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**Midbody Anchoring of SNARE and Exocyst Complexes by Centriolin is Required for Completion of Cytokinesis: A Dissertation**

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A Dissertation Presented

By

ADAM SCOTT GROMLEY

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# MIDBODY ANCHORING OF SNARE AND EXOCYST COMPLEXES BY CENTRIOLIN IS REQUIRED FOR COMPLETION OF CYTOKINESIS.

A Dissertation Presented

By

ADAM SCOTT GROMLEY

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# Abstract

Although much progress has been made in understanding the events that lead to successful cell division, many details of this process remain a mystery. This dissertation presents findings which help to explain events that occur in the latest stages of cytokinesis, with an emphasis on the role of centrosome proteins. The first chapter introduces the novel centrosome protein centriolin. We show that this protein is localized specifically to the subdistal appendages of the maternal centriole in interphase, and it localizes to the midbody during cytokinesis. Disruption of this protein results in a unique cytokinesis defect in which cleavage furrow formation and ingression appear normal, but the cells remain connected by a thin intracellular bridge for extended periods of time. These results lead us to the conclusion that centriolin has an important function in cytokinesis. The second chapter describes our attempt to identify centriolin interacting partners. A yeast two hybrid screen was performed, and the results of this screen revealed an interaction between centriolin and proteins involved in vesicle target specificity and fusion. Further studies of these proteins revealed a novel localization to the midbody in cycling cells and a novel function in the final stages of cytokinesis, similar to centriolin. The third chapter discusses my attempts to clone and characterize a novel GTPase Activating Protein (GAP), which was also discovered in the screen for centriolin interacting proteins.

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## Introduction:

The centrosome is the major microtubule organizing center in animal cells. It is composed of a pair of centrioles surrounded by a pericentriolar material. One of the centrioles is older than the other and is termed the "mother" or "maternal" centriole. Its orthogonally-positioned partner centriole is called the "daughter" centriole. The mother centriole serves as the template for the formation of a new daughter centriole during the centriole duplication cycle. Structurally, the mother centriole can be distinguished from the daughter by the presence of appendages at its distal end. Electron microscopy studies have demonstrated that these appendages appear to be the focus of the minus ends of microtubules (Chretien et al., 1997), suggesting that these structures might be anchorage sites for nucleated microtubules. Furthermore, studies using L929 cells, which have well-separated mother and daughter centrioles, have shown that microtubules nucleate from both centrioles, but only the mother centriole appears to have the ability to anchor these nucleated microtubules (Piel et al., 2000). The pericentriolar material surrounding the centriole pair contains the microtubule nucleating activity of the centrosome, consisting of the γ-tubulin ring complex (γ-Turc) and pericentrin (Doxsey et al., 1994; Job et al., 2003) .

Although the role of the centrosome was traditionally believed to be only as a microtubule organizing center, there is emerging evidence that suggests this organelle may have important functions in many cellular processes including primary cilia

formation, DNA damage checkpoint, cell cycle progression, mitotic exit, and cytokinesis (Doxsey, 2001a; Doxsey, 2001b; Rieder et al., 2001).

### *Primary cilia formation*

Primary cilia are solitary, nonmotile microtubule structures which are believed to have a function in sensory perception. These structures form through the outgrowth of microtubules from the distal, or plus end, of the basal body (mother centriole), which occurs by a motor-driven process called intraflagellar transport (IFT). Although cellular processes such as interphase microtubule organization (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Rodionov and Borisy, 1997) and mitotic spindle organization (Debec et al., 1995; Khodjakov et al., 2000; Walczak et al., 1998) can occur in the absence of a functional centrosome, the centrosome appears to be essential for formation of the primary cilium (Rieder et al., 2001). Recent studies suggest that a specific component of the centrosome, pericentrin, is required for proper formation of primary cilia (Jurczyk et al., 2004), as well as proper function of motile cilia (Martinez-Campos, 2004).

### *DNA damage checkpoint*

Another function of the centrosome appears to be in monitoring DNA damage. Studies of early *D melanogaster* embryogenesis demonstrate that embryos respond to exposure to DNA damaging agents by inactivating their centrosomes in mitosis (Sibon et al., 2000). Similarly, embryos that are mutant for components of the DNA

replication checkpoint exhibit mitotic centrosome inactivation. It was further demonstrated that this DNA damage monitoring process is dependent on the Drosophila checkpoint 2 kinase homologue, DmChk2 (Takada et al., 2003). The authors postulate that this centrosome inactivation may be a mechanism by which cells inhibit mitotic progression in the presence of DNA damage to prevent subsequent genomic instability.

### Cell cycle progression

Disruption of the centrosome has been demonstrated to cause an arrest early in the cell cycle, as these cells fail to enter a subsequent S phase. Laser ablation (Khodjakov and Rieder, 2001), physical removal with microsurgical techniques (Hinchcliffe et al., 2001), and even disruption of individual centrosome components (Gromley et al., 2003; Keryer et al., 2003) are all sufficient to induce a pre-S phase arrest. Several possible mechanisms for this arrest have been suggested. One possibility is that the centrosome serves as a site for concentration or sequestration of catalytic components that are required for the G1-S transition. Perturbation of this organelle could lead to a mislocalization and, therefore, inactivation of proteins or complexes important for entry into S-phase. Another possibility is that centrosome disruption activates a cellular checkpoint that senses aberrations in the physical composition of the centrosome. In this model, any change in the centrosome's status, whether it be as extreme as the removal of the entire centrosome or as subtle as the absence of a single component, would trigger a signaling cascade which would

ultimately lead to cell cycle arrest. The fact that disruption of a single centrosomal protein is sufficient to cause arrest argues for the latter possibility.

It has been suggested that this arrest may be dependent on an intact p53 pathway, as cells with disrupted p53 do not arrest (Gromley et al., 2003; Keryer et al., 2003). Our unpublished results using cell lines with disruptions in either the p53 or Rb/p16INK4a pathway suggest a much more complicated story. In SAOS-2 cells, which are p53 null and Rb (-) but retain a functional p16INK4a, we do not see this G1 arrest. Similarly, in U2OS cells, which are wild type for p53 and Rb but are negative for p16INK4a, this arrest is also absent. These results present the possibility that the signals that cause G1 arrest are not p53 specific and can work through either the p53 or Rb/p16INK4a pathway, or perhaps both.

*Cytokinesis*

Cytokinesis is the final event in the cell cycle, resulting in the physical separation of the two daughter cells. This process begins in anaphase and is initiated by formation of the future division plane, termed the cleavage furrow. Inward movement, or ingression, of this cleavage furrow followed by resolution of the midbody, or abscission, ultimately results in the division of the two daughter cells. It has been demonstrated that the cleavage furrow ingression is due to constriction of a ring structure around the inner surface of the plasma membrane at the site of the previously determined cleavage plane. This contractile ring is composed of actin and

myosin II (Carter, 1967; Fujiwara et al., 1978) and is commonly referred to as an actomyosin contractile ring. Some centromere-associated proteins, known as chromosomal passenger proteins, translocate from the centromere to the central spindle in mitosis (Adams et al., 2001). This translocation appears to be important for cleavage furrow function, as disruption of these proteins leads to cytokinesis failure and formation of multinucleate cells (Bischoff and Plowman, 1999; Li et al., 1999; Mackay et al., 1998).

A recently discovered complex, termed the centralspindlin complex, has been shown to be important for cytokinesis (Jantsch-Plunger et al., 2000). This complex is composed of equimolar amounts of the mitotic kinesin-like protein MKLP-1/CHO1 and the GTPase Activating Protein (GAP) MgcRacGAP. During cytokinesis, this complex forms a ring just inside the actomyosin contractile ring and is thought to control ring constriction through modulation of the GTP status of the GTPase RhoA (Saint and Somers, 2003).

The importance of membrane insertion and vesicle fusion events in animal cell cytokinesis has been previously demonstrated. In the *C. elegans* embryo, inhibition of golgi secretory function using the drug brefeldin A (BFA) results in a late stage cytokinesis defect (Skop et al., 2001). In these studies, the early events of cytokinesis, such as cleavage furrow formation and ingression, appeared to be normal, but the embryos failed to complete separation at the midbody stage. Because BFA does not

inhibit furrow ingression, the authors suggest that targeted post-golgi vesicle secretion to the midbody is the only process disrupted in these experiments, and that this secretion event is important for the terminal stage of cytokinesis. Furthermore, the authors suggest that this process is homologous to cytokinesis in plant cells, in which a structure, termed the phragmoplast, develops in the middle of the spindle midzone and targeted secretion of post-golgi vesicles to this region results in outward growth of the cell plate and eventual separation of the daughter cells.

Recently, the SNARE complex components syntaxin-2 and endobrevin were shown to be required for the terminal step of cytokinesis in mammalian cells (Low et al., 2003). The SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor) complex is a set of transmembrane proteins which mediate the fusion event between a vesicle and its target membrane (Jahn and Sudhof, 1999). This complex consists of a transport vesicle-associated SNARE, or v-SNARE, and a target membrane SNARE, or t-SNARE. Interaction of these two components brings the vesicle close to the target membrane and mediates the fusion event. Overexpression of mutants of syntaxin-2 (t-SNARE) or endobrevin (v-SNARE) that lack the transmembrane domain resulted in an accumulation of binucleate cells. Timelapse imaging revealed that cleavage furrow formation and ingression were fine in these cells, but abscission of the midbody did not occur. These results suggest that membrane fusion in a specific location, the midbody, at a very discreet moment in cytokinesis is a requirement for successful abscission.

Studies in both the budding and fission yeast suggest a cytokinesis function for the exocyst. The exocyst is a multiprotein complex that functions in targeting of secretory vesicles to distinct sites of secretion on the plasma membrane (Bowser and Novick, 1991; Hsu et al., 1999; Potenza et al., 1992; Roth et al., 1998). It is composed of eight separate proteins, designated Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. The mammalian exocyst has been studied in polarized epithelial cells (Yeaman et al., 2004), but its localization in cycling cells is not known. In the budding yeast *S. cerevisiae*, members of the exocyst complex have been shown to localize to the mother-bud neck (Finger et al., 1998; Mondesert et al., 1997), suggesting that the exocyst may have a function in cytokinesis. Moreover, cells overexpressing the exocyst component Sec15 accumulate vesicles that are positioned toward the mother-bud neck (Salminen and Novick, 1989), the site of cytokinesis in the budding yeast. In the fission yeast *S. pombe*, the exocyst components Sec6, Sec8, Sec10, and Exo70 have been shown to localize to the actomyosin contractile ring in mitosis (Wang et al., 2002). Similar to the results from *S. cerevisae* studies, mutants for the exocyst component Sec8 accumulate 100nm "presumptive" secretory vesicles in the vicinity of the division septum during cytokinesis. The authors proposed a cytokinesis function for the exocyst, as these mutants were shown to be defective in division septum cleavage and physical separation of the two daughter cells.

