occupied by the chromatin) was quantified.

**Statistical analysis.** Data were analyzed using the unpaired t-test on a Graphpad software package for Windows (Prism). p values of 0.05 were considered statistically significant.
Figure 1. Survivin Silencing Increases Microtubule Dynamics.

(A) RPE cells were transfected with survivin-specific S4 or control VIII dsRNA oligonucleotides (siRNAs), harvested after 48 h and analyzed by immunoblotting. (B, C) Analysis of microtubule growth and shrinking. RPE cells stably expressing GFP-α tubulin were transfected with control VIII (B) or survivin-specific S4 dsRNA oligonucleotide (C) and imaged by time-lapse video microscopy (see movies 1, 2). The extent of growth and shrinking (red arrows) was measured for individual microtubules in interphase. (D, E) Quantification of microtubule dynamics observed in B and C. The length of microtubule polymer growth or shrinking (distance in micrometers) was examined over time in control cells (D) or survivin-depleted cells (E). Increasing values represent microtubule growth; decreasing values represent shrinking. Transitions from growth to shrinking (catastrophe), shrinking to growth (rescue), periods of no net growth (pause) and other parameters of microtubule dynamics are quantified in Table 1. Symbols represent 5 individual microtubules from two cells for both D and E.
Figure 1

A

VIII S4

Survivin

14-3-3

B

0 sec 6 sec 12 sec 18 sec 24 sec 30 sec 36 sec 42 sec

C

D

Control siRNA

Distance (µm)

Time (sec)

0 15 30 45 60 75

-2.7 -5.3 -8

E

Survivin siRNA

Distance (µm)

Time (sec)

0 15 30 45 60 75

-2.7 -5.3 -8
Figure 2. Survivin Silencing Increases the Number of Growing Microtubules and Decreases the Duration of Microtubule Growth.

(A, B) Number of EB1 foci. GFP-EB1-expressing RPE cells were transfected with control dsRNA (A) or survivin-specific S4 dsRNA (B) and EB1 foci were examined in composite images made from three consecutive frames of each movie (see movies 3, 4). C. GFP-EB1 foci, representing individual microtubules, are quantified from fields covering 50-70% of a cell’s area (boxes in A and B) from 5 cells in two separate experiments (C, each bar represents the average number of EB1 tracks). (D, E) GFP-EB1 movements. GFP-EB1-expressing RPE cells were transfected with control dsRNA (D) or survivin-specific S4 dsRNA (E) and EB1 movements were examined over one minute (see movies 3, 4). All GFP-EB1 movements, representing microtubule growth, are displayed as linear tracings in D and E. The first 15 seconds of microtubule growth are represented in yellow and the final 45 seconds in red. Bar in E, 5 μm for D and E. Examples of microtubules used for analysis are displayed as green to blue instead of yellow to red. (F, G) Higher magnification images of individual growing microtubules in D (control, VIII transfectants) and E (survivin, S4 transfectants) at times indicated. (H) Quantification of GFP-EB1 tracking distances after transfection of dsRNA VIII (control) or survivin-directed S4 (survivin) oligonucleotide. Length in micrometers. Data represent 10 measurements from each of ten cells from two separate experiments. Examples of microtubules analyzed for H are shown as green (first 15 sec) and blue (next 45 sec) in D and E. Bars in C and H represent the mean +/- standard deviation.
Figure 2

(A) and (B) show images before and after treatment. (C) Graph showing the comparison of GFP-EB1 foci number between Survivin siRNA and Control siRNA. (D) and (E) show the distribution of GFP-EB1 foci after treatment.

(F) and (G) show the movement of GFP-EB1 foci over time with Control siRNA and Survivin siRNA. (H) Graph showing the comparison of distance traveled by GFP-EB1 foci.
Figure 3. Survivin Modulates the Number of Growing Microtubules Emanating from the Centrosome.

(A) Maximum projection of z-series taken of individual centrosomes in interphase RPE cells treated with nocodazole and then allowed to regrow microtubules for 2 minutes. Cells had been treated with either survivin-specific S4 dsRNA (A, left) or control dsRNA (A, right). Cells were stained by immunofluorescence using an antibody to EB1 (red) and γ-tubulin (green) (A, left and right) and foci were quantified in each z-plane and compared to adjacent planes to ensure that individual foci were not counted multiple times (graph, A, far right). (B) Maximum projection as in A where cells had been transduced either by pAd-surivin (B, left) or pAd-GFP (B, right). Cells were stained by immunofluorescence and quantified as in A (B, far right). (C) Maximum projection as in A and B where cells had been treated with either survivin-specific S4 dsRNA (C, left) or control dsRNA (C, right) in the presence of Hesperadin. Cells were stained by immunofluorescence and quantified as in A and B (C, far right).
Figure 3

A  
Svv siRNA  
Control siRNA

B  
Svv-GFP OX  
GFP OX

+ Hesperadin

C  
Svv siRNA  
Control siRNA
Figure 4. Increased Levels of Survivin Suppress Microtubule Growth in Interphase and Mitotic Cells.

(A, B) Individual frames from a movie of GFP-EB1 in an interphase COS-7 cell co-expressing β-galactosidase (control) showing multiple foci at time zero (A) that became more visible after growth (displayed as tracks that extend for long distances over one minute, B, see movie 5). First 15 sec of microtubule growth are in yellow and last 45 sec are in red. (C) Taxol treatment. The same cell as in B treated with 10μM Taxol for 31 min; no detectable GFP-EB1 foci are seen (see movie 6). (D) Higher magnification of a growing microtubule in B at 0, 30 and 60 sec of filming. (E) Suppression of microtubule dynamics by survivin. Cell co-expressing survivin and GFP-EB1 showing no detectable GFP-EB1 foci (see movie 7). (F) Quantification of spindles with detectable GFP-EB1 foci (or short tracks) in living cells expressing β-galactosidase or survivin. Data are the average of two separate experiments. n>20 cells per bar. (G, H) Individual frames from movies of GFP-EB1 in mitotic cells expressing either survivin (G, see movie 11) or β-galactosidase (H, see movie 10). Bar in C, 5 μm for A-H. (I-N). Cells co-expressing GFP-EB1 together with β-galactosidase (I-K) or survivin (L-N) were analyzed for GFP-EB1 foci (I, L), β-galactosidase and HA-surivin expression (J, M) or microtubules (K, N). Insets, DNA labeled with DAPI. For all injection studies, an average of 91% of cells survived injections and 96% of those expressed both GFP-EB1 and either survivin or β-galactosidase at levels that do not affect microtubule dynamics or organization (surivin). Bar in N 10μm for I-N.
Figure 5. Increased Levels of Survivin Suppress Microtubule Growth in Midbodies During Cytokinesis.

(A-C) GFP-EB1 staining at midbodies in telophase cells expressing control protein (β-galactosidase) or survivin (F-H). Midbody GFP-EB1 labeling is significant in control cells (AC), with movements (representing microtubule growth) both away from the central midbody region (D, arrow) and toward the central midbody (E, arrow) (see movies 8 and 9 in supplemental material). Schematic in D shows midbody region examined in this figure. In survivin-expressing cells (F-H), little GFP-EB1 labeling is observed at the midbody (F) although the cell is at a similar stage of cytokinesis to that in A. Insets (A-C), (F-H), DNA labeled with DAPI. Images in A and F represent enlargements of midbodies seen in B and G, respectively. Bar in H 10 μm for B, C, G, H and 5 μm for A, and F. Bar in E, 5 μm for D and E.
Figure 5

A. GFP-EB1
B. β-gal
C. MT

D. 1:46
E. 2:38
F. GFP-EB1
G. HA-Survivin
H. MT
Figure 6. Increased Levels of Survivin Increase Microtubule Stability.

(A, B) Survivin increases acetylated tubulin content. COS-7 cells transduced by pAd-GFP (A) or pAd-GFP-survivin (B) were stained by immunofluorescence using an antibody to a stabilized acetylated form of α tubulin. Bar in B, 5 μm for A and B. Individual cells outlined. (C, D) Quantification of acetylated tubulin signal in individual cells (C, total integrated fluorescence of a single representative experiment) and in all cells (D, expressed as an average, n>2 x 10^3 measurements from optical sections taken from >200 cells/bar. All data for A-D was acquired from interphase cells). (E, F) Survivin increases acetylated microtubules at midbodies. Cells were transduced with pAd-GFP (E) or pAd-GFP-survivin (F) and analyzed with an antibody to acetylated tubulin by fluorescence microscopy. Bar in F, 5 μm for E, F. (G, H) Quantification of acetylated tubulin signal at midbodies in individual cells (G) or whole cell population (H). (I, J) Nocodazole resistance. Cells were transduced with pAd-GFP or pAd-GFP-survivin, exposed to nocodazole for 20 minutes (I, J) and analyzed for α-tubulin staining by fluorescence microscopy. I, J represent high magnification images of cells acquired from random fields for analysis. Bar in J, 5 μm for I, J. (K) Quantification of nocodazole resistance of microtubules (α-tubulin staining) in cells expressing GFP or survivin at 20 min. Fluorescence intensity is in arbitrary units.
Figure 6

Acetylated tubulin
Interphase

Acetylated tubulin
Midbody

GFP

OX Survivin

100
75
50
25
0

Fluorescence intensity

Individual cells

Acetylated tubulin

GFP

OX Survivin

100
75
50
25
0

Midbody fluorescence

Individual cells

Nocodazole resistance

10 µM nocodazole

100
75
50
25
0

Fluorescence intensity
Figure 7. Aurora B Silencing Has No Detectable Effect on Microtubule Dynamics.

(A) RPE cells were transfected with a survivin-specific siRNA or an Aurora B specific siRNA, harvested 48 h later and analyzed by immunoblotting. (B) Binucleated cells were quantified 24 and 48 h after transfection of Aurora B and control siRNAs (n = 300 cells/bar). (C) Image showing two binucleated cells after treatment with Aurora B siRNA (arrows). Nuclei, blue, microtubules, red. (D) Quantification of microtubule acetylation following Aurora B or control siRNA treatment (n = 200 cells for each bar). (E, F) Images of acetylated microtubules in cells treated with Aurora B (E) or control (F) siRNAs. Nuclei, blue, acetylated microtubules, red. Bar in C, 5 μm, bar in F, 5 μm for E, F.
Figure 7

A

siRNA:
S4 AurB

IB:

Aurora B

survivin

14-3-3

B

Binucleated cells

% Binucleates

24 48

AuroraB siRNA

Control siRNA

D

Acetylated tubulin

Fluorescence intensity

Aurora B siRNA

Control siRNA
Figure 8. Aurora B Silencing Has No Detectable Effect on Microtubule Dynamics.

(A, B) RPE cells stably expressing GFP α-tubulin were transfected with Aurora B (B) or control (A, VIII) siRNAs. Images of microtubules in interphase cells were acquired every three seconds (sec) and the resulting time-lapse movies were used to construct history plots of individual microtubules. No significant differences were observed in microtubule catastrophe or rescue frequencies (6 microtubules from 2 cells are shown), the duration of microtubule pause, growth or shrinking or the rate of microtubule growth or shrinking (see Table 1). (C, D) Silencing of Aurora B has no detectable effect on the distance traveled for GFP-EB1 foci (C, tracking distances) or the number of GFP-EB1 foci per unit area (D) (n = 16 microtubules from 3 or more cells for each bar). See legends to Figs. 1 and 2 and Table 1 for more details. (E) Survivin was immunoprecipitated from asynchronous cultures of HeLa cells using previously characterized antibodies (Giodini et al., 2002) and probed for survivin, Hsp 90 and Aurora B as indicated. While Aurora B can be detected in the cell lysates it is undetectable in the survivin immunoprecipitation. IgG is nonimmune rabbit IgG used for immunoprecipitation control. Asterisk (*) represents the IgG band.
Figure 8

A. Control siRNA

B. AuroraB siRNA

C. AuroraB siRNA vs Control siRNA

D. Number of GFP-EB1 foci

E. Western blot analysis of Pellet and Supernatant of Survivin and IgG.
Figure 9. Chemical Inhibition of Aurora B Activity by Hesperadin Does Not Affect
Microtubule Dynamics in Interphase or Mitotic Cells.

(A) RPE cells treated with 100 nM Hesperadin or a nonfunctional analog for six hours
were used to quantify the phosphorylation of the Aurora B target, histone H3 (A, phos-
H3). Graph, n = 10 randomly chosen mitotic cells/bar. Representative images are shown
below bars. DAPI panel shows DNA pattern, phos-H3 panel shows staining with
antibody specific for phosphorylated H3. DAPI staining was used to identify regions
occupied by chromatin given the drastic loss of phos-H3 in Hesperadin-treated cells. (B-
D) Mitotic spindles in Hesperadin treated cells are narrower (C) and angled (D)
compared with controls (B). Microtubule growth as measured by the distribution of GFP-
EB1 foci (red) in mitotic cells treated with Hesperadin (C, D) or the control analog (B)
show no significant differences. Large images represent merge of microtubules (green)
GFP-EB1 (red) and DNA (DAPI, blue). Smaller images below show, from left to right, α
tubulin staining (microtubules), GFP-EB1 and DNA. Bar in D, 5 μm for B-D. (E, F)
Microtubule dynamics in interphase cells treated with Hesperadin (E) or the analog (F)
show no significant differences (6 microtubules from two cells shown; also see Table 1).
Figure 9

A. Bar graph showing fluorescence intensity comparison between Hesperadin and its analog. The graph includes DAPI and phosphH3 markers.