Recent studies have suggested an essential role for the centrosome in cytokinesis. Physical removal of the centrosome using microsurgical techniques (Piel et al., 2001) or laser ablation of the centrosome (Khodjakov and Rieder, 2001) results in failure of cytokinesis. Furthermore, it has been observed that an acentrosomal *D melanogaster* cultured cell line appears to have difficulties in performing the final abscission event in cytokinesis, as they remain connected by a thin intracellular bridge for extended periods of time, often separating only after a subsequent mitosis. Further experiments suggest that this cytokinesis function is specific for the mother centriole of the centrosome. In their studies using GFP-labeled centrin, a component of both centrioles that labels the mother centriole more intensely, (Piel et al., 2001) observed that the daughter centriole was highly motile during interphase while the mother centriole was rather stable and centered in the focus of the microtubule aster. Interestingly, it was found that the mother centriole specifically moved toward the midbody during cytokinesis, and movement of this centriole away from the midbody and back to the center of the cell corresponded with the final separation event, termed abscission. Unfortunately, these results are merely an interesting observation, as the authors were unable to show evidence for a mechanistic explanation of this phenomenon.

Studies involving the disruption of individual centrosome components, such as centriolin (Gromley et al., 2003) and AKAP-450 (Keryer et al., 2003), have shown that perturbation of a single centrosome protein is sufficient to cause cytokinesis

defects. When centriolin levels are reduced using siRNA, cells are unable to efficiently complete the final abscission event in cytokinesis. Time-lapse microscopy showed that the cells remained connected by a thin intracellular bridge for extended periods of time before they crawled apart to break the bridge, fused back together to form binucleate cells, or blebbed and died in a process which appeared morphologically similar to apoptosis. In cells overexpressing the C-terminus of AKAP 450, which mislocalizes endogenous AKAP 450 as well as PKA from the centrosome, cytokinesis defects were also observed. Although timelapse images were not presented, the authors state that cells overexpressing this construct took significantly longer to complete abscission, as measured by the time spent from anaphase onset to abscission.

### Mitotic Exit

In both *S. cerevisiae* and *S. pombe*, a signaling pathway exists to coordinate successful chromosome segregation with exit from mitosis/cytokinesis (Bardin and Amon, 2001; McCollum and Gould, 2001). In *S. cerevisiae*, this pathway is termed the Mitotic Exit Network, or MEN. Its counterpart in *S. pombe* is known as the Septation Initiation Network, or SIN. Components of these pathways are localized to the spindle pole body, the yeast equivalent of the centrosome, and consist of scaffolding proteins (Nud1p in *S. cerevisiae*; Sid4p and Cdc11p in *S. pombe*) which anchor proteins involved in a signaling cascade. These signaling pathways are

regulated by modulation of the GTP status of a central GTPase, Tem1 (*S. cerevisiae*)

or Spg1 (*S. pombe*), by a unique two-component GTPase Activating Protein (GAP),

Bub2p/Bfa1p (*S. cerevisiae*) or Cdc16p/Byr4p (*S. pombe*). Mutations in MEN genes

result in a late anaphase arrest, and the cells fail to initiate cytokinesis. In contrast,

SIN mutants fail to initiate contractile ring constriction, but the cells continue to

cycle, resulting in long, multinucleate cells which eventually lyse (Balasubramanian

et al., 1998; Fankhauser et al., 1995). Although putative mammalian homologues of

some MEN/SIN components have been identified (Gromley et al., 2003; Li et al.,

1997; Luca and Winey, 1998; Moreno et al., 2001), an equivalent vertebrate pathway

has not been described.

Studies of the MEN and septins reveal a potential link between the exocyst and

mitotic exit. Septins are a class of GTPases that assemble into two distinct ring

structures at the bud neck in telophase, between which the actomyosin contractile ring

forms. In a recent report by (Dobbelaere et al., 2003), the dynamics of the septin

rings is described. During bud growth and telophase, the rings exhibit an immobile,

or "frozen", state in which the turnover of septin subunits within the ring is stable.

However, at the onset of cytokinesis, the septin ring exhibits a rapid turnover of

subunits, producing a "fluid" state. In cells mutant for the Rts1 protein, the B'

regulatory subunit of PP2A, this "fluidity" of the septins is not observed, and the cells

fail to complete cytokinesis. The authors conclude from these studies that Rts1 is

required for septin ring fluidity. Because the localization of Rts1 to the bud neck is

dependent on activation of the MEN pathway, the authors propose a link between septins and the MEN mediated by the protein Rts1.

An interaction between septins and the exocyst complex has previously been described (Hsu et al., 1998). In studies of rat brain extracts, it was found that the septin complex coimmunoprecipitates with the exocyst complex. Furthermore, subcellular localization using indirect immunofluorescence revealed a partial colocalization, supporting the idea that these complexes interact. Taken together, these studies suggest a link may exist between mitotic exit and the exocyst in which a cytokinesis function of the exocyst may be regulated by the activation of the MEN/SIN.

Individually, the centrosome, exocyst, SNARE complex, and MEN/SIN have been shown to influence events leading to successful cell separation, or cytokinesis. However, it is not known how all of these components are linked molecularly. In this dissertation, I introduce the novel centrosome protein centriolin. Centriolin functions in a late stage event of cytokinesis, abscission. The amino acid sequence of centriolin reveals a domain with homology to a component of the MEN/SIN, Nud1p/Cdc11p. Furthermore, a yeast two-hybrid screen reveals an interaction between centriolin and members of the exocyst and SNARE complexes. These complexes colocalize with centriolin to a novel ring-like structure at the midbody, and disruption of these complexes produces a cytokinesis defect similar to the centriolin phenotype.

Additionally, the yeast-two hybrid screen identified a novel GTPase Activating Protein (agGAP), which may represent a homologue of the GAP found to interact with Nud1p/Cdc11p in the yeast MEN/SIN. These results suggest that centriolin may represent the molecule that mediates the interactions among the centrosome, exocyst, SNARE complex, and MEN/SIN to coordinate successful completion of cytokinesis.

# CHAPTER I

# A NOVEL HUMAN PROTEIN OF THE MATERNAL CENTRIOLE IS REQUIRED FOR THE FINAL STAGES OF CYTOKINESIS AND ENTRY INTO S-PHASE

## Introduction

Centrosomes are the major microtubule-nucleating organelles in most vertebrate cells (Doxsey, 2001b). In mitosis, they contribute to spindle organization and function, and in interphase, they organize microtubule arrays that serve as tracks for transporting proteins, organelles, and chromosomes. The centrosome also anchors regulatory molecules and may serve as a central site that receives, integrates, and transmits signals that regulate fundamental cellular functions. The core of the centrosome is comprised of a pair of centrioles, microtubule barrels that appear to anchor microtubules (Chretien et al., 1997; Piel et al., 2000). Each centriole is surrounded by pericentriolar material or centrosome matrix, which nucleates the growth of new microtubules and seems to be organized by the centrioles(Bobinnec et al., 1998). Although best known for their role in microtubule nucleation, recent data suggest that centrosomes also play key roles in cytokinesis and cell cycle progression. A role for centrosomes in defining the site of cell cleavage during cytokinesis has been suggested for some time(Rappaport, 1986). Recent studies with vertebrate cells provide evidence for a direct link between centrosome activity and completion of cytokinesis. Elimination of centrosomes from interphase cells by removal with a microneedle (Hinchcliffe et al., 2001)or from mitotic cells by laser ablation (Khodjakov and Rieder, 2001) caused cytokinesis defects, arrest, or failure. In another study, it was shown that during the final stages of cytokinesis, the maternal centriole moved to the intercellular bridge, the microtubule-filled interconnection

between nascent daughter cells (Piel et al., 2001). Centriole repositioning correlated with bridge narrowing and microtubule depolymerization, while movement of the centriole away from the bridge correlated with cell cleavage or abscission. The authors suggested that the maternal centriole might anchor a regulatory pathway that controls the final stages of cell division in vertebrate cells. This would be analogous to regulatory pathways anchored at spindle pole bodies (the centrosome equivalent) in budding and fission yeasts that control mitotic exit and cytokinesis (for reviews see (Bardin and Amon, 2001); McCollum and Gould, 2001; (Pereira and Schiebel, 2001)). However, no vertebrate pathway analogous to the mitotic exit network (MEN)* in budding yeast or septation initiation network (SIN) in fission yeast has been identified (Glotzer, 2001; Guertin et al., 2002). Moreover, the role of centrosome-associated molecules in the process of cytokinesis is poorly understood.

In addition to their role in cytokinesis, centrosomes appear to have a role in cell cycle progression. Recent evidence demonstrates that vertebrate cells lacking centrosomes do not initiate DNA replication (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). The authors suggested that centrosomes controlled entry into S phase by recruiting or concentrating "core" centrosome molecules required for this process or that they indirectly activated a cellular checkpoint that monitored aberrant centrosome number. In another experimental system, vertebrate cells treated with cytochalasin D to inhibit actin-mediated cell cleavage also arrested cells in G1 as binucleate cells with supernumerary centrosomes(Andreassen et al., 2001). Although these results

suggest that changes in centrosome number can affect entry into S phase, the precise role of centrosomes in cell cycle progression in vertebrate cells will require identification of the molecular components and pathways that control these events. In this paper, we identify a novel component of the vertebrate maternal centriole called centriolin. Abrogation of centriolin function by small interfering RNA (siRNA) silencing, overexpression, or antibody inhibition produces cytokinesis failure and G1/G0 arrest, just as seen when centrosomes are experimentally eliminated from cells. Centriolin silencing produces a novel cytokinesis phenotype in which dividing cells remain interconnected by long strands of cytoplasm and fail to cleave. The cytokinesis activity lies in a centriolin domain that is homologous to the MEN/SIN components Nud1p/Cdc11p and binds the Nud1p-interacting GTPase activating protein Bub2p. We conclude that centriolin is required for a distinct step in the final stages of vertebrate cytokinesis and can influence entry into S phase.