B. Fluorescence microscopy images of Hesperadin analog showing distinct fluorescent patterns.

C. Fluorescence microscopy images of Hesperadin showing similar fluorescent patterns.

D. Close-up images of Hesperadin highlighting specific areas of interest.

E. Graph showing distance versus time for Hesperadin, with markers at various time points.

F. Graph showing distance versus time for the analog, with markers at various time points.
Table 1. Microtubule Dynamics in Survivin Depleted Cells.

(A) Catastrophe is increased in survivin depleted cells when compared with cells treated with control siRNAs or siRNAs targeting Aurora B (1.66-1.99-fold increase, respectively; values indicated by asterisks). (B) The average time microtubules spent shrinking, pausing or growing is not significantly perturbed. There is no significant difference in growth or shrinkage rates in survivin depleted cells compared to controls. Dynamicity is increased in cells with depleted survivin. Here, dynamicity represents the exchange of dimer at the microtubule tip over time (sec), see materials and methods. Pauses represent periods between growth and shrinking or vice versa (< 0.5 μm change).

All data shown was acquired from 25 microtubules in 5 cells per experimental condition. All differences described are statistically significant (t-test: p< 0.005). Cat, catastrophe, freq, frequency, ave, average.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Avg. frequency rescue (s⁻¹)</th>
<th>Avg. frequency catastrophe (s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Control siRNA</td>
<td>0.070 ± 0.0148</td>
<td>0.025 ± 0.0047</td>
</tr>
<tr>
<td>Survivin siRNA</td>
<td>0.094 ± 0.0207</td>
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Supplemental data available online:

http://www.molbiolcell.org/cgi/content/full/E05-08-0723/DC1

Supplemental Movies

Movies 1 and 2. High magnification images of individual GFP-labeled microtubules in cells treated with control siRNAs (1) or survivin-treated cells (2).

Movie 3 and 4. GFP-EB1 foci traces in cells treated with control siRNAs (3) or survivin-treated cells (4).

Movies 5, 6, 7. GFP-EB1 foci formation in a β-galactosidase-expressing control cell (5) but only Brownian movement in a cell treated with Taxol (6) or a survivin-expressing cell (7).

Movies 8 and 9. GFP-EB1 foci showing movement inward (8) and outward (9) from the midbody. The zone that does not stain for microtubules at asterisk.

Movies 10 and 11. GFP-EB1 foci moving in mitotic cells expressing either β-galactosidase (10) or survivin (11).
Supplementary Figure 1. Data taken from two sets of time lapse movies. A background reading outside the cell was used to subtract extraneous fluorescence and a threshold range (yellow) was used to define specific areas for quantification. (In the case of quantifying cytoplasmic signal, the threshold was assigned to exclude the morphologically distinct region of the spindle. Spindle fluorescence was quantified after subtracting cytoplasmic fluorescence.)
Supplementary Figure 2.

Data shows reduction of survivin levels after RNAi and increased prometaphase
Supplementary Figure 3.

Note fewer acetylated microtubules (MT) in survivin (SVV) depleted cells when compared with controls (Con). This is in contrast to the increase in acetylated tubulin in survivin over-expressing cells and consistent with the idea that survivin modulates microtubule dynamics.
Supplementary Figure 4.

Changes in microtubule parameters observed in survivin-depleted cells occurred in the absence of changes in total cellular tubulin levels.
Supplementary Figure 5.

In cells over-expressing survivin the majority of EB1 was lost from the spindle. Although EB1 foci were present in some spindles (~15%, n = 20), their number never exceeded ~10% of control spindles. Control cells expressing βgal showed robust EB1 localization (above) with multiple GFP-EB1 foci moving away from both spindle poles (see movie 10).
Supplementary Figure 6.

Cells over-expressing either a GFP vector control or the pAD-survivin were analyzed by immunofluorescence for multinucleate cells and quantified over a 24hr period. Consistent with previous observations (Speliotes et al., 2000; Uren et al., 1999), survivin-expressing cells (which have reduced microtubule dynamics) often generated multinucleated cells.
Supplementary Figure 7.

Hela cells synchronized by thymidine block were harvested in mitosis. Extracts were immunoprecipitated with polyclonal anti-survivin (Survivin-IP) or control IgG (Control IP). Immune complexes were probed with anti-Hsp90 or anti-Aurora B.
Chapter 3:

Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody is Required for Secretory-Vesicle-Mediated Abscission
Contributions and Acknowledgements

The data presented in Chapter 3 is published in Cell: Gromley, A., Yeaman, C., Rosa, J., Redick, S., Chen, C. T., Mirabelle, S., Guha, M., Sillibourne, J., and Doxsey, S. J. (2005). Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. Cell 123, 75-87. Adam Gromley, Ph.D. conducted siRNAs/ Brefeldin A treatments and subsequent westerns, immunoprecipitations, live analysis documenting cytokinesis defects and immunofluorescent localization (figures 3-7). Charles Yeaman, Ph.D. provided exocyst fractionation data (figure 2). Jack Rosa provided images of midbody morphology by phase-contrast (figure 1) and live analysis of cytokinesis describing the asymmetric delivery of vesicles and the immediate post-abscission-fate of the midbody ring (figure 8). Chun-Ting Chen provided immunofluorescent examples of multiple, persistent midbody rings in mammalian cells (figure 8). Stephanie Mirabelle provided localization of γ-tubulin to both the lateral ends of the intracellular bridge and the midbody ring (figure 1). Manakshi Guha provided live localization of GFP-GAPCENA, illustrating the dynamic nature of the midbody and the midbody ring (figure 1). James Sillibourne, Ph.D. contributed his skills to the design and implementation of yeast two-hybrid screens.
Introduction

Cytokinesis is a fundamental process that results in division of a single cell with replicated DNA into two daughters with identical genomic composition (Glotzer, 2001; Glotzer, 2005; Guertin et al., 2002). Early events in animal cell cytokinesis include assembly and contraction of the actomyosin ring to form the cleavage furrow. Continued furrowing results in constriction of the plasma membrane to form a narrow cytoplasmic bridge between the two nascent daughter cells. Within this intercellular bridge are bundled microtubules and a multitude of proteins that together form the midbody. In a poorly understood final step called abscission, the cell cleaves at the intercellular bridge to form two daughter cells.

Membrane trafficking is required for late stages of cytokinesis (Albertson et al., 2005; Finger and White, 2002; Jurgens, 2005; Papoulas et al., 2004; Strickland and Burgess, 2004). In C. elegans embryos, inhibition of Golgi secretion by brefeldin A (BFA) resulted in late-stage cytokinesis defects (Skop et al., 2001). More recent studies in mammalian cells using dominant-negative approaches showed that the membrane fusion-inducing SNARE components, syntaxin-2 and endobrevin/VAMP8, are required for a final step in cell cleavage (Low et al., 2003). Endocytic traffic also plays a role in cytokinesis. Recycling endosomes and associated components localize to the midbody and are required for cell cleavage (Monzo et al., 2005; Thompson et al., 2002; Wilson et al., 2005). However, little is known about the spatial and temporal control of dynamic membrane compartments and molecules during abscission or how these activities are
coordinated to achieve cell cleavage. The role of membrane-vesicle-tethering exocyst complexes in animal cell abscission is poorly understood. The exocyst is a multi-protein complex that targets secretory vesicles to distinct sites on the plasma membrane. In the budding yeast *S. cerevisiae*, exocyst components localize to the mother-bud neck, the site of cytokinesis (Finger et al., 1998; Mondesert et al., 1997). Exocyst disruption results in accumulation of vesicles at this site (Salminen and Novick, 1989) and impairs actomyosin-ring contraction and cell cleavage (Dobbelaere and Barral, 2004; Verplank and Li, 2005). In the fission yeast *S. pombe*, exocyst components localize to the actomyosin ring (Wang et al., 2002). Mutants for the exocyst component Sec8 accumulate 100 nm “presumptive” secretory vesicles near the division septum and cannot complete extra-cellular separation of the two daughter cells. A screen for cytokinesis mutants in *Drosophila melanogaster* identified the exocyst component sec5 (Echard et al., 2004), and proteomic analysis of the midbody in mammalian cells showed that the exocyst protein sec3 is at the midbody (Skop et al., 2004). Mammalian exocyst components are involved in secretion in polarized epithelial cells (Yeaman et al., 2004) and localize to the midbody (Skop et al., 2004; Wilson et al., 2005), but the function of the exocyst during cytokinesis is unclear. Components of membrane-vesicle-tethering and -fusion complexes have been identified in some organisms and linked to cytokinesis, but the pathway that integrates these complexes with vesicle trafficking during cell cleavage is unknown. Little is known about how SNAREs and the exocyst are anchored at the midbody or how they modulate membrane-vesicle organization and fusion to coordinate abscission. Moreover, the origin and dynamics of membrane compartments involved in abscission have not been investigated. In this chapter a multi-step pathway
for abscission that requires a scaffold protein to anchor SNARE and exocyst complexes at a unique midbody site and also requires asymmetric transport and fusion of secretory vesicles at this site is described.

**Results**

**Centriolin Is Part of a Ring-like Structure at the Central Midbody during Cytokinesis.**

We previously showed that centriolin localized to the midbody during cytokinesis (Gromley et al., 2003). Using high-resolution deconvolution microscopy, we now demonstrate that centriolin is part of a unique ring-like structure within the central portion of the midbody, which we call the midbody ring (observed in ~75% of all telophase cells, Figures 1A–1C). The midbody ring was 1.5–2 μm in diameter (Figure 1C), contained α-tubulin (Figure 1D), and co-localized with the phase-dense Flemming body (Figure 1B, inset) (Paweletz, 1967). In fact, high-magnification phase-contrast imaging revealed that the Flemming body was organized into a ring-like structure (Figure 1E). The midbody ring was flanked by Aurora B kinase, which co-localized with microtubules on either side of the ring (Figure 1B, inset). Several other proteins localized to the midbody ring including ectopically expressed GFP-GAPCenA, a GTPase-activating protein previously shown to localize to centrosomes (Cuif et al., 1999). Time-lapse imaging of GFP-GAPCenA and other proteins in living cells showed that the midbody ring was dynamic, moving between cells and tipping from side to side to reveal the ring structure (Figure 1F; see also Movie S1 in the Supplemental Data available with
this article online). In addition, midbody-ring localization of GFP-GAPCenA confirmed the ring structure seen by immunofluorescence microscopy and demonstrated that there were no antibody penetration problems in this midbody region as seen for other antigens (Saxton and McIntosh, 1987). The midbody ring was distinct from the actomyosin ring and did not change in diameter during cytokinesis (Figures 1A and 1B). It appeared during the early stages of actomyosin-ring constriction and persisted until after cell cleavage (see below). The centralspindlin components MKLP-1/CHO1/ ZEN-4 (Figure 1G) and MgcRacGAP/CYK-4 (data not shown) also localized to the midbody ring and appeared earlier than centriolin during actomyosin-ring constriction. Depletion of MKLP-1 by RNAi to 18% of control levels (n = 2 experiments) prevented recruitment of centriolin to the ring (Figures 1H and 1I). In contrast, depletion of centriolin had no effect on the localization of MKLP-1 or MgcRacGAP (data not shown). These data suggested that centralspindlin anchored centriolin to the midbody ring.

Centriolin Interacts with the Exocyst Complex and the SNARE-Associated Protein Snapin and Is in Membrane-Associated Cytoplasmic Fractions.