**Results**

**Identification and cloning of a novel protein localized to the maternal centriole and intercellular bridge**

Using sera from patients with the autoimmune disease scleroderma that react with centrosomes (Doxsey et al., 1994), we screened a human placenta gt11 cDNA

expression library to identify genes encoding the autoantigens. Of the 3106 clones

screened, only one of 1.7 kb was identified, indicating that the mRNA for this

molecule was rare. The full-length cDNA was obtained, and the protein encoded by

the cDNA was called centriolin (see below and Materials and methods). The amino

acid sequence of centriolin predicted a protein with several coiled-coil regions

interrupted by noncoiled domains (Lupas et al., 1991)(Fig. 1 a). Two domains within

the centriolin sequence shared homology with human oncogenic transforming acidic

coiled-coil proteins (TACCs), which localize to centrosomes and are implicated in

microtubule stabilization and spindle function(Lee et al., 2001). Another domain of

centriolin was homologous to human stathmin, an oncogenic protein involved in

microtubule destabilization (Andersen, 2000). The carboxy terminus of centriolin was

identical to CEP110, a naturally occurring fusion to the fibroblast growth factor

receptor that localizes to centrosomes, is oncogenic, and is of unknown function

(Guasch et al., 2000). The relationship of CEP110 to centriolin is unknown, although

we did not identify a cDNA corresponding to this protein in our library screens. A

region of centriolin near the amino terminus shared homology with Nud1p and

Cdc11p, budding and fission yeast spindle pole body proteins that anchor components

of the yeast MEN and SIN, respectively, and are required for completion of

mitosis and cytokinesis (Bardin and Amon, 2001; McCollum and Gould, 2001;

Pereira and Schiebel, 2001;(Guertin et al., 2002)). The 120–amino acid region of

shared homology between the Nud1 domain, Nud1p, and Cdc11p all have leucine-

rich repeats and are predicted to form ß helix structures in tertiary structure prediction

programs. Antibodies raised against recombinant centriolin recognized a band of

~270 kD on Western blots of isolated centrosome fractions, whereas preimmune sera

showed no specific bands (Fig. 1 b, Centrosome fractions). In vitro translation and

overexpression of the protein in mammalian cells using the full-length cDNA

produced a protein with a molecular weight similar to the endogenous protein (Fig. 1

b, In vitro translated and Overexpressed) and to a protein predicted from the cDNA

sequence (see Materials and methods). Immunofluorescence microscopy

demonstrated that centriolin was localized to centrosomes in a wide variety of

species, including human, monkey, hamster, mouse, and *Xenopus* (Figs. 1 and 2;

unpublished data). Centrosome localization was confirmed by showing that an HA-

tagged centriolin protein ectopically expressed in COS cells localized to centrosomes

(Fig. 2 a). The endogenous protein was present on the centrosome throughout the cell

cycle. In late G1/early S phase, centrosomes begin to duplicate, and by G2/M,

duplication is usually completed. During the duplication process, centriolin was

present on only one of the two duplicating centrosomes, although other proteins, such

as γ-tubulin, were found on both (Fig. 1 c, G2 cell). Beginning at late

G2/prometaphase, dim staining was observed next to a brightly stained centrosome.

By metaphase, when centrosomes become "mature," both centrosomes had equally

high levels of centriolin and were more brightly stained than at any other cell cycle

stage. This demonstrates that centriolin is a marker for centrosome maturation, a

characteristic shared with cenexin(Lange and Gull, 1995) and ninein(Mogensen et al.,

2000). At the metaphase to anaphase transition, centriolin staining diminished at

centrosomes and reached its lowest levels by late anaphase/telophase. During

cytokinesis, centriolin sometimes appeared as one or two dots adjacent to the

intercellular bridge, suggesting that the centrosome/centriole had moved to this site

(Fig. 1 c, Telo early). This staining pattern was consistent with recent time-lapse

imaging experiments showing that the maternal centriole translocates to the

intercellular bridge during cytokinesis (Piel et al., 2001). Centriolin next appeared as

diffusely organized material within the intercellular bridge and ultimately became

concentrated at the midbody (Fig. 1 c, Telo late). The organization of centriolin at the

centrosome was more precisely determined by serum starving cells to induce growth

of a primary cilium from the maternal centriole (Vorobjev and Chentsov Yu, 1982).

In these cells, centriolin staining was confined to the maternal centriole underlying

the cilium (Fig. 2 b). Immunogold electron microscopy on centrosome fractions

(Doxsey et al., 1994; (Blomberg-Wirschell and Doxsey, 1998)) confirmed

localization to the maternal centriole (Fig. 2 e, M) and further demonstrated that the

protein was concentrated on subdistal appendages, specialized substructures of the

maternal centriole implicated in microtubule anchoring (Fig. 2, c–e) (Chretien et al.,

1997; Piel et al., 2000). Based on its centriolar localization, the protein was named

centriolin. Centriolin was also found at noncentrosomal apical bands of material in

specialized epithelial cells that lack proteins involved in microtubule nucleation and

appear to anchor the minus ends of microtubules (Mogensen et al., 1997) (Fig. 2, f

and g).

**Centriolin silencing by siRNA induces cytokinesis failure and a novel cytokinesis phenotype**

To determine the function of centriolin, we reduced its levels using siRNAs ((Fire et al., 1998);(Elbashir et al., 2001)). Treatment of telomerase-immortalized diploid human retinal pigment epithelial (RPE-1) cells(Morales et al., 1999) with centriolin-specific siRNAs caused a significant reduction in centriolin mRNA levels (Fig. 3 a). Although we were unable to examine protein levels by Western blotting of whole cell lysates due to the rare nature of this and other centrosome autoantigens (Doxsey et al., 1994), immunofluorescence staining demonstrated that centriolin was undetectable, or greatly reduced, at centrosomes in most cells (86%; $n$ =1,012). Quantitative analysis showed that immunofluorescence signals at individual centrosomes were significantly below those in cells treated with control lamin A/C siRNA, despite severe disruption of the nuclear lamina in the latter (Fig. 3 b) (Elbashiret al., 2001). Midbody staining of centriolin was alsoreduced in cells treated with siRNAs targeting centriolin. Because centriolin shares homology with proteins known to affect microtubule organization and cytokinesis, we examined cells with reduced centriolin for defects in these functions. The most obvious cellular change detected in RPE-1 cells with reduced centriolin was a dramatic increase in the percentage of late-stage mitotic cells ( ~70-fold increase; Fig. 3 c). In addition, we observed an increase in the percentage of binucleate cells in three different cell lines, suggesting that a certain proportion of cells failed to cleave (Fig. 3 d; see below). The incidence of binucleate

cells was significantly greater than controls, although somewhat lower than that observed for some other proteins involved in cytokinesis (Matuliene and Kuriyama, 2002; Meraldi et al., 2002; (Mollinari et al., 2002)). A similar cytokinesis phenotype was observed with a second set of siRNAs targeting a different centriolin sequence and with morpholino antisense DNA oligonucleotides targeting centriolin. The dramatically high percentage of cells in late mitotic stages suggested a unique cytokinesis defect in these cells. When carefully analyzed by immunofluorescence microscopy, cells with reduced centriolin appeared to be arrested or delayed in the final stages of cytokinesis. Most cells retained intercellular bridges of varying length and thickness (Fig. 3, m and n, arrowheads). In some cases, cells remained connected even though one or both of the future daughter cells had reentered mitosis (Fig. 3 n, M). Some cells failed to cleave, forming syncytia with two, three, of four cells remaining interconnected (Fig. 3, m and n). During the early stages of cytokinesis, midbodies appeared normal. A more complete understanding of the mechanism of cytokinesis failure was obtained by imaging live HeLa cells treated with centriolin-specific siRNAs (Fig. 4; see Videos 1–3, available at http://www.jcb.org/cgi/content/full/ jcb.200301105/DC1). As expected, control cells (lamin siRNA) performed a distinct cell cleavage event with normal timing (average 2 h after mitosis) and immediately flattened and crawled apart (Fig. 4 a). Cells silenced for centriolin progressed normally through mitosis (Fig. 3, g–j; Fig. 4 e) and sometimes cleaved normally, but most failed to cleave or cleaved after prolonged periods of time (up to 23.2 h after metaphase; Fig. 4, b–d and f). These cells arrested

or delayed in a unique post-telophase state. Most were unusually elongated, each with a persistent intercellular bridge of variable diameter that was often dynamic. Bridges alternated between thin threads of interconnecting cytoplasm to very thick interconnections of large diameter that appeared able to produce membrane ruffles (Fig. 4 b, 5:50, arrow). Midbodies were not detected within persistent interconnections between cells, suggesting that they were lost sometime during the protracted period spent in cytokinesis. Interconnected cells sometimes coalesced to form single cells and then quickly moved apart again (Fig. 4 d). They sometimes made multiple failed attempts at cleavage, but in no case did we observe a cell that formed a stable binucleate. This suggested that binucleate cells observed in fixed cells (Fig. 3 d) were transient intermediates in a process that involved multiple failed attempts at cytokinesis. Cells that retained intercellular connections for long periods of time continued to progress through the cell cycle. To our surprise, some cells reentered the next mitosis while still interconnected and produced interconnected "progeny" that formed two- to four-cell syncytia, thus confirming the cell–cell interconnections observed by indirect immunofluorescence (Fig. 3, m and n). In some cases, cells that remained interconnected for long periods of time appeared to undergo apoptosis. They showed extensive blebbing, increased phase density, and decreased size and lifted from the substrate (Fig. 4 b, upper cell, 7:20). Microtubule organization in cells with reduced centriolin appeared normal at all cell cycle stages. This included microtubules of the spindle midzone in anaphase and the midbody in telophase (Fig. 3, e–j). Microtubule nucleation from centrosomes also appeared

normal (Fig. 3, k and l), although a slight delay was sometimes observed within the first minute or two. $\gamma$-Tubulin, a marker for centrosome-associated microtubule nucleation, was localized normally to centrosomes (Fig. 3 b), as were several other centrosome antigens, including GCP-2 (Murphy et al., 1998) and cNap-1 (Fry et al., 1998) (unpublished data). Midbody markers, such as anillin (see Glotzer, 2001) and $\gamma$-tubulin(Shu et al., 1995), were also localized normally. At later stages of cytokinesis in cells with long intercellular bridges, midbodies were no longer detected. These data indicate that cytokinesis failure did not result from disruption of microtubules, centrosomes, or midbodies.

## Overexpression of centriolin and its Nud1 domain induces cytokinesis failure in a microtubule-independent manner

We next tested the effect of ectopic expression of centriolin and its Nud1 domain on cytokinesis and microtubule organization (Fig. 5, a–g). The most striking defects in COS-7 cells expressing HA-tagged centriolin were an increase in the proportion of telophase cells and the formation of binucleate cells (Fig. 5 g). Microtubule bundles were observed within intercellular bridges in telophase cells (Fig. 5a), but they did not appear to cause binucleate cell formation. We found that overexpression of the 120–amino acid GFP-tagged Nud1 domain of centriolin was sufficient to induce cytokinesis failure and binucleate formation (Fig. 5, e and g) in the absence of detectable changes in microtubule

organization (Fig. 5, c and d) and without disrupting endogenous centriolin from centrosomes (Fig. 5 f). The results from gene silencing and protein overexpression support a microtubule-independent mechanism for cytokinesis failure.