To determine the molecular function of centriolin in cytokinesis, we performed a yeast two-hybrid screen using a 120 amino acid domain of centriolin that is required for the cytokinesis function of centriolin and shares homology with budding- and fission-yeast genes (Nud1/Cdc11) involved in cytokinesis and mitotic exit (Gromley et al., 2003). A screen of approximately 12 million clones from a human testis cDNA library yielded two potential interacting proteins: sec15, a member of the exocyst complex, and snapin, a
SNARE-associated protein. Additional biochemical analysis confirmed the yeast two-hybrid interactions and demonstrated that centriolin was in a large complex associated with membranes (Figure 2). The centriolin Nud1 domain fused to the DNA binding domain (DBD) and sec15 fused to the activation domain (AD) were co-expressed in the same yeast cells. Immunoprecipitation of the Nud1 fusion protein effectively co-precipitated the sec15 fusion protein (Figure 2C). To test whether other members of the exocyst complex were bound to centriolin, we immunoprecipitated endogenous centriolin from HeLa cell lysates with affinity-purified centriolin antibodies and showed that sec8 and sec5 co-precipitated (Figure 2D). Gel filtration experiments (Superose 6) using MDCK cell lysates demonstrated that centriolin co-eluted with fractions containing the exocyst complex (detected with antibodies to sec8 and sec3, Figure 2A). Centriolin was eluted as a single peak that overlapped with peaks of sec3 and sec8. We next asked if centriolin co-immunoprecipitated with the exocyst. Antibodies to sec8 were added to each of the fractions from the gel filtration column, and immune complexes were collected and probed with affinity-purified centriolin antibodies as described (Gromley et al., 2003). Centriolin was found only in fractions containing exocyst components (Figure 2A). The centriolin-containing fractions eluted earlier than the peak of sec 3 or sec8, suggesting that the exocyst fraction to which centriolin was bound was different from the cytosolic and lateral plasma-membrane fractions of the exocyst (Yeaman et al., 2004). The exocyst-centriolin fractions did not co-fractionate with the bulk of the cellular protein and eluted considerably earlier than thyroglobulin (MW 669,000) suggesting it was part of a large complex. Since the exocyst associates with membrane vesicles, we next tested whether centriolin was also present in membranous fractions. Cell
homogenates were prepared in the absence of detergent and underlain at the bottom of linear iodixanol gradients. Isopycnic centrifugation was performed, and fractions were probed for both centriolin and the exocyst component sec8. Centriolin "floated up" to fractions lighter than the cytosol having a buoyant density of δ ~ 1.14 g/ml (Figure 2B). The centriolin peak co-fractionated with a major peak of Sec8 that was slightly less dense than the junction-associated peak of Sec8 described previously in confluent MDCK cells (δ ~ 1.16 g/ml; Yeaman et al., 2004). Little to no centriolin was observed at other positions in the gradient or in the major protein peak, suggesting that most if not all centriolin was associated with membranes. Taken together, the density gradient, immunoprecipitation, and chromatography data support the conclusion that centriolin associates with the exocyst in a very large complex bound to cellular membranes. The yeast two-hybrid interaction between centriolin and the low-abundance protein snapin was confirmed by showing that endogenous centriolin co-immunoprecipitated a His6-tagged snapin fusion protein expressed in HeLa cells (Figure 2E) and by the centriolin-dependent midbody localization of snapin (see below).

The Exocyst Complex Co-localizes with Centriolin at the Midbody Ring.

Further support for the centriolin-exocyst interaction was obtained by showing that exocyst-complex components localized to the midbody ring with centriolin. HeLa cells were co-labeled with antibodies against one of several exocyst components (sec3, sec5, sec8, sec15, exo70, or exo84) and either microtubules or centriolin (Figure 3A). We
found that all these exocyst components localized to the midbody ring during cytokinesis and formed a ring-like structure similar to that seen for centriolin. In fact, double-stained images revealed considerable overlap between sec8 and centriolin, indicating that they were part of the same structure (Figure 3A, panel 1). We also showed that a myc-tagged form of sec8 localized to the midbody ring when expressed in HeLa cells (Figure S1), confirming the localization seen with antibodies directed to the endogenous protein.

Midbody Localization of the Exocyst Is Disrupted in Cells Depleted of Centriolin.

We next tested whether centriolin was required for midbody-ring localization of the exocyst. siRNA-mediated depletion of centriolin resulted in a ~70% reduction in centriolin protein levels and complete loss of midbody staining in 24% of cells compared with control cells treated with lamin siRNA (Figures 3B and 3E). Immunofluorescence quantification of midbody signals performed as in our previous studies (Gromley et al., 2003) demonstrated that many of the remaining centriolin-depleted cells had lower levels of midbody staining than controls (48%, n = 23 cells), bringing the total percentage of midbody depleted cells to 72%. Cells that lacked detectable midbody-associated centriolin usually lacked midbody labeling of sec8 (10/10, Figure 3B, panels 1 and 6). Although other exocyst components could not be co-stained with centriolin because all were detected with rabbit antibodies like centriolin, all were lost from or reduced at midbodies in centriolin-depleted cells (Figure 3B, panels 2–5). For example, Exo84 was undetectable at midbodies in 22% of centriolin-depleted cells (n = 9 cells) or had levels
below the lowest control midbody staining in 42% of centriolin-depleted cells (n = 19 cells). Significant reduction in midbody staining of centriolin and other exocyst components was observed with a second siRNA targeting a different centriolin sequence (Gromley et al., 2003) (data not shown). To test whether centriolin was dependent on the exocyst complex for localization to the midbody, we initially targeted sec5 for siRNA depletion. Recent studies showed that mutants of sec5 in *D. melanogaster* disrupted exocyst function (Murthy and Schwarz, 2004) and that RNAi-mediated depletion of sec5 inhibited exocyst-dependent processes in vertebrate cells (Prigent et al., 2003). We found that depletion of sec5 resulted in loss of midbody-associated sec5 as well as other exocyst components, including sec3, sec8, and sec15 (Figures 3C and 3E). These results show that sec5 depletion disrupts midbody-ring localization of the exocyst. In contrast, neither sec5 nor sec8 loss from the midbody affected the association of centriolin with the midbody ring (Figures 3D and 3E). These data demonstrate that centriolin is required for midbody localization of the exocyst, while localization of centriolin appears to be independent of the exocyst.

**Disruption of the Exocyst Causes Failure at the Final Stages of Cytokinesis**

Localization of the exocyst to the midbody and its interaction with centriolin suggested that the complex might play a role in cytokinesis. To examine this, we disrupted the midbody-associated exocyst using siRNAs targeting sec5 and examined cytokinesis by time-lapse imaging over a 20 hr time period. We found that over half the cells exhibited severe cytokinesis defects, including failure in the final abscission step (42%, Figures 4B
and 4C, Movie S3) and delays during cytokinesis (24%, n = 18) compared with control lamin siRNA-treated cells (Figures 4A and 4C, Movie S2). Some cells remained interconnected by thin cytoplasmic bridges (Figure 4B, panel 17:05 and Movie S3) and sometimes entered one or more additional rounds of mitosis while still connected to their partner cells. Sec5-depleted cells viewed for an additional 24 hr showed a similar level of cytokinesis defects (data not shown), suggesting that nearly all cells in the culture experienced cytokinesis problems over time. Cytokinesis defects were also observed when the exocyst was disrupted by siRNA depletion of sec15 and sec8 (data not shown). Cells remained healthy, as no differences in cell morphology or mitochondrial function were observed. These data show that disruption of the exocyst produces late-stage cytokinesis defects similar to centriolin (Gromley et al., 2003) and demonstrates a requirement for the exocyst in the final stages of animal cell cytokinesis.

**Snapin and SNARE Components Localize to the Midbody Ring in a Centriolin-Dependent Manner.**

Snapin was originally considered to be a neuron-specific protein, but recent studies demonstrated that it is also expressed in non-neuronal cells (Buxton et al., 2003). Snapin may facilitate assembly of SNARE complexes and may define a limiting step in vesicle fusion mediated by PKA phosphorylation (Chheda et al., 2001). Although the role of snapin in neurotransmission has been questioned (Vites et al., 2004), recent results indicate that it is essential for this process (Thakur et al., 2004). The role of snapin in cytokinesis is currently unknown. Using previously characterized antibodies to snapin
(Thakur et al., 2004), we demonstrated that the protein localized to the midbody ring at the same time as the exocyst and shortly after centriolin (Figure 5A, panel 2). Previous immunofluorescence studies showed that the v-SNARE endobrevin/VAMP8 and t-SNARE syntaxin-2 were enriched in the region of the midbody flanking the Flemming body and coincident with microtubules and Aurora B staining (Carvalho et al., 2003a). Using the same antibodies, we confirmed the localization pattern of endobrevin/VAMP8 (Figure 5A, panel 1) and syntaxin-2 (data not shown). Very late in cytokinesis, the intercellular bridge narrows to 0.5 μm, and microtubule bundles are reduced in diameter to 0.2–0.5 μm. At this time, endobrevin/VAMP8 and syntaxin-2 joined centriolin, snapin, and the exocyst at the midbody ring (Figure 5A, panels 3 and 4). siRNA depletion of centriolin eliminated the midbody-ring localization of snapin (>35% of cells, Figure 5B), endobrevin/VAMP8 (>20% of cells, Figure 5C), and syntaxin-2 (Figure 5C). Of the remaining cells, 24% and 36% showed midbody staining levels below those of controls for snapin (n = 22) and endobrevin/VAMP8 (n = 25), respectively. As shown earlier, midbody-ring integrity was not compromised under these conditions, as MKLP-1 and MgcRacGAP remained at this site in cells with reduced centriolin. These results indicated that centriolin was required for midbody-ring localization of v- and t-SNARE proteins and the SNARE-associated protein snapin.

Snapin Depletion Mislocalizes the Protein from the Midbody and Induces Cytokinesis Defects.
Midbodies in 41% of snapin-depleted cells showed no detectable snapin staining (Figure 5D). Time-lapse imaging over a 22 hr period showed that 40% of snapin-depleted cells experienced late-stage cytokinesis failure (Figure 5E, Movie S4). Other cells showed long delays and often remained connected by a thin intercellular bridge (data not shown). When cultures were imaged for an additional 24 hr, we observed multi-cellular syncytia resulting from multiple incomplete divisions and additional individual cells undergoing cytokinesis failure. This suggested that most cells in the population ultimately failed cytokinesis and that some failed multiple times. Occasionally, cells separated when one of the attached daughters re-entered mitosis, possibly due to tensile forces generated by cell rounding during mitosis (Figure 5E, Movie S4). These results demonstrated that snapin was necessary for abscission and suggested that it functioned by anchoring SNARE complexes at the midbody.

Disruption of the Exocyst Results in Accumulation of Secretory Vesicles at the Midbody.

We next tested whether the late-stage cytokinesis defects observed in this study resulted from changes in membrane trafficking to the midbody. As a first test of this idea, we used brefeldin A, which disrupts cytokinesis in C. elegans presumably due to inhibition of post-Golgi secretory-vesicle trafficking (Skop et al., 2001). In HeLa cells treated with brefeldin A, we observed late-stage cytokinesis defects (Figure 6A) that were similar to those observed following depletion of centriolin. Many cells were delayed in or failed cytokinesis (n = 9/13 cells in two separate experiments). This suggested that post-Golgi
vesicle trafficking was involved in late stage cytokinesis events in vertebrate cells, although brefeldin A is known to affect other membrane-trafficking pathways (Antonin et al., 2000). Based on the localization of the exocyst to the midbody ring, we reasoned that the vesicle-tethering function of the complex might be operating at this site to facilitate fusion of v-SNARE-containing vesicles at the late stages of cytokinesis. To test this idea, we depleted cells of sec5 to disrupt exocyst complexes and examined the localization of v-SNARE (endobrevin/VAMP8) containing vesicles. We observed a collection of small, spherical endobrevin/VAMP8-containing structures resembling vesicles at the midbody (Figure 6B, panel 1, arrows) that were positioned around the phase-dense Flemming body (Figure 6B, arrowhead, panel 2). Although these structures were occasionally seen in control lamin A/C siRNA-treated cells, they were significantly increased in sec5-depleted cells (Figure 6B, graph).

To determine whether the endobrevin/VAMP8-containing structures were secretory vesicles, we used a more specific marker for the secretory pathway. We expressed a GFP-tagged construct containing an amino-terminal signal peptide that targets the protein to the lumen of the ER (lum-GFP) (Blum et al., 2000) and lacks retention and retrieval motifs, so it would not be expected to target to endosomes, multi-vesicular bodies, or lysosomes. The lum-GFP was efficiently secreted from non-dividing MDCK cells following a 19°C trans-Golgi network block and release from the block in the presence of protein-synthesis inhibitors (C.Y., unpublished data). When we expressed lum-GFP, numerous GFP-containing vesicles were observed in the cytoplasm. Following fixation and staining for endobrevin/ VAMP8, we found that most of the endobrevin/VAMP8
vesicles co-labeled with lum-GFP throughout the cytoplasm (Figure 6C) and within the intercellular bridge during late stages of cytokinesis (Figure 6C, insets). This observation demonstrates that the v-SNARE-containing vesicles that accumulated following disruption of the exocyst are secretory vesicles, an observation similar to that seen in studies in exocyst mutants of *S. cerevisiae* where vesicles dock normally but fail to fuse with the plasma membrane (Guo et al., 2000).