**The centriolin Nud1 domain interacts with the yeast Bub2p in vitro**

Budding yeast Nud1p anchors the MEN to the spindle pole body through direct interactions with Bub2p and perhaps other MEN components ((Gruneberg et al., 2000); (Pereira et al., 2000)). To determine if the centriolin Nud1 homology domain (Fig. 5 h) had similar properties, we tested its ability to bind Bub2p by directed two-hybrid analysis and immunoprecipitation. Because no vertebrate Bub2p homologue has been unequivocally identified(Cuif et al., 1999), we examined the ability of the centriolin Nud1 domain to interact with yeast Bub2p. Both two-hybrid analysis and immunoprecipitation from yeast cells coexpressing the two proteins revealed a strong and specific interaction between the centriolin Nud1 domain and Bub2p (Fig. 5, i and j). No signal was observed when either protein was used alone, and no binding was detected between the centriolin Nud1 domain and the budding yeast MEN component Bfa1p, consistent with interaction observations in budding yeast (Pereira and Schiebel, 2001).

## Cleavage failure is observed in *Xenopus* embryos injected with centriolin antibodies

A third approach was used to examine centriolin function. When affinity-purified anticentriolin antibodies (Fig. 1 b) were microinjected into one cell of two-cell *Xenopus* embryos (Doxsey et al., 1994), the injected cell failed to cleave, or cleaved a few times and then arrested; uninjected cells or preimmune IgG-injected cells divided normally (Fig. 6, a and d). Centriolin antibody–injected cells arrested with two nuclei and two well-organized microtubule asters, indicating that karyokinesis and microtubule organization were normal, but cells failed to complete the final event of mitosis, cell cleavage (Fig. 6 c). Preimmune IgG-injected cells had a single nucleus with one or two microtubule asters, depending on their cell cycle stage, as would be expected for cells that had undergone normal cell cleavage (Fig. 6 b). Taken together, the results from gene silencing, antibody injection, and protein overexpression in several experimental systems all demonstrate that centriolin plays an important role in the late stages of cytokinesis.

## siRNA-induced gene silencing of centriolin causes G1/G0 arrest

Cytokinesis defects and delays induced by centriolin silencing were observed at early times after treatment of RPE-1 cells (18–24 h). At later times (48–72 h after treatment), a reduction in the mitotic index was observed, suggesting that the cells

were arrested at some other stage of the cell cycle. This was directly tested by treating

cells with nocodazole to induce mitotic arrest and quantifying mitotic cells in DAPI

stained preparations. Under these conditions, most lamin siRNA–treated control RPE-

1 cells were arrested in mitosis (71%), whereas only a small fraction of centriolin

siRNA– treated cells arrested at this cell cycle stage (<1%). To determine the cell

cycle stage of arrest, cells were analyzed by flow cytometry. In the presence of

nocodazole, control cells showed a significant shift from the G1 peak to the G2/M

peak (Fig. 7 a, red). In contrast, cells treated with siRNAs targeting centriolin did not

significantly shift into the G2/M peak in the presence of nocodazole but remained

largely in G1 (Fig. 7 a, blue). The inability to undergo a nocodazole induced shift into

the G2/M peak was a feature shared by cells driven into G0 by serum starvation (Fig.

7 b, blue). The proportion of cells in S phase was either unaltered or slightly

decreased in cells silenced for centriolin both in the presence of nocodazole

(centriolin, 13%; lamin, 23%) or in its absence (centriolin, 13%; lamin, 19%). These

results demonstrate that cells with reduced centriolin arrest before S phase, possibly

in G1/S, G1, or G0. Ki-67 staining was also used to examine the stage of cell cycle

arrest. Ki-67 is an antibody directed against a nuclear protein that stains cycling cells

or cells arrested in cycle (e.g., G1/S or S phase; (Gerdes et al., 1984)) but not cells

that are quiescent (G0) or differentiated. As expected, nearly all untreated RPE-1 cells

or control cells treated with siRNAs targeting GFP or lamins A/C were positive for

Ki-67 (Fig. 7, c and d). However, most cells with reduced centriolin had undetectable

levels of Ki-67 staining (Fig. 7, c and d). Taken together, results from mitotic index

assays, flow cytometry, and Ki-76 staining in RPE-1 and HME-1 (human mammary epithelia) cells (unpublished data) demonstrated that reduction of centriolin levels prevented cells from entering S phase and appeared to drive them out of cycle into a G0-like state. This cell cycle arrest effectively prevents the initiation of additional rounds of centrosome duplication in cells compromised by having diminished levels of centriolin.

**Discussion**

In this paper, we characterize centriolin, a maternal centriole component whose disruption induces cytokinesis failure and G1/G0 arrest. The ability of centriolin to induce this phenotype may provide a molecular explanation for the cytokinesis defects and G1 arrest previously observed in vertebrate cells after experimental removal of entire centrosomes (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Moreover, association of centriolin with the maternal centriole may be a molecular requirement for activation and completion of the final stages of cytokinesis, functions recently suggested for the maternal centriole in vertebrate cells (Piel et al.,2001). The cytokinesis phenotype observed in cells treated with siRNAs targeting centriolin is uncommon among proteins that function in cytokinesis. The predominant phenotype observed after functional abrogation of most proteins that cause cytokinesis failure is coalescence of dividing cells into a single cell with two nuclei. This has been shown for proteins of the central spindle, regulatory proteins

that control cytokinesis, microtubule-associated proteins, and the centrosome proteins γ-tubulin and centrin (Shu et al., 1995; (Kaiser et al., 2002); (Manabe et al., 2002); (Matuliene and Kuriyama, 2002); (Meraldi et al., 2002); Mollinari et al., 2002; (Salisbury et al., 2002); (Seong et al., 2002)). In contrast, the predominant cytokinesis phenotype observed in cells with reduced centriolin is failure to resolve persistent intercellular bridges between dividing cells. The apparent consequences of this event are generation of multicellular syncytia, continuous attempts at cell cleavage that include binucleate cells as intermediates, completion of cytokinesis, perhaps artificially, by traction-mediated cytokinesis(Burton and Taylor, 1997), and apoptotic cell death. Although we have not determined the precise mechanism of cytokinesis failure in cells with abrogated centriolin function, we are testing whether membrane is incorporated normally into the cleavage site ((Finger and White, 2002); (Thompson et al., 2002)). Persistent intercellular bridges have also been observed between dividing vertebrate cells overexpressing the Cdc-14A phosphatase (Kaiser et al., 2002). This uncommon phenotype has thus far been observed only with proteins (centriolin and Cdc14A) that share homology with components of yeast regulatory pathways that control mitotic exit and cytokinesis (MEN/SIN) and localize to the same intracellular sites as the yeast proteins (centrosome/spindle pole bodies). Persistent intercellular connections may also be present in cells lacking centrosomes and may reflect a similar type of cytokinesis defect (Doxsey, 2001a). Although the mechanism of cytokinesis failure appears to be different in SIN mutants, the outcome is the same, production of bi and multinucleate cells. These observations support the

idea that vertebrate cells may possess regulatory pathways that function in much the same way as those in yeast to coordinate the final stages of cytokinesis with chromosome segregation. Several domains of centriolin share sequence homology with proteins implicated in tumorigenesis, including the TACCs and oncoprotein 18/stathmin. In this paper, we show that the domain homologous to Nud1p/Cdc11p can induce aneuploidy through cytokinesis failure. This event could generate further genetic instability through the organization of multipolar spindles by supernumerary centrosomes and could thus facilitate the accumulation of activated oncogenes and loss of tumor suppressor genes ((Pihan et al., 2001); (Doxsey, 2002)). We are currently investigating the role of centriolin in human oncogenesis. Our results suggest that minor changes in centrosome composition, such as loss of centriolin, can arrest cells in G1/G0. Because cells with reduced centriolin have difficulty completing the final stages of cytokinesis, it is tempting to suggest that G1/G0 arrest is a consequence of improper cytokinesis. However, it has been suggested that G1 arrest in cells lacking centrosomes may also result from the loss of core centrosome components, improper spindle alignment, or the presence of excess DNA in binucleate cells (Andreassen et al., 2001; Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Meraldi et al., 2002). We are currently testing a model in which the loss of core centrosome components activates a checkpoint involved in monitoring "centrosome integrity" and thus prevents centrosome duplication by arresting cells in G1/G0, as we and others have proposed (Doxsey, 2001a; (Quintyne and Schroer, 2002)).

**Figure 1. Centriolin is an ~270-kD coiled-coil protein localized to mature centrosomes and the midbody.** (a, top) Schematic showing centriolin domains that share homology with budding and fission yeast proteins Nud1p and Cdc11p, human stathmin, and human and *Drosophila* TACCs. Percentages represent identities and similarities of centriolin to the homologous proteins described above. (a, bottom) Schematic showing centriolin regions predicted to be coiled coil. Below both diagrams are centriolin amino acid numbers. (b, In vitro translated) [$^{35}$S]methionine-labeled HA-tagged centriolin produced by in vitro translation of cDNA and resolved directly (Cen) or after immunoprecipitation with HA antibodies (Cen IP); empty vector (Vec). (b, Overexpressed) Western blots probed with anti-HA antibody showing overexpressed HA-tagged centriolin (HA-Cen) and its absence from cells overexpressing β-galactosidase (β gal). (b, Centrosome fractions) Centrosome fractions prepared from HeLa cells and *Xenopus* tissue culture (XTC) cells blotted with antibodies to centriolin (Cen) or preimmune sera (PreI). Arrowheads show position of centriolin. In XTC cells, the bands below centriolin appear to be degradation products as they are sometimes observed in protein fractions produced by in vitro translation or overexpression. Bars represent positions of molecular weight markers (x10$^3$). (c) Immunofluorescence images of endogenous centriolin in RPE-1 cells at different cell cycle stages. The top panels show merged images of centriolin (green), γ-tubulin (red), and nuclei (blue) from cells in G1, G2, proM (prometaphase), M (metaphase), and anaphase (A). Insets, higher magnifications of centrosomes stained for γ-tubulin (left) and centriolin (right). Centriolin localization to one of the

two centrosomes is demonstrated most clearly in the G2 cell. Middle panels (Telo early) and bottom panels (Telo late) show separate images of γ-tubulin staining (left) and centriolin (right). γ-Tubulin marks both mother and daughter centrioles, which are separated in some cases. Centriolin staining is confined to one of two centrioles, which sometimes appears at the intercellular bridge (Telo early). Centrioles lacking centriolin are indicated by arrowheads in right panels. At later stages of telophase (Telo late), centriolin is also on the midbody. All immunofluorescence images (here and elsewhere) are two-dimensional projections of three-dimensional reconstructions to ensure that all stained material is visible. C, centriole; MB, midbody. Bar in c, 10
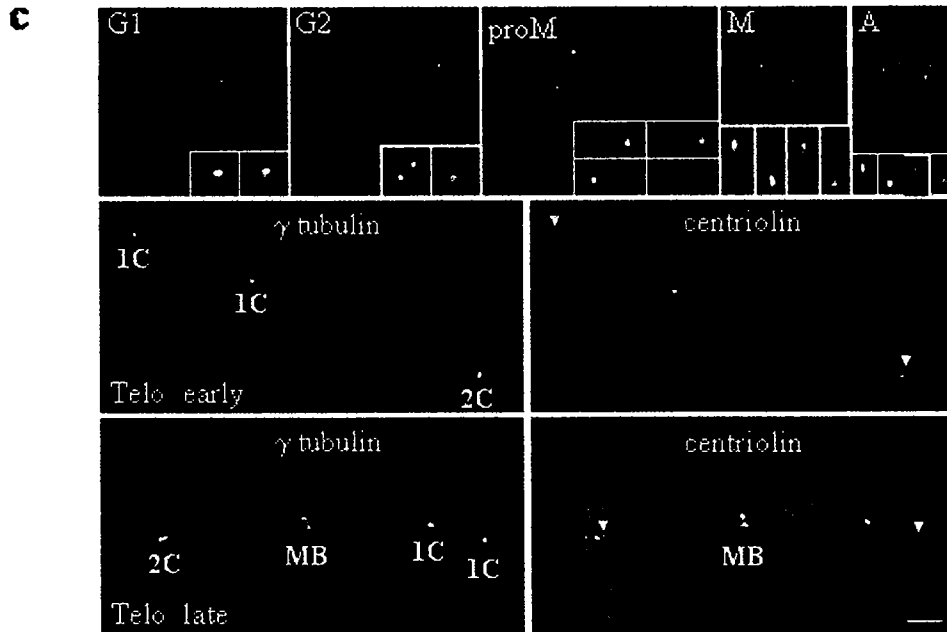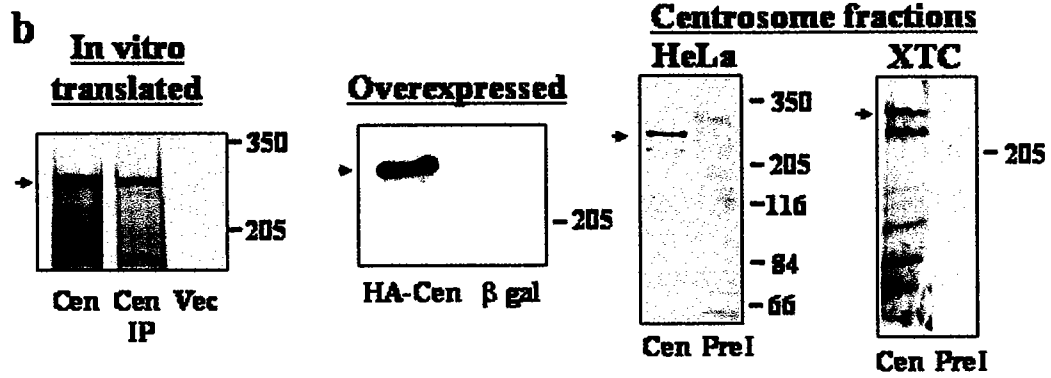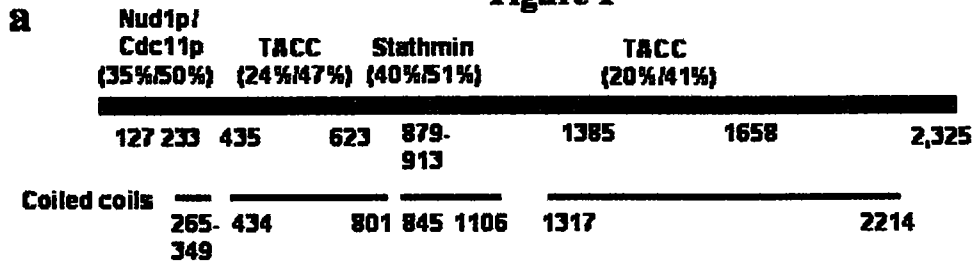
# Figure 1

**Figure 2. Centriolin is localized to maternal centrioles and noncentrosomal sites of microtubule anchoring.**

(a) HA-tagged centriolin overexpressed in COS-7 cells localizes to the centrosome (anti-HA, green) at the convergence of microtubules (red, anti–α-tubulin). (b) An RPE-1 cell immunostained with an antibody to polyglutamylated tubulin (GT335) (Bobinnec et al., 1998) to label centrioles and the primary cilium (red), and for centriolin (green), which is localized to the maternal centriole (m) associated with the primary cilium (yellow in merge) but not on the daughter centriole (d). n, nucleus. (c–e) Electron micrographs showing specific immunogold labeling of centriolin on subdistal appendages found on maternal (e, bottom, M) but not daughter centrioles (e, top, D). c and d are longitudinal and cross sections, respectively, through maternal centrioles, and e is a longitudinal section through both centrioles. Arrowheads show striations characteristic of subdistal appendages. (f and g) Centriolin is found at noncentrosomal sites of microtubule anchoring in pillar cells of the mouse cochlea (arrows) (Mogensen et al., 1997). (f) Schematic representation of a pillar cell. (g) Centriolin immunofluorescence staining overlaid with phase contrast image. Centrosome and associated cilium are shown schematically at top of cell in f. Bars: (a and g) 10 μm; (b) 2 μm; (c–e) 100 nm.

**Figure 2**



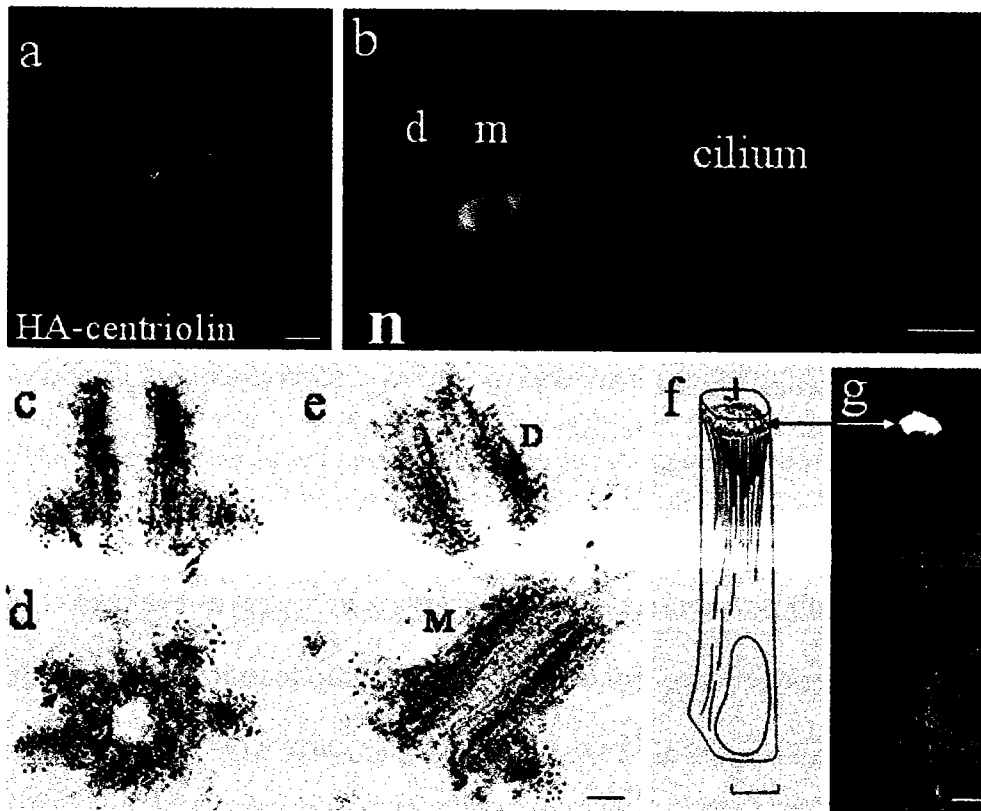HA-centriolin
d  m  cilium
n
c
e D
d
M
f
g

**Figure 3. RPE-1 cells treated with siRNAs targeting centriolin retain persistent intercellular connections and fail in cytokinesis.**

(a) RT-PCR analysis shows that centriolin mRNA is reduced in RPE-1 cells treated with centriolin specific siRNAs (top right) but is unaffected in cells treated with siRNAs targeting lamins A/C (top left, sequence identity confirmed). Control ($\alpha$-tubulin) RT-PCR was performed in the same reaction mixtures with centriolin and lamin (bottom panels). (b) Immunofluorescence images (top right; $\alpha$-tubulin, red; centriolin, green) and quantification of centriolin levels in centrosomes from cells treated with siRNAs as indicated. The top right panel shows a cell with undetectable centriolin at the centrosome/ centriole, and the top left panel shows a cell that is unaffected by the treatment and stains for centriolin (green/white). Graph shows the average centriolin fluorescence intensity/pixel at individual centrosomes (bars) in cells treated with lamin or centriolin siRNAs. The centrosome fluorescence in most centriolin siRNA–treated cells (83%) was below the lowest values observed in control cells. (c) Graph showing a dramatic increase in the percentage of cells in telophase/cytokinesis after siRNA targeting of centriolin (~70-fold). n, total number of cells counted (c and d). (d) Graph showing the increased percentage of binucleate cells in HeLa, U2OS, and RPE-1 cell lines after treatment with centriolin siRNAs (4–15-fold greater than controls). In c and d, values represent data from single experiments representative of three to four experiments. The time analyzed was as follows: HeLa, 48 h and 72 h; U2OS, 72 h; RPE-1, 24 h. (e–l) Microtubule organization (e–j) and microtubule nucleation (k and l) are not detectably altered in