**Asymmetric Delivery of Secretory Vesicles to the Midbody Is Followed by Abscission.**

At early stages of cytokinesis, we observed numerous GFP-labeled secretory vesicles in Golgi complexes and cell bodies of nascent daughter cells but few within intercellular bridges (Figure 7A, panel 1). However, at a late stage of cytokinesis when the intercellular bridge narrowed to a diameter of ~2 μm and the midbody microtubule bundle was reduced to a diameter of 0.5–1 μm, GFP secretory vesicles accumulated in the intercellular bridge near the midbody ring (Figure 7A, Movie S5). Higher-magnification imaging of another cell at a similar cell-cycle stage revealed labeled secretory vesicles moving suddenly and rapidly (within 20 min) from the cell bodies into the intercellular bridge and up to the midbody ring (Figure 7B, Movie S6). In 11/11 cells, the vesicles were delivered primarily if not exclusively from one of the nascent daughter cells (Figure 7B, center panels). Vesicles packed into the region adjacent to the phase-dense Flemming body (Figure 7B, panels 2 and 3, large arrowhead; Movie S6). Within 20 min, the GFP signal disappeared (Figure 7B, last panel and Figure 7A, last panel),
suggesting that the vesicles fused with the plasma membrane, releasing the GFP signal into the extra-cellular space where it was free to diffuse. Loss of the GFP signal was not due to photo-bleaching because GFP-labeled vesicles in cell bodies adjacent to the intercellular bridge and in the Golgi complex retained the signal. We next examined the relationship between vesicle delivery to the midbody and abscission. We found that, shortly after the GFP signal was lost from the midbody region, the cell cleaved on the side of the Flemming body that received the GFP vesicles (6/6 cells from four experiments, Figure 7C). The cell on the opposite side received the Flemming body (Figure 7C, 70'–95' and Figure 7D). In some cases, the Flemming body moved around rapidly after abscission on the cell surface (Movie S7), suggesting that the structure was not anchored at a discrete point on the new daughter cell. Post-division midbodies contained multiple midbody-ring components and retained microtubules from both sides of the midbody ring (Figure 7E, panel 1). They persisted for some time after abscission, consistent with previous results (Mishima et al., 2002), and were often present in multiple copies, suggesting that they were retained through several cell cycles (Figure 7E, panels 2–4). These structures were seen on ~35% of HeLa cells and often retained features of the Flemming body and midbody ring, including MKLP-1 staining, Aurora B staining, phase-dense Flemming bodies, and localization to the plasma membrane (Figure 7E, data not shown). This suggested that supernumerary midbodies represent structures from previous divisions similar to the bud scars observed in yeast (Chen and Contreras, 2004).
Discussion

A Model for the Final Stage of Cytokinesis

This study defines several distinct molecular and structural steps during the late stages of cytokinesis (Figure 8). During cleavage-furrow ingression, MKLP-1 and MgcRacGAP arrive at the midbody ring (Figure 8A). When the intercellular bridge forms, centriolin localizes to the ring (Figure 8B), followed by snapin and exocyst proteins (Figure 8C). When the diameter of the midbody microtubule bundle and the intercellular bridge are reduced to $\sim 0.5-1 \, \mu m$, endobrevin/VAMP8 (v-SNARE) and syntaxin-2 (t-SNARE) move to the midbody ring. The v-SNAREs are part of secretory vesicles that move asymmetrically into the intercellular bridge predominantly from one nascent daughter cell; binding to v-SNAREs may incorporate t-SNAREs into this organization. The vesicles pack into the area adjacent to the ring and appear to fuse, releasing their contents into the extra-cellular space (lum-GFP, Figures 8D and 8E). Vesicle fusion with the plasma membrane may be initiated near the midbody ring where v- and t-SNAREs are localized. This could be followed by additional fusion events between vesicles and the plasma membrane as well as vesicle-vesicle fusion events (homotypic) mediated by SNAP23/25, a v-SNARE involved in compound exocytosis (Takahashi et al., 2004) (Figures 8F and 8G). Abscission then occurs at the site of vesicle fusion, and the entire midbody remains with the daughter cell opposite the fusion site (Figure 8H). Abscission could be triggered by arrival of v- and t-SNAREs at the midbody ring; release of SNAP23/25 from lipid rafts (Takahashi et al., 2004; Takeda et al., 2004); phosphorylation of snapin by PKA, which mediates its binding to the t-SNARE complex
(Buxton et al., 2003; Chheda et al., 2001); or another event. Dynamic movement of the post-abscission midbody ring suggests connections to motile forces within the cell, although this remains to be determined.

**Asymmetric Delivery of Secretory Vesicles Marks the Site of Abscission**

It is remarkable that secretory vesicles loaded with luminal GFP move into the intercellular bridge from only one of the two prospective daughter cells. The mechanism of this asymmetric vesicle delivery is unknown. It is tempting to speculate that a signal, negative or positive, emanates asymmetrically from one centrosome in the dividing cell. Centrosomes in the two prospective daughter cells are different in that one was “born” from the older centriole in the previous cell division during the centrosome duplication process (Doxsey, 2001). Consistent with this idea is the asymmetric spindle pole body (SPB) localization of budding- and fission yeast proteins that control mitotic exit and cytokinesis (Doxsey et al., 2005a; Grallert et al., 2004; Molk et al., 2004). In *S. pombe*, inhibitors of mitotic exit (Cdc16p and Byr4p) localize to the “old” SPB while activators of mitotic exit (Cdc7p and presumably Sid1p and Cdc14p) localize to the new SPB (Grallert et al., 2004). The relevance of this localization in both yeasts is still unknown. Further studies will be required to determine the role of centrosome protein asymmetry in the unidirectional delivery of secretory vesicles and abscission in animal cells. It has been suggested that the mother centriole moves to the intercellular bridge in telophase cells to coordinate the final steps in cytokinesis (Piel et al., 2001), although this was not consistently observed in this study (data not shown) or another that investigated several
cell lines (RPE-1, Ptk-1, CV-1, NRK-52E; A. Khodjakov, personal communication). The final stages of cytokinesis in animal cells share features with cell division in higher plants. Higher plant cells cannot divide using an actomyosin-based cleavage furrow due to the presence of a non-pliant cell wall, so they accomplish cell division by constructing a new membrane at the division plane, called the cell plate, that is independent of the plasma membrane and is established by microtubule-dependent delivery and fusion of vesicles at this site (Albertson et al., 2005; Finger and White, 2002; Jurgens, 2005). Our data show that the coordinated delivery of vesicles to the midbody ring during the late stages of cytokinesis is also required for the final stages of cell division in animal cells. However, we still do not understand the mechanism of secretory-vesicle delivery to the midbody, the role of microtubules in this process, or the precise contribution of vesicle transport and fusion to abscission. The presence of vesicles with heterogeneous diameters adjacent to the midbody ring prior to abscission is consistent with a model in which some vesicles fuse together prior to fusion with the plasma membrane. This would be analogous to the cell plate in plant cells. The endocytic pathway also appears to play a role in cell cleavage as components (dynamin, FIP3, Rab11) and compartments (endosomes) involved in this pathway affect the late stages of cytokinesis (Thompson et al., 2002; Wilson et al., 2005). Recycling endosomes have been shown to move from both prospective daughter cells to the midbody during cytokinesis then return to the daughter-cell cytoplasm (Wilson et al., 2005). It is still unclear how recycling endosomes participate in abscission and how the bidirectional movement of endosomes into the intercellular bridge is related to the unidirectional movement of secretory vesicles to this site in our study.
Materials and Methods.

Cell culture and transfections. The cells used primarily in this study were diploid, telomerase-immortalized human RPE cells (hTERT–RPE-1s; CLONTECH Laboratories, Inc.) (Morales et al., 1999) and Hela cells. All cells were grown as previously described (American Type Culture Collection). Hela cells were transfected as previously described (Lipofectamine; Invitrogen).

Immunofluorescence. Cells were prepared for immunofluorescence, imaged, and deconvolved (Metamorph; Universal Imaging Corp) using either formaldehyde, formaldehyde followed by methanol or methanol alone as previously described (Dictenberg et al., 1998). All immunofluorescence images are two-dimensional projections of three-dimensional reconstructions to ensure that all stained material was visible in two-dimensional images. Quantification of signals produced by immunofluorescence staining for various midbody antigens was performed as described for centrosome protein quantification in our earlier studies (Gromley et al., 2003).

Antibodies. Antibodies to the following proteins were used: sec3, sec5, sec8 sec10, exo70, exo84, and sec15 (Yeaman, 2003), centriolin (Gromley et al., 2003); α-tubulin, γ-tubulin, α-6His, and α-myc (Sigma-Aldrich); aurora B (Transduction Laboratories); MKLP-1, GAL4 trans-activation domain (AD), GAL4 DNA binding domain (DBD) (Santa Cruz Biotechnology, Inc.) and GT335 for stabilized microtubules (Gromley et al., 2003).
Yeast Two Hybrid Screen. Yeast two-hybrid library screens were performed following the manufacturers instructions using a human testis Matchmaker Pre-Transformed Two-Hybrid Library (Matchmaker GAL4 Yeast Two Hybrid System; Clontech). False positives were eliminated by mating each clone with strains expressing either lamin C or the DNA Binding Domain alone and plating on quadruple dropout media.

siRNAs. Two siRNAs targeting centriolin and one targeting lamin A/C were used as described (Gromley et al., 2003). Additional siRNAs targeted nucleotides in the following proteins: MKLP-1, 189-207, sec5, 260-278, sec8, 609-627, snapin, 312-330. Cells were examined 24-48 hours after siRNA treatment. siRNAs were used at 10-50 nM and lipofectamine was the delivery agent (Gromley et al., 2003).

Brefeldin A treatment. Hela cells were treated with 5-10 μg/ml brefeldin A (Sigma-Aldrich) and imaged.

Immunoprecipitations. Antibodies to centriolin or exocyst were added to hTERT-RPE cell extracts and incubated at 4°C overnight. The lysis buffer included 50mM Tris HCl (pH 7.5), 10 mM Na2HPO4 (pH 7.2), 1 mM EDTA, 150 mM NaCl, 1% IGEPAL CA-1630 and protease inhibitors (Mini tablets, Roche Diagnostics, Mannheim Germany). Superose 6 samples were incubated with antibodies to sec3 and sec8, bound to protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4°C for 2 hours (Yeaman, 2003) then exposed to SDS-PAGE and immunoblotting (Harlow, 1988).
**Time-lapse imaging.** Time-lapse imaging of cytokinesis was performed using a wide field microscope (Gromley et al., 2003) and images were taken every 5 minutes for 18-24 hours. For lum-GFP expressing cells (Figure 7B), two concurrent time-lapse programs were used (GFP, phase contrast) and images were taken every 2 minutes for 3-4 hours. A Perkin-Elmer spinning disc confocal microscope was used for Figure 7A, C, D; images were taken 5 minutes using. Images of GFP-GAPCenA-expressing cells were taken every ten minutes using wide field microscopy. Mitochondria function was assessed by Mitotracker staining (Molecular Probes).

**Exocyst fractionation.** For isopycnic centrifugation, membrane compartments containing exocyst fractions were prepared as described (Yeaman, 2003). For size exclusion chromatography, cells were extracted with MEBC buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl) containing protease inhibitors (0.1 mM Na3VO4, 50 mM NaF, 1 mM Pefabloc (Boehringer Mannheim), and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A) for 10 min at 4 °C. Lysates were first sedimented in a Microfuge (Beckman Instruments, Fullerton, CA) for 10 min then for 30 min at 100,000 x g, passed through a 0.22-μm filter (Millipore) and loaded on a Superose 6 HR 10/30 column (200 μl, 10 mm x 30 cm; Pharmacia Biotech, Inc.) equilibrated in MEBC buffer and 1 mM dithiothreitol with 0.1 mM Pefabloc. Proteins were eluted (0.3 ml/min) at 17 °C in 0.5-ml fractions their concentration determined then used for various assays (fractions 7-30).
Figure 1. Centriolin Localizes to a Midbody Ring

(A) Immunofluorescence/phase image of HeLa cell during cytokinesis showing the phase-dense Flemming body within the larger diameter of the plasma membrane (arrowheads in [B]). (B and C). Boxed region enlarged with insets (B) to show the centriolin ring (Centr, enlarged in [C]) as part of the Flemming body (phase) and flanked bilaterally by Aurora B (Aur B). (D) α-tubulin localizes to the midbody ring (inset, Flemming body) and sites of presumed microtubule minus ends (arrowheads). (E) The Flemming body forms a ring. (F) GFP-tagged GAPCenA localizes to the midbody ring and is highly dynamic (time in s). (G–I) MKLP-1 localizes to the midbody ring (G) and, upon depletion, mis-localizes centriolin from the midbody (I). Immunoblots (IB) from cells treated with siRNAs targeting MKLP-1 or lamin A/C (control) (H). α–tubulin, loading control. Scale bars in (A), 10 μm; (B), 5 μm; (C), (E), and (G), 1 μm.
Figure 2. Centriolin Interacts with Exocyst Components and Snapin

(A) Gel filtration (Superose 6) using MDCK cell lysates shows that centriolin co-elutes with peak exocyst fractions (top). Immunoprecipitation (IP) of sec8 co-precipitates centriolin. Graph, total protein profile; markers a–e are indicated. (B) Following isopycnic centrifugation (iodixanol), centriolin co-migrates in peak fractions containing sec8 (upper panels). Graph shows sec8 levels, iodixanol density, and total protein. (C) Immunoprecipitation (IP) of Nud1-DBD (DBD antibody) pulls down sec15-AD (left). DBD, DNA binding domain; AD, activation domain; Con, control beads; Lys, lysate. (D) Endogenous exocyst components co-immunoprecipitate with endogenous centriolin (Cen IP). (E) Endogenous centriolin immunoprecipitates (Cen IP) over-expressed His6-tagged snapin.
Figure 2

A

- Proteins a, b, c, d, e are labeled.
- a: thyroglobulin (MW = 669,000)
- b: apoferritin (MW = 443,000)
- c: catalase (MW = 232,000)
- d: bovine serum albumin (MW = 66,000)
- e: cytochrome c (MW = 12,400)

B

- Centrin blot
- Sec8, Sec3, Centrin
- Sec8 IP-> centrin blot

C

- IB: Nud1 DBD IP Con Lys
  - Sec15AD
  - Nud1DBD

D

- IB: Can IP Con Lys
  - Centrin
  - Sec8
  - Sec5

E

- IB: Can IP Con Lys
  - Snapin (6 His)
Figure 3. Exocyst Localization to the Midbody Ring Is Centriolin Dependent

(A) Immunofluorescence images of exocyst components (green) co-stained with centriolin antibodies (panel 1) or with anti-α-tubulin antibody (red) to visualize microtubules (MTs, panels 2–6). Panel 1 inset: top, sec8; bottom, centriolin. (B) Cells depleted of centriolin lack midbody-associated exocyst. Images labeled as in A1–A5; B1 inset, Flemming body. Graph, percentage of midbodies (MB) without (w/o) sec8 signal following treatment with siRNAs targeting lamin A/C or centriolin; other cells have reduced levels (see text). (C) siRNA depletion of sec5 disrupts the exocyst from midbodies co-stained with two exocyst proteins (C1 inset, phase) or one exocyst protein and microtubules (C2–C3). Graph, percentage of midbodies (MB) lacking sec5 staining in cells treated with lamin A/C or sec5 siRNAs. (D) Exocyst disruption by siRNAs does not affect centriolin midbody localization. Graph, percentage of midbodies (MB) lacking centriolin stain following treatment of indicated siRNAs. Scale bar equals 1 μm (all panels). (E) Immunoblots showing reduction of proteins targeted by siRNAs. α-tubulin (γ-tub), loading control. Cen, centriolin.
Figure 3

A

B Centriolin siRNA

C Sec5 siRNA

D Sec8 or Sec5 siRNA
Figure 4. Exocyst Disruption Induces Cytokinesis Defects

(A) Time-lapse images of a HeLa cell treated with lamin A/C siRNAs showing a mitotic cell entering mitosis (arrow), forming a cleavage furrow, and cleaving into two separate cells in 3 hr. Time, hr:min. (B) A cell depleted of sec5 enters mitosis (arrow), forms a cleavage furrow with normal timing (~50 min), and remains interconnected by a thin intercellular bridge for over 17 hr (panels 1:50 through 17:05). (C) Graph shows percentage of mitotic cells that fail cytokinesis; many others are delayed (see text).
Figure 5. Centriolin siRNA Mis-localizes Midbody-Ring-Associated SNAREs and Snapin, which Disrupts Cytokinesis When Depleted

(A) Endobrevin/VAMP8 (1) localizes adjacent to the midbody ring when snapin is on the ring (2). Later, when the midbody diameter is thin (0.5–1 μm), endobrevin/VAMP8 and syntaxin-2 localize to the ring (3 and 4). (B) Centriolin-depleted cell shows loss of snapin from the midbody ring (green). Graph, percentage of midbodies lacking snapin after siRNA depletion of proteins. (C) Centriolin-depleted cells lose SNARE proteins from the midbody ring. Graph, percentage of midbodies lacking endobrevin/VAMP8 staining after indicated siRNAs treatments. Endo, endobrevin. (D) Snapin-depleted cells show loss of snapin from the midbody ring. Graph, percentage of midbodies lacking snapin after indicated siRNA treatments. (E) A snapin-depleted cell in cytokinesis (0) remains connected by a thin intercellular bridge for >17 hr before separating (20:25) (time, hr:min). Graph, percentage of mitotic cells that failed cytokinesis.
Figure 6. Disruption of the Exocyst Results in Accumulation of Secretory Vesicles at the Midbody Ring

(A) A mitotic cell (0, arrow) treated with BFA exits mitosis and forms a cleavage furrow with normal timing but arrests with a thin intercellular bridge that connects the two daughters (panels 2:35 through 6:45). (B) sec5 siRNA-treated cells accumulate endobrevin/ VAMP8-containing vesicle-like structures (arrows) at the Flemming body (arrowhead, panel 2). Dotted lines, plasma membrane. Graph, percentage of cells with endobrevin/VAMP8 vesicles at the midbody following indicated siRNA treatments. Scale bars, 2 μm. (C) Endobrevin/VAMP8 (green) localizes to luminal-GFP secretory vesicles (red). Box at midbody is enlarged in insets. Endo, endobrevin/ VAMP8.
Figure 6

(A) Sequential images showing the time course of a process over 6:45 minutes.

(B) Imaging of Endobreven/Aur B and Sec5 siRNA with a bar graph indicating the percentage of MB with vesicles.

(C) Fluorescent imaging of a cellular structure with phase, GFP, Endo, and Merge images.
Figure 7. Asymmetric Delivery of Secretory Vesicles to One Side of the Flemming Body Is Followed by Abscission at This Site

(A) A dividing HeLa cell expressing luminal GFP accumulates secretory vesicles on one side of the Flemming body (arrows in 2 and 3, inset). In panel 1, most luminal-GFP signal is in Golgi complexes (G1 and G2). The signal appears transiently at one side of the midbody (2 and 3, arrows; Movie S5) and is lost, although Golgi signal remains (4). Scale bar in panel 1, 10 μm. (B) Higher-magnification images of another cell (see Movie S6) showing unidirectional delivery of luminal-GFP-containing vesicles from one nascent daughter cell to one side of the Flemming body (arrowhead). GFP vesicles move to the Flemming body from the cell on the right (1:18 and 1:40, arrows; see Movie S6) and quickly disappear (1:52), presumably due to vesicle fusion with the plasma membrane and diffusion of the signal into the extra-cellular space. Phase and GFP signals are overlaid. Time, hr:min. Scale bar in panel 1, 1 μm. (C) Lum-GFP vesicle delivery to the Flemming body (0'–30', arrows) followed by signal loss (60', at arrow) and abscission (80' and 95'). Phase-contrast images were taken after disappearance of GFP signal. Enlargements of Flemming body are shown to the right of each low-magnification image in 70'–95'. Scale bar at 70': 10 μm for 0'–95' and 2 μm for enlargements in 70'–95'. (D) Lum-GFP vesicle delivery to one side of the midbody (panels 1–3) followed by disappearance of the GFP signal (panel 4) and abscission (loss of intercellular bridge, panels 5–7, arrows). The box in panel 5 is enlarged in panel 6. Solid and dotted lines show cell boundaries. (E) Post-mitotic cell (1) showing microtubules (green, GT335 antibody) of the intercellular bridge (phase-contrast image, inset) attached to one of the two daughter cells; no detectable midbody microtubules are seen on the other cell.
Microtubules are on both sides of the midbody ring (arrow, red, MKLP-1) and Flemming body (inset, phase), showing that the midbody with attached microtubules was delivered to one daughter cell. Prophase HeLa cell (2) with condensing chromatin (blue) and two centrosomes (green) has a midbody ring and lateral material stained with MKLP-1 (arrow, red) and in enlargement (bottom right); the Flemming body with flanking material is enlarged at upper right. Metaphase cell (3) with two midbody rings stained for MKLP-1 (red). Inset, two Flemming bodies corresponding to the two MKLP-1-stained structures. Centrosomes, green; DNA, blue. Interphase cell (4) showing four MKLP-1-stained midbody rings (red). Two are enlarged in lower inset and co-localize with phase-dense Flemming bodies (upper inset). DNA, blue; microtubules, green.
Figure 7
Figure 8. Model Depicting Vesicle-Mediated Abscission during Cytokinesis

(See text for details.)

(A) MKLP-1 and MgcRacGAP (green) arrive at midbody ring after cleavage furrowing has progressed. Microtubules, brown; plasma membrane, upper and lower lines. (B and C) Centriolin moves to ring ([B], blue) and anchors sec15, other exocyst components, and snapin ([C], red). (D) When midbody microtubules are reduced and the membrane constricted, v- and t-SNAREs ([D], black) move to the midbody ring from one prospective daughter cell. v-SNAREs presumably move with vesicles and bind there in a centriolin-dependent manner; t-SNAREs on the plasma membrane could bind through v-SNAREs. (E) Vesicles heterogeneous in diameter pack asymmetrically into the intercellular bridge adjacent to the midbody ring. (F and G) Vesicles adjacent to the ring containing SNARES and exocyst fuse with the plasma membrane (F) as well as at other plasma membrane sites and with one another (G). (H) Abscission follows at the site of membrane fusion, and the midbody is retained by the daughter cell opposite the fusion site. The released midbody ring contains multiple midbody-ring proteins and usually retains microtubule bundles from both sides of the ring. (In this model, the apparent “layering” of components is a simplification to depict arrival of different components at the midbody.)
Figure 8
Chapter 4:

A Transactivation-deficient p53 Mutant Has the Potential to Induce Severe Tumor Phenotypes and Centrosome-specific Abnormalities.
Contributions and Acknowledgments

MEFs were cultured and sent to the Doxsey lab by Michelle Beeche from the laboratory of Geoffrey M. Wahl, Ph.D. Paraffin embedded tumor tissue was provided by Monica Nister, M.D., Ph.D. of the Karolinska Institute, Stockholm, Sweden. Subsequent tissue culture maintenance, immunofluorescence, FACS sample preparation and FISH analysis were performed by Jack Rosa. Immunoperoxidase and In Situ Hybridization-peroxidase experiments were also performed by Jack Rosa in the lab of Dr. German Pihan, M.D., with technical supervision by Jan Wallace. Quantification of DNA content by FACS was conducted at the University of Massachusetts Medical Center FACS facility.
Introduction

The tumor suppressor p53 is a vital component in the defense against developing cancer. This is clearly illustrated by the degree of incidence of mutations in p53 found in a majority of human tumor types (Hollstein et al., 1991; Levine et al., 1991; Nigro et al., 1989). Furthermore, functional disruption of p53 signaling in mice leads to the early and spontaneous development of tumors and subsequent death (Donehower et al., 1992; Jacks et al., 1994). Though the role of p53 as a tumor suppressor is firmly established, the mechanism by which it is acting is still fervently debated.

p53 is most often described as a gatekeeper or caretaker. p53 essentially monitors the cells progression through the cell cycle and is activated by a broad range of cellular stresses and insults such as DNA damage, oncogene expression and hypoxia among others. These, in turn, lead to p53-activated pathways which include cell cycle arrest, senescence, DNA repair or apoptosis (Vousden and Lu, 2002; Wahl and Carr, 2001).

Molecularly, p53 is predominantly characterized as a transcription factor (Yu et al., 1999; Zhao et al., 2000). When tetramERICALLY assembled, the central domain of p53 exhibits sequence specific DNA binding. Once bound to the promoters of these p53-target genes, the gene is activated via interactions between the N-terminal domain of p53 and the transcriptional machinery. Both the abundance and regulation of p53 are predominantly regulated by an E3 ubiquitin ligase MDM2, in addition to some more recently characterized proteins (Dornan et al., 2004; Haupt et al., 1997; Leng et al., 2003). MDM2
binds the N-terminal transcriptional-activation domain of p53, directly blocking components of the transcriptional machinery and its E3 ligase activity can ubiquitinate p53 for protein degradation. Furthermore, MDM2 is itself activated by p53 resulting in a negative feedback loop that maintains p53 at low levels in unstressed cells. Upon introduction of a stressor (DNA damage, abnormal oncogenic expression etc.) p53 is activated, usually in the form of post-translational modifications such as N- and C-terminal serine phosphorylation or acetylation of the C-terminus (Appella and Anderson, 2001; Brooks and Gu, 2003). Alternatively, modifications of MDM2, which accelerate its degradation, result in p53 stabilization and subsequent activation of downstream targets (Stommel and Wahl, 2004).