cells treated with centriolin siRNA (f, h, j,and l) compared with cells treated with lamin siRNA (e, g, i, and k). Arrows indicate position of centrosomes; no centrosome staining is observed in centriolin siRNA–treated cells. Insets in k and l are enlargements of centriolin staining at centrosomes (or microtubule convergence sites) at arrows. Cells in interphase (e and f, lower cell), prometaphase/metaphase (proM/M; g and h), and telophase (i and j) were labeled for microtubules (red), centrosomes (green/yellow), and nuclei (blue). MB, midbody. (m and n) Cells treated with siRNAs targeting centriolin were stained for microtubules (red) and DNA (blue). Arrowheads indicate contiguous connections between two or more cells. (m) Three interconnected cells form a syncytium. (n) One daughter of an interconnected pair of cells has reentered mitosis (M). Bar in n: (e–j) 5 µm; (k, l, and insets) 3.5 µm; (m and n) 15 µm.
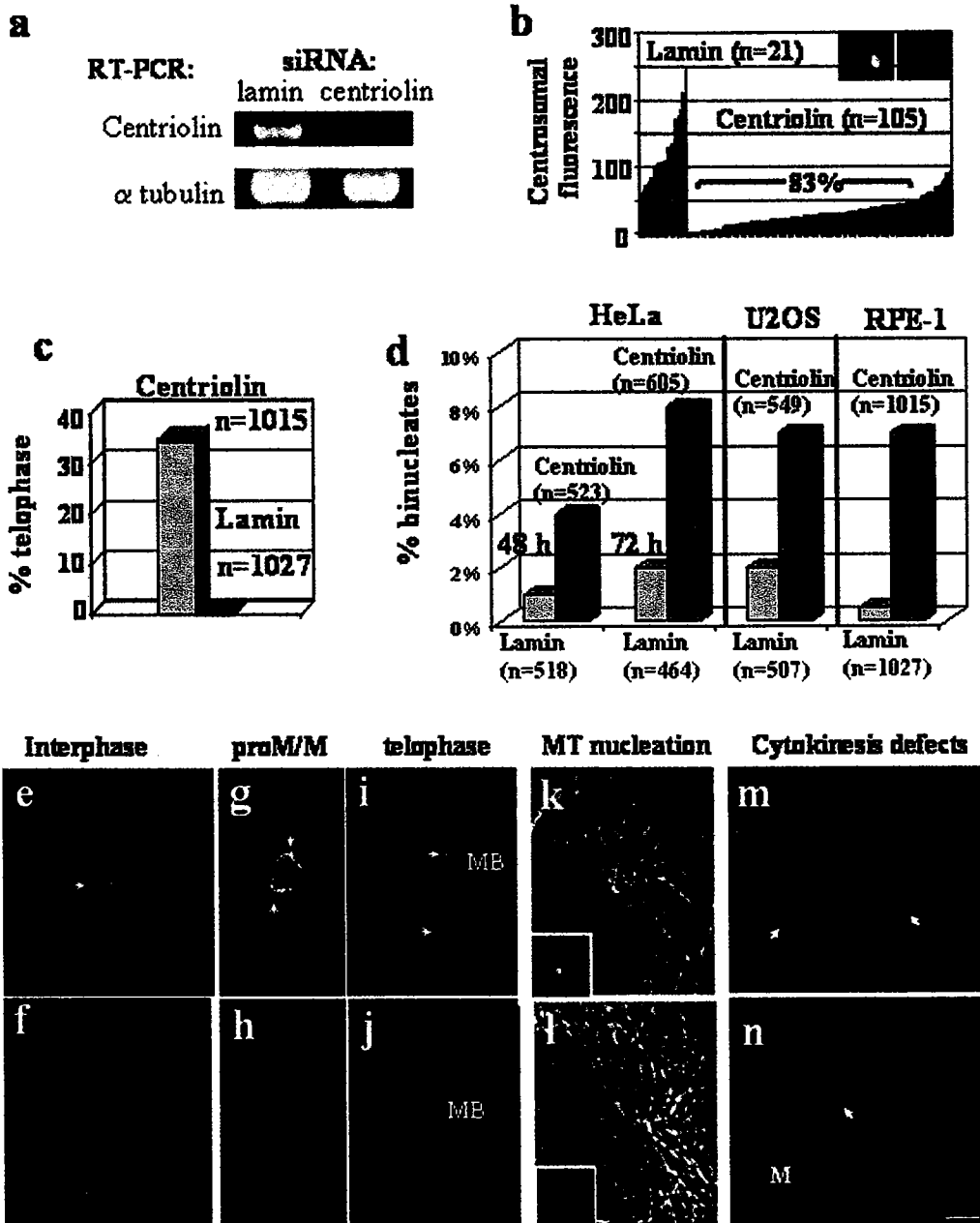
# Figure 3

**Figure 4. Time-lapse images of HeLa cells treated with centriolin siRNAs reveal unique cytokinesis defects.**

(a) A cell treated with control siRNAs targeting lamin moves apart, forms visible midbodies (arrow), and completes the final cleavage event with normal timing (1–3 h after metaphase). (b–d) Centriolin siRNA–treated cells remain attached for extended periods of time through persistent intercellular bridges and sometimes do not show visible midbodies. (b) A cell that remains attached by a long intercellular bridge for at least 8 h. The cell cleaves, both daughter cells round up, and at least one appears to undergo apoptotic cell death (upper cell, 7:20, extensive blebbing and decrease in size). (c) A dividing cell that has not completed cleavage reenters the next mitosis. One cell rounds up and is drawn to the other. The other rounds up and both undergo the early stages of cytokinesis to form a total of four cells; these progeny often remain attached by intercellular bridges forming syncytia. (d) Cell showing three failed attempts at cell cleavage over a 9.5-h time period. (e) Cells treated with siRNAs targeting centriolin progress from nuclear envelope breakdown (NEB) to anaphase with normal timing, similar to lamin siRNA controls. Vertical bars represent recordings from single cells (e and f). (f) Centriolin siRNA–treated cells are delayed in cytokinesis (~70%) compared with control lamin siRNA– treated cells, a value consistent with a 70–80% silencing efficiency. Results represent recordings of individual cells from several independent experiments. Time in hours and minutes is included in each panel in a–d. Bar, 10 μm. Timelapse videos (Videos 1–3) of the

series of images shown in a–c are available at

http://www.jcb.org/cgi/content/full/jcb.200301105/DC1.
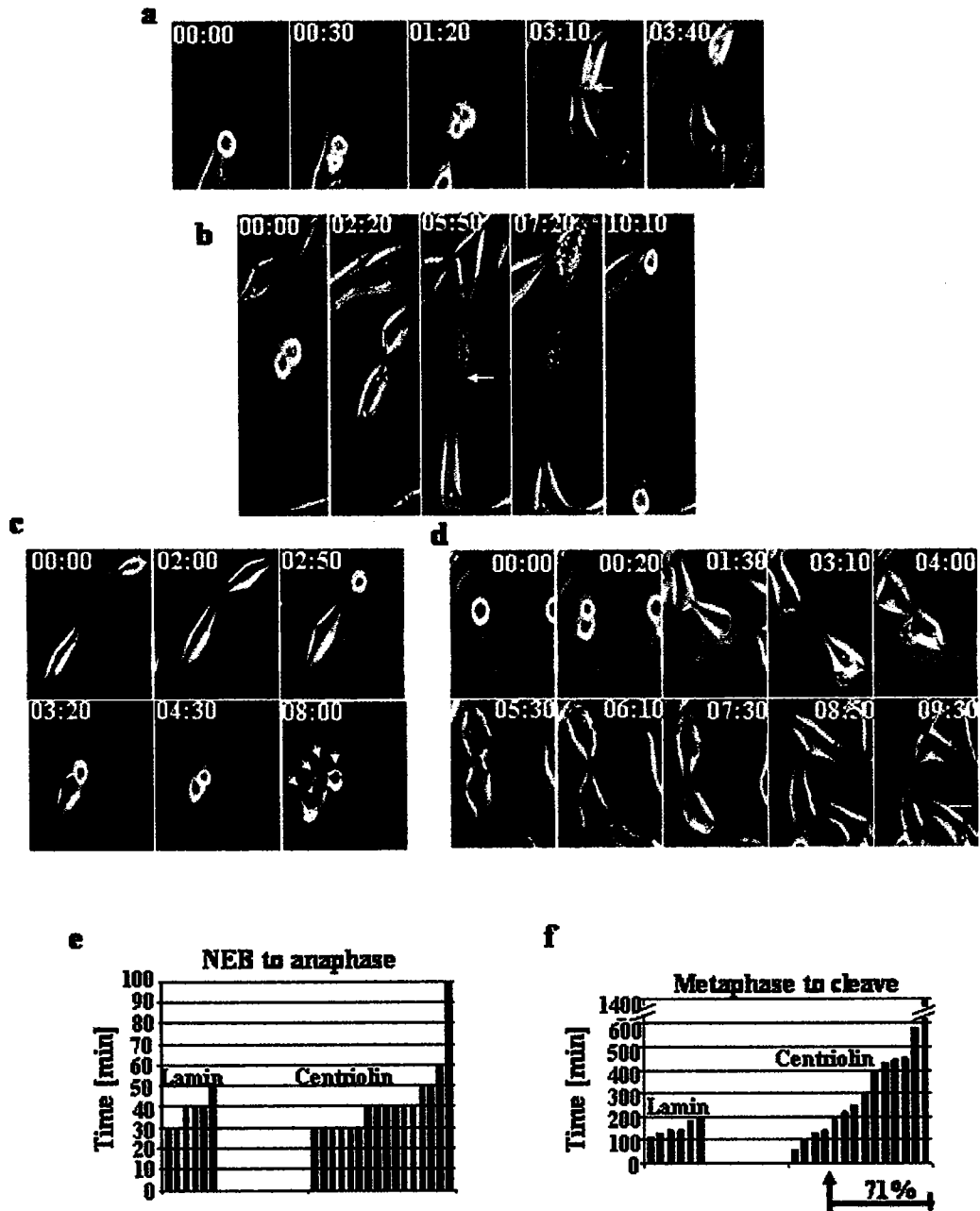
## Figure 4

**Figure 5. Cells expressing centriolin fail in cytokinesis, as do cells expressing the centriolin Nud1 domain that interacts with yeast Bub2p.**

(a) COS-7 cell overexpressing HA-tagged centriolin (left inset) showing persistent microtubule bundles in the intercellular bridge during cytokinesis (main panel, α-tubulin staining) despite reformation of the nucleus and decondensation of chromatin (DNA, right inset). (b) Control COS-7 cell (expressing HA alone, bottom center) at a similar cell cycle stage based on nuclear morphology (bottom right) shows a narrow intercellular bridge and diminished microtubule polymer (center), characteristic of cells that have reformed nuclei and decondensed DNA. (c–f) COS-7 cells expressing a GFP-tagged Nud1 domain of centriolin (c and d, insets, green; DNA, blue) have normal intercellular bridges (d, telophase, arrowhead) and normal microtubule organization (c, interphase) as in controls. However, the cells (yellow) often become binucleate (blue) by a mechanism that does not involve disruption of the endogenous centrosome-associated centriolin (f; right inset shows enlargement of centrosome, left inset shows transfected cell). (g) Quantitative analysis showing that HA–centriolin and the GFPtagged Nud1 domain both induce significant binucleate cell formation compared with controls (HA and GFP). Values are from a single experiment and are representative of three experiments. Bar, 7μm (for all panels). n, total number of cells counted. (h) Alignment of the centriolin Nud1 domain with the yeast Nud1p and Cdc11p proteins. (i) Direct yeast two-hybrid analysis demonstrates an interaction between the human Nud1 domain with one of the two components implicated in GTPase activating protein activity, Bub2p, but not Bfa1p. The blue colony in the blue

box and increased β-galactosidase activity (bar 2 of graph) demonstrate a specific interaction between the human centriolin Nud1 domain (hNud1) and yeast Bub2p. Bar 1, LexA–BUB2 x TAD; bar 2, LexA–BUB x TAD–hNud1; bar 3, LexA–BFA1 x TAD; bar 4, TAD–hNud1 x LexA–BFA1; bar 5, TAD-hNud1 x LexA. (j) Specific coimmunoprecipitation of HA-tagged hNud1 and LexA-tagged yeast Bub2p from yeast cells. Coprecipitation was observed using antibodies to either protein and only when both were coexpressed (top middle panel in each group). Lane 1, TAD–HA–hNud1 x LexA; lane 2, TAD–HA–hNud1 x Bub2p–LexA; lane 3, TAD–HA x Bub2p–LexA.

# Figure 5



**Centriolin full-length**

**Centriolin Nud1 domain**

**g** HA-centriolin (n= 2835)  Nud1-GFP (n=251)

Binucleate cells

HA (n=3420)  GFP (n=253)

**h**
Cdc11p
Nud1p
Centriolin

**i**

β-galactosidase units

TAD hNud1

LexA  BUB2  BFA1

**j**

Blot:

HA IP
LexA
LexA (Supnt)
HA
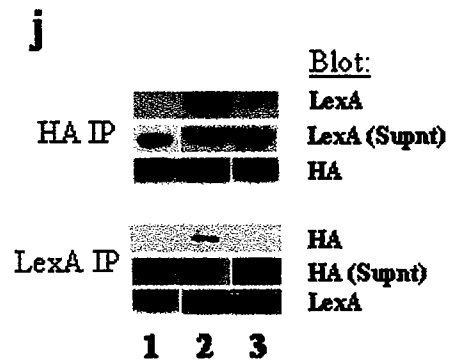
LexA IP
HA
HA (Supnt)
LexA

1  2  3
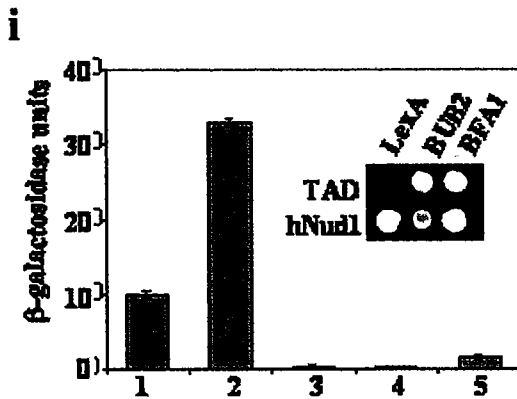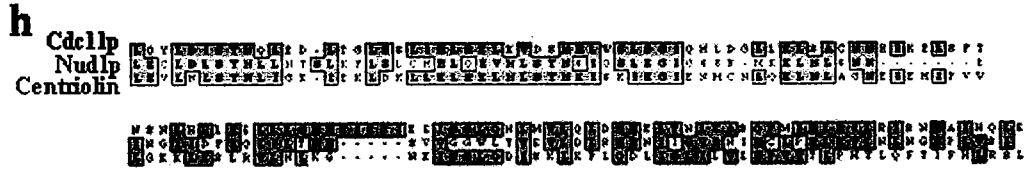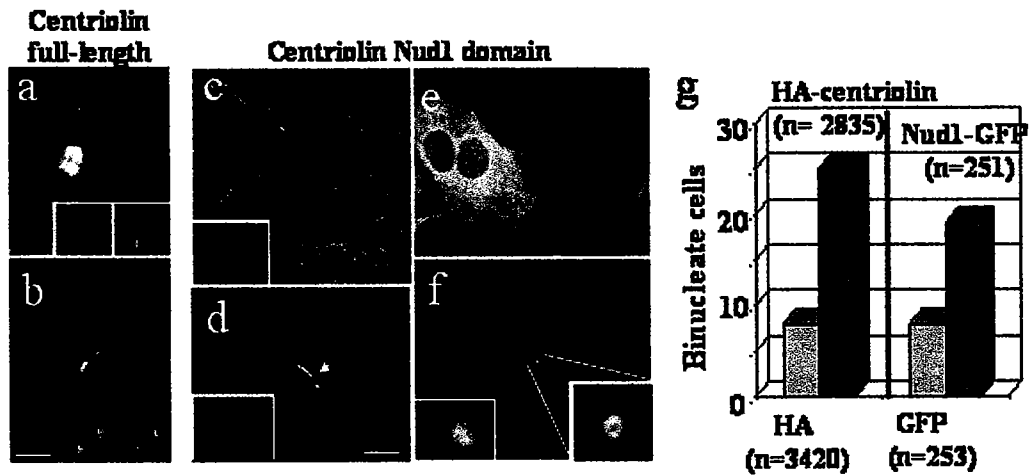
**Figure 6. Cytokinesis failure in *Xenopus* embryos injected with centriolin antibodies.** (a) *Xenopus* two cell embryos injected into one cell with ~50 nl anticentriolin antibodies (2 mg/ml affinity purified) fail to cleave (arrows), whereas noninjected cells (side opposite arrows) and cells injected with control rabbit IgG (2 mg/ml, top) cleave normally. (b and c) Immunofluorescence images showing two microtubule asters near a single nucleus (N) in a cell from an embryo injected with control IgG (b), and two nuclei and two asters in a cell from a centriolin antibody–injected cell (c, microtubules stained with anti–α-tubulin). Bar, 10μm (for b and c). (d) Quantification of results from injection experiments showing an approximately eight fold increase in cleavage failure (representative of three experiments). n, number of embryos examined.

**Figure 6**
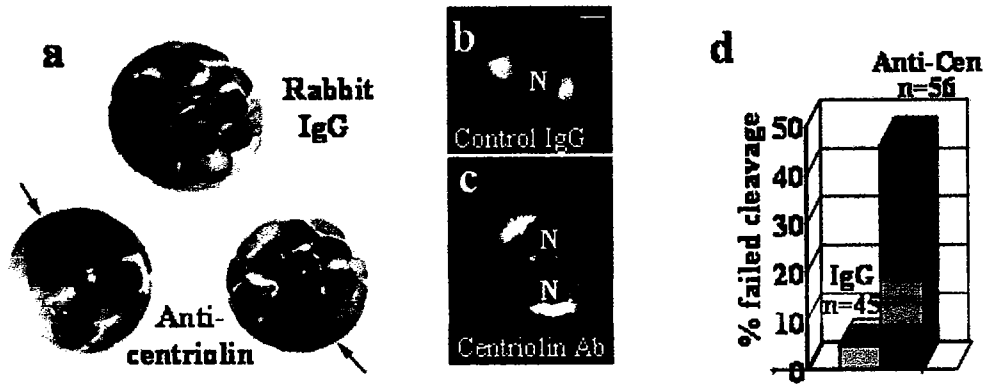
**Figure 7. siRNAs targeting centriolin induce G1/G0 arrest.**

(a) RPE-1 cells treated with centriolin siRNAs (blue) do not shift into the G2/M peak after nocodazole treatment as seen for control lamin siRNA–treated cells (red). (b) The inability of cells treated with centriolin siRNAs to shift into the G2/M peak is similar to that observed in serum-starved cells (blue, serum starved; red, not starved). (c) Ki-67 staining of control cells (left cell, green) occurs together with centriolin staining (left cell, red at arrow); both are gone in centriolin siRNA– treated cells (right, arrowhead). Bar, 7.5 µm. (d) Cells treated with siRNAs targeting centriolin lack Ki-67 staining (~70%), whereas most control cells stain positively (GFP siRNAs). a, b, and d are representative experiments from five experiments for a, two for b, and three for d. n, total number cells counted.

**Figure 7**



a

Centriolin

-noc    +noc

% of max

Relative DNA fluorescence

b

Serum starved

+noc

% of max

Relative DNA fluorescence

c

DNA, blue
Ki-67, green
Centriolin, red

d

Centriolin

n=1031

% Ki-67 negative (noncycling cells)

GFP
n=1040
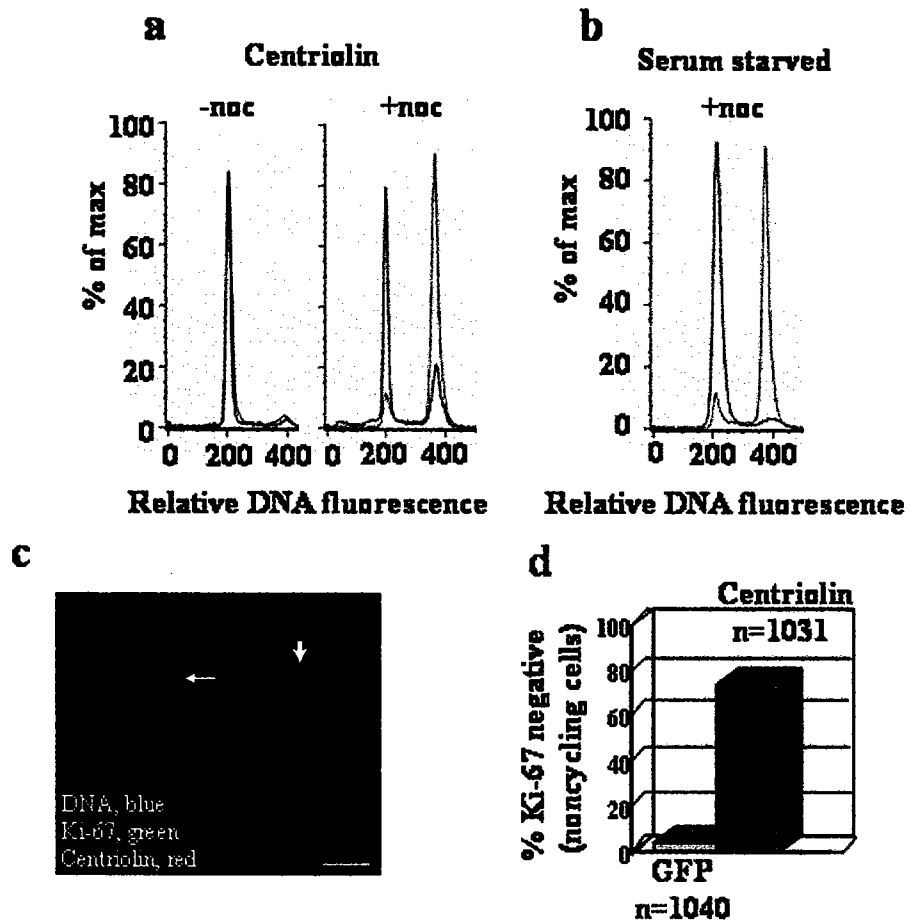
# CHAPTER II

# CENTRIOLIN-ANCHORING OF EXOCYST AND SNARE COMPLEXES AT THE MIDBODY IS REQUIRED FOR LOCALIZED SECRETION AND ABSCISSION DURING CYTOKINESIS.