Nearly all p53 mutants derived from tumors contain point mutations within the DNA binding domain that abolish or alter the recognition of p53 binding sites. These p53 mutants therefore lack the ability to activate target genes normally responsive to the wild-type (Prives, 1994; Vogelstein and Kinzler, 1992). Likewise, the identification of p53-inducible target sites suggests that the transcriptional function of p53 is likely the driving mechanism behind tumor suppression. The cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, one of the first p53 targets identified, is a prime example. The deletion of $p21^{Waf1/Cip1}$ abrogates the ability of p53 to induce a G1 arrest in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995). More recently, the BH3 domain protein PUMA has been shown to be vital in p53-mediated apoptosis by genetic knock-out and knock-down techniques (Jeffers et al., 2003; Villunger et al., 2003). In fact, the bioinformatic mining
of consensus sequences for p53-responsive elements helps drive the detection of new p53 targets (el-Deiry et al., 1992).

However, the contributions made by the transactivation domain to tumor suppression are not clearly understood. Some studies have shown that p53 is capable of a stress-response independent of its transactivation domain through a direct interaction with mitochondria, activation of pro-apoptotic proteins or inactivation of anti-apoptotic proteins (Chipuk et al., 2004; Mihara et al., 2003). p53 may even directly contribute to DNA double-strand break or base repair to maintain genetic stability (Rubbi and Milner, 2003; Zhou et al., 2001).

The design and cultivation of genetically engineered animals offer the ability to analyze whether the suppression of tumor formation requires transcription-dependent or transcription-independent p53 activity. Specifically, the mutation of two adjacent amino acid residues in the transactivation domain of p53, L25QW26S in mice (referred to as p53Qs), disrupts transactivation of downstream targets (Jimenez et al., 2000). In vitro, p53Qs/Qs mouse embryonic fibroblasts (MEFs), are indistinguishable from p53null MEFs in terms of p21 activation, cell cycle arrest and in DNA damage induced apoptosis. p53Qs/Qs MEFs are also transformed in vitro by cooperative oncogenes and develop tumors when injected into athymic nude mice. Also, in vivo analysis in mouse models demonstrated that p53Qs/Qs and p53null mice were equivalent with respect to spontaneous tumor development, in both incidence and spectrum when evaluated histopathologically (Nister et al., 2005).
Despite these abundant similarities our study demonstrates a compelling and distinguishing phenotype in p53\(^{QS/QS}\) MEFs and tumors. Previous work from our lab, and others, have shown a significant correlation between centrosomal defects and malignant, genetically unstable tumors (Ghadimi et al., 2000; Hartwell and Kastan, 1994; Lingle et al., 2002; Pihan et al., 1998; Pihan et al., 2001; Pihan et al., 2003). In order to determine whether p53\(^{QS/QS}\) mutants have any specific centrosomal defects MEFs were analyzed by immunofluorescence for the centrosomal proteins pericentrin and \(\gamma\)-tubulin. In a blind study, p53\(^{^{\text{wildtype}}}\), p53\(^{^{\text{null}}}\), and p53\(^{QS}\) were successfully distinguished from each other based on centrosome number, structure and spindle abnormalities, the most severe of which are associated with p53\(^{QS}\). Consistent with the centrosome defects, the DNA content of MEFs were analyzed by both flow cytometry and in situ hybridization of DNA probes and revealed that p53\(^{QS}\) MEFs contain a greater degree of aneuploidy. Given the distinct phenotypes found in the p53\(^{QS}\) MEFs we next investigated paraffin embedded tumor samples from p53\(^{null}\) and p53\(^{QS}\) transgenic mice. Using the same antigens and DNA probes, sectioned tumor tissue was immunoperoxidase stained. As expected, tumors derived from p53\(^{QS}\) mice exhibited distinct centrosomal defects and an elevated level aneuploidy. Based on the data, we propose that p53 either directly or indirectly contributes to the stability of centrosome structure and function. Also, a fully functional p53 may be requisite for the maintenance of a proper centrosome.
Results

Centrosomal and spindle Defects in p53^{Qs/Qs} MEFs.

For this study, four vials, labeled only as “1” through “4” were thawed and the cultures expanded to provide a pool of cells for analysis. As mentioned earlier, centrosomal defects are associated with malignancy and genetic instability. Operating under the assumption that the homozygous mutant exhibits more severe centrosomal anomalies, samples taken from each of the four pools were stained for the centrosomal proteins pericentrin and γ-tubulin. The predominant phenotype was a deviation from the wild-type number of centrosomes, that being either a single focus or two foci. As expected, wild-type fibroblasts maintained the most stable profile, maintaining dominant populations containing one focus (indicative of G1) and two foci (representing the cells transition into G2). In addition, p53^{null} MEFs showed a considerable increase in cells carrying two foci and a concomitant increase in cells with γ-tubulin staining of three foci or greater. Remarkably, abnormal centrosome number is exacerbated in MEFs that are p53^{+/Qs} and p53^{Qs/Qs} (Graph. 1). The percentage of cells carrying a single γ-tubulin focus is severely depleted while the population of cells carrying two foci increases.

Furthermore, there is a significant increase in the population of cells having greater than the wild-type number of foci; over 30% of the population contains an aberrant number of centrosomes in MEFs carrying a p53^{Qs} mutation, with the most severe cases being associated with the homozygous mutant (Fig. 1A-H). Naturally, as the precursor to the spindle poles, a concurrent increase in spindle abnormalities should be observed. Indeed, when we characterized the structure and number of foci in the spindle there was a
significant increase in abnormalities in p53\textsuperscript{QS/QS} MEFs (Graph. 2). In addition, p53\textsuperscript{QS/QS} MEFs contained more severe abnormalities in number and structure of the spindle poles (Fig. 1 I-P). Interestingly, in p53\textsuperscript{QS} MEFs the quality of abnormalities in spindles was not limited to the number of spindle poles. In several instances the structure of the poles themselves was abnormal, appearing as a “crescents” or “threads” which apparently retained the ability to interact with microtubules and participate in spindle formation.

\textbf{p53\textsuperscript{QS/QS} MEFs Exhibit an Abnormal Ploidy and Chromosomal Content.}

Due to the centrosome’s role in mitotic chromosome segregation, it contributes significantly to the maintenance of genomic stability. Also, a relationship between centrosomal abnormalities and chromosomal instability has been demonstrated in other model systems. We therefore asked whether the obvious centrosome defects we observed specifically in the p53\textsuperscript{QS} mutants had a corresponding relationship to its DNA content. Samples drawn from each of the four pools were processed and analyzed for their DNA content by flow cytometry. The resultant DNA profiles were then compared by the distribution of their 2N and 4N populations (Fig. 2A). p53\textsuperscript{wt}, p53\textsuperscript{null} and even p53\textsuperscript{+/-QS} show a dominant 2N (G1) peak and a cycling 4N (G2/M) peak, representing the duplication and segregation of their genome. On the other hand, the p53\textsuperscript{QS/QS} mutant has a dominant 4N peak and a subsequent 8N peak in addition to a diminished 2N peak (Fig. 2A). To determine the nature of the chromosomal content in these cells, chromosome specific point probes were used for in situ hybridizations. Upon quantifying the amount of hybridized probe in each cell, the distribution is consistent with our flow cytometry...
(Fig. 2B, compare signal number= 2, 4 and >4). In the wild-type MEF line most cells carried only two copies of the point probe in most cells, with a smaller, duplicated population carrying four. Although the \( p53^{\text{null}} \) and \( p53^{+/QS} \) MEFS exhibit a degree of variability when compared to the wild-type, they both maintain a dominant population with only two copies of the chromosome per cell. In contrast, the distribution of the chromosomal probe in \( P53^{QS/QS} \) MEFs represents a higher degree of variability of chromosome copy number per cell. (Fig. 2A, compare signal number 1, 3 and >4). Therefore, not only does the \( p53^{QS/QS} \) mutant carry severe centrosomal abnormalities, these structural defects can also be associated with increased chromosomal variability.

**Tumors from \( p53^{QS/QS} \) Mice have Distinct Centrosomal Phenotypes and Greater Chromosomal Instability.**

The initial characterizations of \( p53^{QS} \) mice relied on in vitro methods and subsequent analysis focused on tumor kinetics and gross histology (Jimenez et al, Nister et al). Here we immunohistochemically probe tumor tissue-sections for centrosomal defects. Tumor sections, predominantly soft tissue sarcomas embedded in paraffin, were immunoperoxidase stained for pericentrin and counterstained. Centrosomal labeled could be observed as a dense brown dot and, in the case of mitotic cells, two dots, one at each pole. Centrosome number per cell was quantified within each tissue section of \( p53^{QS/QS} \) and \( p53^{\text{null}} \) tumors for comparison. \( P53^{\text{null}} \) tumors exhibit a relatively normal profile with most of their population having only one focus where as \( p53^{QS/QS} \) cells had clusters of foci. (Graph 3, Fig. 4 A-D). Upon closer inspection, \( p53^{QS/QS} \) tumors typically had
pericentrin staining resembling aggregates of foci of varying size, an amorphous region of staining or both, with the cluster localized within a region of diffuse staining in the cytoplasm. (Fig. 3AI, BII, III, IV; Graph 4 A). Conversely, p53\textsuperscript{null} tumors typically had a single pericentrin focus with diameters twice that of foci from surrounding normal tissue or greater. (Fig. 3 CV, BVI; Graph 4 B). In cases where p53\textsuperscript{null} tumor tissue had multiple foci they were usually very discrete, dense structures that did not aggregate. The foci themselves did not appear to be composed of smaller aggregates. Associated with the aberrant centrosomal structure, the homozygous mutant tumors also appeared to have a greater heterogeneity in its nuclear morphology (Fig. 3 AI and III, Graph 4 C). Cells could be seen to have large, lobular nuclei and showed a greater degree of variability in their nuclear dimensions. To determine if the drastic differences in centrosome structure and nuclear morphology are associated with any changes in chromosome stability, tumor sections were also hybridized with chromosome specific probes. When compared to p53\textsuperscript{null} tumors, p53\textsuperscript{QS/QS} tumors showed a much greater distribution of chromosome copy number (Fig. 4, Graph 5 A, B). In p53\textsuperscript{null} tissue the majority of the signal represented a diploid population, two copies per cell. The p53\textsuperscript{QS/QS} tumors exhibit a dramatic redistribution of copies per cell, reducing the percentage of cells carrying two copies per cell and showing increases from three to nine copies per cell. Taken as a whole, these data suggest that p53 plays a critical role in the structure and function of the centrosome. The presence of a transactivation-deficient p53 appears to impact the level and organization of pericentriolar components resulting in ectopic and aberrant complexes that lead to abnormal spindle and missegregation events.
Discussion

Prior to this study, the transactivation deficient p53 mutant (p53\textsuperscript{QS}) has been the target of other investigations that have endeavored to define a specific role, either dependent upon or independent from, the transcriptional regulation function of p53. The initial analysis of p53\textsuperscript{QS} employed in vitro molecular and biochemical techniques to test the ability of p53\textsuperscript{QS} to activate known downstream targets or respond to specific stimuli (Jimenez et al., 2000). Subsequent analysis focused on the function of transcriptional regulation by p53 on the suppression of tumors and looked mostly at the gross morphology of tumor tissue (Nister et al., 2005). Logically, the introduction of mutations at codons 25 and 26 interferes with the ability of p53 to interact with the transcriptional machinery and should result in a crippled regulatory molecule. Though not completely unexpected, p53\textsuperscript{QS} has proven to be very similar, if not identical, to p53\textsuperscript{null}.

In contrast, using immunofluorescence and immunoperoxidase labeling techniques, we have successfully identified distinguishing phenotypes between p53\textsuperscript{null} and p53\textsuperscript{QS} MEFs. Furthermore, these distinguishing features persist in the development of tumor tissue in transgenic mice. The p53\textsuperscript{QS} MEFs missegregate chromosomes and exhibit severe centrosomal abnormalities beyond the level of p53\textsuperscript{null} MEFs. The subsequent tumors in p53\textsuperscript{QS} mice are also distinguishable from p53\textsuperscript{null} tumors. Specifically, in p53\textsuperscript{QS} tumor tissue, centrosomal abnormalities were not only more severe and abundant but also structurally distinct from tumors derived from p53\textsuperscript{null} mice. While p53\textsuperscript{null} tumors had discrete centrosomes of considerable diameter, p53\textsuperscript{QS} tissue presented with aggregates of
smaller pericentrin-staining foci or with localized regions of diffuse staining. The centrosome has been shown to be structurally and numerically abnormal in most cancers, particularly those of a malignant and invasive nature. However, although these studies have shown that supernumerary centrosomes are observed in cells lacking p53 (Hinchcliffe et al., 1999), centrosomal abnormalities can be observed in cells with wild-type levels of p53 (Lengauer et al., 1997; Pihan et al., 1998). Our data represents a unique case where centrosomal defects and perturbations in ploidy have been characterized within the context of a specific p53 mutation.

Though p53Q5 is indistinguishable from P53null with regards to activation of specific, down-stream, molecular targets, it does not exclude the possibility that the transactivation domain maybe required to, perhaps, regulate an epigenetic effect through the centrosome. Loss of transactivation activity induced centrosomal abnormalities in both number and structure, which also occur in p53null cells. However, our study clearly shows that the severity of these phenotypes is specific to the homozygous mutant. Furthermore, these p53Q5 specific phenotypes can be observed in both embryonic fibroblast culture and in the adult mouse. Though there may not be significant differences in the tumor spectra or kinetics between p53null and p53Q5 mice, it may be interesting to propagate explants of these tumors in subsequent nude mice. The centrosomal defects associated with p53Q5/Q5 tumors may be an early indicator of particularly malignant characteristics (i.e. metastasis or chemotherapeutic resistance). The severity of the phenotype in MEFs and the abundance and quality of the abnormalities in p53Q5 tumors correlate with an increase in chromosome missegregation. When coupled with the selection pressures that the tumor
experiences in vivo, the missegregation events provide a means by which cells can accumulate tumor-promoting genes and lose copies of necessary tumor suppressors. Consequently, these cells could have a significant predisposition to accumulate additional genetic lesions, leading to a malignant phenotype.

Pericentrosomal proteins such as γ-tubulin and pericentrin form a highly organized structure at the centrosome, typically referred to as the pericentrosomal matrix (PCM). Specifically, pericentrin forms a three-dimensional, reticular lattice at the centrosome which increases in both size and complexity as the cell progresses from G1 into mitosis (Young et al., 1999). The pericentrin lattice then acts as an efficient scaffold onto which protein complexes can be tethered. Among these proteins is γ-tubulin, which is essential for linking the centrosome to microtubules and for microtubule nucleating complexes. Abnormal levels of pericentrin have been observed in cancer cell lines and tumor tissue. Typically, elevated levels of pericentrin resulted in multiple foci, sometimes interconnected by filaments. These supernumerary, interconnected structures were often acentriolar yet could still nucleate microtubules (Pihan et al., 1998). In light of these observations, perhaps the transactivation activity of p53 is required for maintaining the integrity of the pericentrosomal lattice. p53null tumors cells maintained discrete supernumerary foci with larger diameters. In contrast, p53Q5/Q5 MEFs and tumor tissue expressed more severe abnormalities in pericentrin structure. The diffuse and aggregate pattern seen in p53Q5/Q5 tumor tissue may represent a disruption or a deficiency in the organization of the pericentrosomal superstructure, where a transactivation-deficient mutant of p53 is present as opposed to p53null cells that lack p53 completely. The
literature has not only localized p53 to the centrosome but has also associated it with molecular chaperones (Ciciarello et al., 2001; de Carcer, 2004; Hut et al., 2005; Tarapore et al., 2001; Tritarelli et al., 2004). Hsp90 and Hsp70 both appear to protect the centrosome from heat induced damage and maintain spindle checkpoints (de Carcer, 2004; Hut et al., 2005). More recently, mortalin, a member of the heat-shock protein family, has been shown to localize to the centrosome in late G1, before duplication. Mortalin remains on the centrosome during S and G2 (during which time pericentrin has been forming a lattice) and dissociates during mitosis. Mortalin also physically interacts with p53 and promotes its dissociation. P53 mutants that lack the ability to bind mortalin remain at centrosomes and suppress duplication, (Ma et al., 2006).

The mechanism and pathway of centrosome amplification and aneuploidy in tumors is still not clearly defined. Part of the current dogma suggests that p53 prevents centrosomal duplication by inhibiting CDK2/cyclinE through the transactivation of p21 (Fukasawa, 2005). If this is true then the transactivation deficient mutant may provide another facet in our understanding of p53 and how it relates to the centrosome. Clearly p53^{QS/QS} cannot activate p21, similar to p53^{null} mutants. Unlike the p53^{null} mutants, p53^{QS/QS} cells still maintain a level of p53 molecule, albeit a p53 with a crippled transactivation domain. In this case p53^{QS/QS} may function as a dominant/ negative where a specific activity is abolished, but it can still interact with and sequester other components. This does not conclusively show that p53 directly interacts with the centrosome although there have been attempts to localize p53 to it, at least in human cell
lines (Ciciarello et al., 2001; Morris et al., 2000; Tarapore et al., 2001; Tritarelli et al., 2004).

Our data provide a compelling contrast to the initial studies on the transcriptionally inactive p53\textsuperscript{QS} mutant. At the molecular and biochemical level, there seems to be little difference between p53\textsuperscript{QS} and p53\textsuperscript{null} cells (Jimenez et al., 2000; Nister et al., 2005). Interestingly, with the appropriate cell biology techniques, it is possible to evaluate tissue and successfully distinguish between the p53\textsuperscript{QS} and p53\textsuperscript{null} mutants. Our classification is based on the increasing severity of centrosomal abnormalities and chromosome instability in p53\textsuperscript{QS/QS} mutants relative to p53\textsuperscript{null}. Also, these characteristics persist in developed murine tumors and give rise to distinct phenotypes in centrosomal structure and elevated aneuploidy. Taken together, the data suggests that p53 may be playing a more integral role in centrosome structure and organization, perhaps in addition to its role in cdk2/cyclinE inhibition. These data introduce another dimension to our knowledge of p53 and illustrate how understanding roles of p53 will require an understanding of its context within the cell.

**Materials and Methods**

**Cell culture.** Primary MEFs were provided by Dr. Geoffrey M. Wahl and were maintained as originally described [Jimenez, 2000 #31]. To remain consistent between experiments, three 10cm plates for each genotype were pooled and re-suspended in 9mls
of room temp phosphate buffered saline (PBS). 3ml samples were reserved for each experiment.

**Antibodies.** Centrosomes were stained with either anti-\(\gamma\)-tubulin (Sigma, cat# T3559; St. Louis, MS) or anti-pericentrin (affinity purified clone 4b or M1) polyclonal antibodies and monoclonal anti-\(\alpha\)-tubulin (Sigma, clone DM1 \(\alpha\), cat# T9026). For immunofluorescence anti-rabbit-FITC and anti-mouse-CY3 secondaries were used (Jackson ImmunoResearch, cat# 711-095-152 and 711-165-152; West Grove, PA and counterstained with DAPI.

**Immunofluorescence.** samples were taken from the pool of cells and resuspended in 5mls of room temperature PBS and cyto-spun onto 12mm glass coverslips. Cells were subsequently fixed in methanol and fixed as previously described (Pihan et al., 1998). Slides were analyzed using wide-field fluorescence microscopy on an inverted microscope (Olympus, JAPAN) and a 100X objective from random fields. All images represent 2D projections of 3D stacks (Metamorph Universal Imaging Corp.) to ensure that all stained structures were captured within the cell. Individual cells were then scored based on the number and morphology of stained structures.

**FACS analysis.** Cells fixed in 90% ethanol overnight at 4\(^{\circ}\)C. Fixed samples were then stained with propidium iodide and analyzed by flow cytometry (FACSCAN\textsuperscript{®}; Becton Dickinson) using Flojo software (Tree Star, Inc.).
**FISH analysis.** Cells were first incubated in hypotonic KCl containing nocodazole at a final concentration of $5\mu g/ml$ at $37^\circ C$ for 15min. Cells were gently pelleted and resuspended Carnoy’s solution (1:3 glacial acetic acid:methanol). Cells were then dropped on glass microscope slides over a $75^\circ C$ bath as described in (Henegariu et al., 2001). Biotin-conjugated point probes for mouse chromosomes 5 (cat# MB1004, $0cM$ from centromere) and 18 (cat# MB1023, $0cM$ from centromere) were purchased from IDlabs (London, Ontario) and visualized by FITC-avidin according to the manufacturer’s protocol. Slides were analyzed by wide-field fluorescence as described for immunofluorescence.

**Immunoperoxidase labeling of tumor tissue for centrosomal antigens.** Paraffin embedded tumor sections mounted on glass capillary slides were provided by Dr. Geoffrey M. Wahl. Tissue was then probed with anti-$\gamma$-tubulin and with anti-pericentrin as described in (Pihan et al., 1998; Pihan et al., 2001). Centrosomes were evaluated based on diameter (greater than or equal to twice the diameter of centrosomes in normal surrounding tissue) and numeration (greater than two foci).

**Immunoperoxidase labeling of DNA in situ hybridization probes in tumor tissue.** Tissue sections parallel to the ones used for centrosomal labeling were used to stain sub-centromeric point probes for mouse chromosome 5 and 18 as described in (Pihan et al., 1998; Pihan et al., 2003). The tissue was then scored based on the number of hybridization signals per nuclei.
Graph 1. P53<sup>QS</sup> increases the number of γ-tubulin in MEFs.

Transgenic mouse embryonic fibroblasts of 4 distinct genotypes of the tumor suppressor p53 (+/+,-/-, +/QS, QS/QS) were stained with anti γ-tubulin and the number of foci in each cell was quantified. As indicated in the graph, cells containing the transactivation-domain mutant of p53 (QS/QS) maintains a greater population of cells with an abnormal number of γ-tubulin foci (~30% of the total population counted.)
Graph 2. P53\textsuperscript{QS} induces abnormal spindles

Transgenic mouse embryonic fibroblasts of 4 distinct genotypes of the tumor suppressor p53 (+/+, -/-, +/QS, QS/QS) were stained with either anti γ-tubulin or anti-pericentrin and anti α-tubulin. Mitotic cells were categorized as either normal or abnormal based on the number of spindle poles and the geometry of the spindle. As indicated in the graph, cells containing the transactivation-domain mutant of p53 (QS) have a greater percentage of spindle abnormalities.
Graph 2

Spindle quality amongst p53QS

% spindle observe

Normal

Abnormal

+/+
/-
+/QS
QS/QS
Figure 1. Centrosomal abnormalities in p53QS mutants.

MEFs stained for γ-tubulin and α-tubulin revealed severely abnormal centrosomes in interphase cells (A-H) and abnormal spindle structure in mitotic cells (I-P). Note that the structure of γ-tubulin staining in some spindle appears not as foci but as strands (arrows, I and J) that contribute to the spindle mass and result in wider poles. The most severe phenotypes are associated with the homozygous mutant p53^{Q8QS}. Indicated by the graphs, the homozygous mutant is associated with increased abnormalities. In addition, the severity of the abnormalities is also greater as demonstrated in the image above. The γ-tubulin staining illustrates approximately 28 foci (Q), each of which appears to be capable of nucleating microtubules (R, overlay in T).
Fig. 2 Flow Cytometry and FISH Analysis of p53<sup>Qs/Qs</sup> MEFs.

(A) Asynchronous MEFs were fixed and stained for DNA content (propidium iodide). A tetraploid peak predominates specifically in the transactivation-deficient mutant p53<sup>Qs/Qs</sup> and appears to have a cycling 8N population. (B) Point probes for chromosome 5 and 18 were used for FISH in fixed MEFs. The probes could be observed as individual foci (D and green overlay in E) within the stained nuclei (C and red overlay in E). Signals were quantified from each nucleus and graphed (B) to illustrate the distribution of chromosome 5 (similar results obtained with probes to chromosome 18). Consistent with the FACS of DNA content, the homozygous mutant (p53<sup>Qs/Qs</sup>) contains a shift towards a tetraploid population.
Figure 2

A

Ungated

2N 4N 8N

B

FISH analysis in p53QS

% pop

signal

1 2 3 4 >4

C

D

E

156
Graph 3. Centrosome number is subsequently altered in tumors derived from p53<sup>Q5/Q5</sup> mice.

γ-tubulin localization was analyzed by immunoperoxidase staining in paraffin-embedded tumor sections derived from homozygous mutant mice (p53<sup>Q5/Q5</sup>). Compared to null mice (p53<sup>+/+</sup>), there is significant increase in pericentrin foci in mutant-derived tumors.
Graph 4. p53^{0s/0s} Induces Structurally Distinct Defects in Centrosomes.

(A) In homozygous mutant tumors, cells contained diffuse staining of pericentrin and pericentrin that appear as localized “clouds” that often contained denser aggregates. This phenotype was striking when compared to p53^{null} tumors (B), which usually contained discrete and dense foci that were greater in diameter than normal centrosomes in neighboring tissue (1.5-2x greater). Consistent with cultured MEFs, the homozygous mutant contained more nuclear abnormalities in the form of larger and fragmented nuclei.
Graph 5

A. Centrosomal structure (clouds/ aggregates)

B. Increased Centrosome

C. Multinucleated and Large

y-axis: Ave %pop
Figure. 3 Structural Abnormalities in p53<sup>Q5/Q5</sup> Tissue.

(A and B) Diffuse staining of pericentrin that appears as localized “clouds”, these often contained denser aggregates (I, II). In p53<sup>Null</sup> tumors (C and D), cells typically contained discrete and dense foci that were greater in diameter than normal centrosomes in neighboring tissue (1.5-2x greater) (V, VI). Consistent with cultured MEFs, the homozygous mutant contained more nuclear abnormalities in the form of larger and fragmented nuclei.
Figure 3
Graph 5. p53^{Q5/Q8} Derived Tumor Tissue has Increased Aneuploid Signal

Quantification of In-situ hybridization signal reveals that p53^{null} sarcomas still maintain a dominant diploid population (B, asterisk) In contrast, the transactivation deficient mutant (A) has a reduced diploid signal and subsequent increases in multi-plaid populations, demonstrating greater variability in its DNA content.
Graph 5

Ave. % Ploidy Among P53\textsuperscript{Q5/Q5} Sarcomas

Ave. % of Pop

Probe Foci

A

Ave. % Ploidy Among P53\textsuperscript{null} Sarcomas

Ave. % of Pop

Probe Foci

B
Chapter 5:

Discussion
The first chapter of this dissertation proposes that survivin functions as a novel regulator of microtubule dynamics and microtubule nucleation throughout the cell cycle, and that this pathway is independent of the expression or activity of the chromosomal passenger protein Aurora B. Time-lapse imaging of living cells using two independent GFP-labeled microtubule markers, the plus end protein EB1 and α-tubulin, combined with quantitative analysis of multiple parameters of microtubule dynamics revealed that survivin affected rates of microtubule catastrophe and the degree of centrosomal microtubule nucleation. By employing multiple techniques and parameters, the data presented in the first chapter is consistent both internally and with current theories that survivin plays a role in microtubule dynamics and microtubule nucleation independent of at least one protein of the chromosomal passenger complex (Aurora B). No effect was observed on microtubule dynamics or nucleation when Aurora B was pharmacologically inactivated during short incubation periods (up to 6 hours) even though inhibitory activity dramatically affected spindle function. The spindle phenotypes that are observed during inhibition of Aurora B activity or depletion of Aurora B levels are dramatically different from survivin-depleted or over-expressing cells. Interestingly, survivin affected microtubule dynamics and nucleation in interphase when Aurora B is thought to be absent or drastically reduced. This would be consistent with previous studies showing that survivin is in multiple separate compartments within cells (Fortugno et al., 2002), whereas Aurora B and other chromosomal passenger proteins have not been localized to these other sites. This is further demonstrated by biochemical studies suggesting that cells possess a fraction of survivin that is not physically associated with the chromosomal passenger complex; even if it was in the complex, survivin could still affect microtubule dynamics and nucleation.
independent of other members of the complex. In light of the data demonstrating
survivin’s distinct modulation of microtubule dynamics, a putative model is proposed that
attempts to unify the variety of survivin phenotypes described in the published literature.
The dynamic nature of survivin, and its existence in multiple and biochemically distinct
cellular pools, allows it to affect the organization/function of the mitotic spindle, the
spindle checkpoint, midbody and the nature of the microtubule network in interphase
cells. In this way survivin may contribute to important microtubule-based functions
throughout the cell cycle.

Two classes of molecules have been defined in the IAP gene family; one class is involved
in cell division while the second suppresses apoptosis by inhibiting the maturation and
proteolytic activity of caspases. However, survivin, being a structurally distinct IAP,
represents an apparent exception to the rule. Evidence suggests that survivin plays a role
in both functions, inhibiting apoptosis cooperatively with other cofactors and shuttling of
a mitochondrial pool (Marusawa et al., 2003) while its role in mitosis remains
controversial. Based on the catastrophic mitotic defects observed by functional
abrogation of survivin, it is typically viewed as indispensable during cell division.
However, the data accumulated in chapter 1 suggest that the complexity of defects
observed by modulation of survivin levels is the result of survivin’s ability to control
microtubule nucleation and dynamics.

In post-anaphase cells, the microtubules of the midzone, once occupied by the metaphase
plate, are actively bundled together by protein complexes such as centralspindlin
(MKLP1 and MgcRacgap) and MAPs (like PRC1). It is suggested that the chromosomal passenger complex (which includes INCENP, survivin and aurora B) plays a role in recruiting the centralspindlin complex to the central-spindle microtubules. A role for survivin in cytokinesis was suggested by experimentation with ancestral IAP proteins in model organisms (Speliotes et al., 2000) and by survivin depletion experiments which led to cleavage furrow regression (Chen et al., 2000). The first chapter makes the interesting observation of midbody microtubules as highly dynamic in normal cells by analyzing GFP-EB1 dynamics and that these dynamics are severely inhibited when survivin levels are altered, accounting for the observed cytokinesis defects. The result is surprising in light of the apparent level of tubulin modification that is observed in the midbody of normal cells (abundance of acetylated tubulin), which typically leads to the assumption that the midbody microtubules are static and stable to begin with. Moreover, the microtubules grow toward the central midbody, as might be expected given the organization of the preceding mitotic spindle, but they also grow away from the central midbody. This suggests that multiple populations of microtubules, differing in polarity, and dynamics, exist in the midbody. They could arise from overlapping microtubules of the central spindle, cytoplasmic microtubules from the cell-proper that invade the midbody or midbody-generated microtubules that are nucleated by γ tubulin at the central midbody. It is important to note that the bi-directional nature of microtubule growth in the midbody correlates with a very late-stage cytokinesis morphology. In the assembly of the central-spindle survivin may have the physiologically relevant role of contributing stabilized microtubules that can be efficiently bundled (Rosa et al., 2006), chapter 1. Consistent with this are FRAP experiments demonstrating that survivin becomes
essentially immobile once it becomes associated with the midbody (Delacour-Larose et al., 2004). By telophase, MKLP1 translocates to the phase-dense midbody ring and the central-spindle itself resembles a diminutive version of the spindle; γ-tubulin localizes to foci at either extreme end of the microtubule bundle in the midbody and to the midbody ring in the center ((Gromley et al., 2005), chapter 2).

Another very interesting event that occurs within the midbody is the delivery of the secretory vesicles themselves, as observed by lum-GFP expression. The luminal GFP vesicles traffic into the midbody predominantly from one cell and accumulate in the intracellular bridge adjacent to the phase-dense midbody ring. The central-spindle acts as a platform or docking site for the organization of membrane trafficking events that result in membrane fusion and abscission. It is at this asymmetrical site that fusion presumably occurs and the daughter cells are separated. The signaling pathway or mechanism of the unidirectional vesicle delivery is currently unknown. However, what can be demonstrated now is a temporally-organized hierarchy of events that are vital to successful abscission (Gromley et al., 2005) chapter 2 figure 8.

Both exocytic and endocytic events have been implicated in the final stages of cytokinesis but with distinct differences. At cytokinesis, RE vesicles traffic to the midbody and can be identified as such by the presence of Rab11-FIP3 and Rab11-FIP4 complexes (Wilson et al., 2005). The recruitment of the Rab11 complexes is regulated by active Arf6 and the ability of the FIP proteins to bind tightly to Arf6 in a GTP dependent manner (Fielding et al., 2005). However, these molecules, though dynamic,
occur with complete symmetry in the prospective daughter cells. Using live imaging techniques and fixed tissue analysis, all the components of the endocytic model localize dynamically to both sides of the midbody. In contrast, the centrosomal protein centriolin localizes specifically to the phase-dense midbody ring (dependent upon MKLP1 localization) and acts as a scaffold for the assembly of secretory pathway components, which include v- and t-SNARES and the exocyst complex. The secretory vesicles move into the intracellular bridge asymmetrically, from one of the prospective daughters, and accumulate adjacent to the midbody ring before fusing. The exact physical mechanism of the fusion event is not understood but may involve a v-SNARE-mediated compound exocytic event. A recent publication proposes a potential link between these two pathways that requires the exocyst. The FIP3/FIP4-Arf6 at the midbody regulates a docking event that involves Exo70p, a component of the mammalian exocyst complex, prior to endocytic membrane fusion (Fielding et al., 2005). In this way, animal cytokinesis becomes increasingly similar to plant cytokinesis which exploits both endocytic and secretory to coordinate and organize complex membrane dynamics.

Several proteins apparently localize to the midbody ring, the phase-dense Flemming body is also organized into the shape of a ring. Earlier ultra-structural studies that describe cytoplasmic channels traversing the central midbody confirm this observation (Mullins and Biesele, 1977). The ring structure may be analogous to the bud scars of S. cerevisiae, which putatively serve as markers for longevity (Chen and Contreras, 2004). The midbody ring in animal cells is inherited by the daughter cell that lies opposite the site of vesicle delivery and appears to persist, as it is often seen in mitotic cells prior to
cytokinesis and found in multiple copies in interphase cells (Figure 7E chapter 2, Gromley et al., 2005) (Mishima et al., 2002). Shortly after abscission, the midbody ring contains microtubules that extend from both sides of the ring. This suggests that dissolution of microtubule bundles adjacent to the midbody ring is not an absolute requirement for the final stage of cytokinesis but rather that abscission can result in transfer of the entire mid body and associated microtubules into one daughter cell.

The asymmetrical nature of the abscission event has never been observed in animal cells until now. The origin or signal that induces the asymmetry is not known though the centrosome presents itself as a tempting candidate. It has been suggested that the mother centriole moves to the intercellular bridge in telophase cells to coordinate the final steps in cytokinesis (Piel et al., 2001), however this is not a consistent observation and may actually be the result of cell line variations or tissue culture conditions. Yet, the centrosomes in the dividing cell are, in fact, different in that one centriole is “born” from the older centriole in the previous cell division during the centrosome duplication process (Doxsey, 2001). As more age-specific centriole markers become available it will become possible to identify which centriole, older or younger, correlates with the site of abscission.

Outside of cytokinesis, the centrosome plays additional roles in coordinating vital signaling events to regulate the cell cycle. The centrosome’s highly organized and specific structure provides a site for anchoring and coordinating regulatory signal molecules. When the structural integrity of the centrosome is disrupted, as in siRNA of
centrosomal proteins, a p53 dependent G1 cell cycle arrest is induced (Doxsey et al., 2005a; Doxsey et al., 2005b). Conversely, the disruption of cell-cycle regulatory molecules can lead to centrosomal abnormalities (Ciciarello et al., 2001; Morris et al., 2000; Tarapore et al., 2001; Tritarelli et al., 2004). As a result, centrosome anomalies and aneuploidy have become benchmarks of cancer in both human and animal models (Nigg, 2002; Pihan et al., 2003).

Supporting evidence can be found in studies of the major tumor suppressor gene p53. Current models propose that p53 prevents centrosomal duplication by inhibiting CDK2/cyclinE through the transactivation of p21 (Fukasawa, 2005). However, most of the analyses of p53 and the centrosome have been carried out under conditions where p53’s abundance exceeds that of normal cells. Additionally, many studies were conducted in transformed cell lines, which can carry genomic alterations that may affect p53’s activation or signaling.

In an effort to develop a biologically relevant system, homologous recombination techniques were employed to develop mouse embryonic fibroblasts and mice that were either null for p53 or carried a transactivation-deficient mutant, p53<sup>Q8</sup> (Jimenez et al., 2000). Though biochemically and molecularly identical to p53<sup>null</sup> MEFs, the p53<sup>Q8</sup> cells could be identified by an increase in centrosomal abnormalities and elevated chromosomal instability. More specifically, tumors derived from p53<sup>Q8</sup> mice exhibited distinct defects in the staining pattern of centrosomal antigens. Diffuse, patchy areas that often times contained denser aggregates were observed. It is tempting to believe that the
p53QS mutant can specifically disrupt the organization of the centrosome. Recently, chaperone molecules have been associated with the centrosome and are believed to protect the integrity of the centrosome’s structure (Ciciarello et al., 2001; de Carcer, 2004; Hut et al., 2005; Tarapore et al., 2001; Tritarelli et al., 2004). Of particular interest is mortalin, a member of the heat-shock protein family, which has been shown to localize to the centrosome in late G1, before centrosome duplication. Mortalin remains on the centrosome during S and G2, as the centrosome accumulates and organizes PCM, and dissociates during mitosis. Mortalin also physically interacts with p53 and promotes its dissociation. P53 mutants that lack the ability to bind mortalin remain at centrosomes and suppress duplication, (Ma et al., 2006). The p53QS mutation not only inhibits transactivation of downstream targets, it also prevents interaction with MDM2, a key regulator of p53 stability. As a result, the p53QS mutant is not degraded and is constitutively present at abundant levels in the cell. In this way the p53QS may be exerting a dominant-negative affect, either disrupting the organization of the PCM directly or perhaps interfering with the activities of chaperone molecules like mortalin.

By using a novel experimental system, the data presented in the third and final chapter of this dissertation adds a new wrinkle to the current understanding of p53 and centrosome function. However, the ability of a cell cycle regulatory molecule like p53 to be associated with the structural organization of the centrosome would be consistent with a “centrosome-centric” model of cell cycle regulation.
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