## Summary

Emerging evidence suggests a function for the centrosome in cytokinesis, but a molecular mechanism for this function has not been described. Recently we identified a novel centriole protein called centriolin that is required for the final stage of cytokinesis. Here we show that centriolin interacts with members of the exocyst and SNARE complexes, which are required for membrane vesicle targeting and fusion, respectively. These complexes colocalize with centriolin to a unique ring structure in the central midbody and localization of both complexes requires centriolin. Disruption of either complex by RNAi results in late stage cytokinesis defects indistinguishable from centriolin downregulation. Moreover, disruption of the exocyst results in an accumulation of SNARE-containing vesicle-like structures at the midbody. In cells expressing a GFP-tagged marker for the secretory pathway, similar vesicles are seen at the midbody. Based on these results, we propose that centriolin functions as a midbody scaffold for protein complexes involved in both vesicle target specificity and vesicle fusion, and mediates the terminal abscission step in cytokinesis.

## Introduction

Cytokinesis is the final stage of the cell cycle, ending in the physical separation of the two daughter cells, in a process known as abscission. The earliest events in

cytokinesis, such as cleavage furrow formation and ingression, have been well studied (for extensive reviews of cytokinesis, see (Glotzer, 2001; Guertin et al., 2002)). It has been demonstrated that cleavage furrow ingression is due to constriction of a ring structure around the inner surface of the plasma membrane at the site of the previously determined cleavage plane. This contractile ring is composed of actin and myosin II (Carter, 1967; Fujiwara et al., 1978) and is commonly referred to as an actomyosin contractile ring. Some centromere-associated proteins, known as chromosomal passenger proteins, translocate from the centromere to the central spindle in mitosis (Adams et al., 2001). This translocation appears to be important for cleavage furrow function, as disruption of these proteins leads to cytokinesis failure and formation of multinucleate cells (Bischoff and Plowman, 1999; Li et al., 1999; Mackay et al., 1998). A recently discovered complex, termed centralspindlin, has been shown to be important in cytokinesis. This complex is composed of equimolar amounts of the mitotic kinesin-like protein MKLP-1 and the GTPase Activating Protein (GAP) MgcRacGAP (Jantsch-Plunger et al., 2000). During cytokinesis, this complex forms a ring inside the actomyosin contractile ring and is thought to control ring constriction through modulation of the GTP status of the GTPase RhoA (Saint and Somers, 2003). The final step of cytokinesis, in which resolution of the midbody results in the physical separation of the two daughter cells, has not been studied in detail.

The importance of membrane insertion and vesicle fusion events in animal cell cytokinesis has been previously demonstrated. In the *C. elegans* embryo, inhibition of Golgi secretory function using brefeldin A (BFA) results in late stage cytokinesis defects (Skop et al., 2001). In these studies, cleavage furrow formation and ingression appeared to be normal, but the embryos failed to complete separation at the midbody stage. Recently, the SNARE complex components syntaxin-2 and endobrevin were shown to be required for the terminal step of cytokinesis in mammalian cells (Low et al., 2003). Overexpression of mutants lacking the transmembrane domain resulted in an accumulation of binucleate cells. Time-lapse imaging revealed that cleavage furrow formation and ingression were unaffected in these cells, but abscission of the midbody and separation of the daughter cells did not occur. Taken together, these results suggest that membrane fusion in a specific location, the midbody, at a very discrete moment in cytokinesis is a requirement for successful abscission. However, little is known about the spatial and temporal control of this event.

The exocyst is a multiprotein complex that targets secretory vesicles to distinct sites on the plasma membrane. It is composed of eight separate proteins, designated Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. The mammalian exocyst has been studied in polarized epithelial cells (Yeaman et al., 2004), but its localization in cycling cells is not known. In the budding yeast *S. cerevisiae*, members of the exocyst complex localize to the mother-bud neck (Finger et al., 1998; Mondesert et al., 1997), suggesting that the exocyst may have a function in cytokinesis. In the

fission yeast *S. pombe*, the exocyst components Sec6, Sec8, Sec10, and Exo70

localize to the actomyosin contractile ring (Wang et al., 2002). Furthermore, mutants

for the exocyst component Sec8 accumulate 100nm "presumptive" secretory vesicles

in the vicinity of the division septum during cytokinesis. The authors proposed a

cytokinesis function for the exocyst, as these mutants were shown to be defective in

division septum cleavage and physical separation of the two daughter cells.

The function of the centrosome was once thought to be limited to microtubule

cytoskeleton organization in interphase and spindle pole microtubule organization in

mitosis, but recent evidence suggests that the centrosome may also have a role in

other important cellular functions, including cytokinesis (Doxsey, 2001a; Doxsey,

2001b). Using laser ablation, Khodjakov and Rieder (2001) demonstrated that

disruption of the centrosome results in cytokinesis defects. Specifically, they noted

that in mitotic cells in which both spindle poles were ablated, mitotic progression was

not altered and a cleavage furrow formed, but the cleavage furrow eventually

regressed, resulting in failed cytokinesis. Interestingly, well-formed midbodies could

be seen in these cells, suggesting that a late stage in cytokinesis was affected.

Furthermore, Piel et al. (2001) observed cytokinesis defects in cells in which the

centrosome was physically removed using microsurgical techniques. These authors

showed that an acentrosomal *D melanogaster* cell line exhibited defects at the final

separation step in cytokinesis, remaining connected by a thin intracellular bridge for

extended periods of time, often separating only after a subsequent mitosis. Disruption

of individual centrosome components, such as centriolin (Gromley et al., 2003) and AKAP-450 (Keryer et al., 2003), also cause cytokinesis defects. When centriolin levels are reduced using siRNA, cells are unable to efficiently complete the final abscission event in cytokinesis (Gromley et al., 2003). Time-lapse microscopy demonstrated that the cells remained connected by a thin intracellular bridge for extended periods of time before they crawled apart to break the bridge, fused back together to form binucleate cells, or blebbed and died in a process which appeared morphologically similar to apoptosis. In cells overexpressing the C-terminus of AKAP 450, which mislocalizes endogenous AKAP 450 as well as PKA from the centrosome, cytokinesis defects were also observed. It was found that cells overexpressing this construct took significantly longer to complete abscission, as measured by the time spent from anaphase onset to abscission. Taken together, these data suggest an important role for the centrosome in cytokinesis.

In this paper, we identify components of the exocyst and SNARE complexes as interacting partners with the centrosome protein centriolin. We show that members of the exocyst and SNARE complexes colocalize with centriolin at the central midbody in telophase. Furthermore, these proteins form a novel ring-like structure around the phase-dense Flemming body. Midbody localization of both exocyst and SNARE proteins requires centriolin. Reduction in the levels of both exocyst and SNARE proteins results in a cytokinesis failure indistinguishable from that seen with centriolin disruption. Further experiments reveal that disruption of the exocyst results

in accumulation of secretory vesicles at the midbody. Based on this data, we propose that centriolin acts as a scaffold at the midbody for localizing complexes required for vesicle fusion, and mediates the final abscission event of cytokinesis.

**Results**

**Centriolin forms a ring-like structure at the midbody during cytokinesis.**

We previously showed that centriolin localized to the midbody during cytokinesis (Gromley et al., 2003). Using higher resolution deconvolution microscopy we showed that centriolin forms a unique ring-like structure within the center portion of the midbody (1 to 2 $\mu$m in diameter). This ring appears to encircle a plaque-like structure containing $\gamma$ tubulin (Fig 1A), and the phase-dense Flemming body (Fig 1B). Costaining with antibodies against actin revealed that this ring was distinct from the actomyosin contractile ring that forms around the plasma membrane between the two daughter cells. This central midbody ring appears during the early stages of actomyosin ring constriction and persists until after cell cleavage (data not shown).

Due to the novelty of this ring structure, we analyzed its composition in more detail. Several proteins that function in cytokinesis localize to this ring, including the centralspindlin components MKLP-1 (Fig 1C) and MgcRacGAP (data not shown).

These components were present at the ring early after anaphase onset, while centriolin arrived at the ring later, after furrow constriction was initiated. Moreover, reduction of MKLP-1 levels by RNAi prevented centriolin from concentrating at the midbody ring (Fig 1C). In contrast, reduction of centriolin had no effect on the localization of MKLP-1 or MgcRacGAP (data not shown). This data suggest that the midbody localization of centriolin is dependent on the mitotic kinesin-like protein MKLP-1. Aurora B kinase/Aim1 was not present at the midbody ring but co-localized with microtubules flanking the ring.

**Identification of centriolin-interacting proteins**

To determine the molecular function of centriolin in cytokinesis, we identified interacting proteins using a yeast two-hybrid screen. We used a domain of centriolin homologous to genes involved in cytokinesis and mitotic exit in budding and fission yeasts (Nud1/Cdc11), which is required for the cytokinesis function of centriolin (Gromley et al., 2003). This domain was used as bait to screen approximately 12 million clones in a human testis cDNA library. After eliminating false positives using several control parameters (see Materials and Methods), we identified two interacting proteins of interest. One of these proteins was sec15, a member of the exocyst complex. The other was snapin, a SNARE-associated protein.

## The exocyst complex and the SNARE-associated protein snapin co-immunoprecipitate with centriolin

To confirm the yeast two-hybrid data, we first asked whether centriolin was present in purified exocyst complexes. Fractions from the final sucrose gradient step of the purification procedure were probed for both the exocyst component sec8 and centriolin. Centriolin co-fractionated with exocyst components in the major peak fractions. Further confirmation of the centriolin-exocyst interaction was provided by immunoprecipitation experiments. In the yeast cells coexpressing the two-hybrid fusion constructs sec15-Activation Domain (AD) and Nud1-DNA Binding Domain (DBD), immunoprecipitation of the Nud1-DBD results in co-immunoprecipitation of sec15-AD (Fig 2B). When endogenous centriolin was immunoprecipitated using affinity purified centriolin antibodies, sec8 was co-immunoprecipitated along with other members of the exocyst such as sec5 (Fig. 2C). Additionally, when individual exocyst components were immunoprecipitated, endogenous centriolin was found to comimmunoprecipitate (Fig. 2D). These data suggest that centriolin binds to the exocyst complex via an interaction with sec15. To confirm for the yeast two-hybrid interaction between snapin and centriolin, centriolin was immunoprecipitated from Hela cells expressing a 6-Histidine (6 His)-tagged snapin construct. In these experiments, 6-His-tagged snapin coimmunoprecipitated with endogenous centriolin (Fig. 2E).

**The exocyst complex colocalizes with centriolin at the midbody during cytokinesis.**

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 the exocyst components (sec15, sec8, exo70, exo84, or sec3) and either microtubules or centriolin (Fig 3A). We found that all of these exocyst components localized to the midbody during cytokinesis, and formed a ring-like structure similar to that seen when using centriolin antibody. In fact, double-stained images revealed considerable overlap between the exocyst and centriolin suggesting that they were part of the same structure (Fig 3A, sec8/centriolin panel). In further support for midbody localization of the exocyst, a myc-tagged construct of sec8 was found to localize to the midbody when expressed in Hela cells (Fig 3B).

**The exocyst is mislocalized in cells with reduced centriolin.**

We next addressed the co-dependence of centriolin and exocyst components on localization to the midbody ring. Centriolin, sec5, or sec8 protein levels were reduced by siRNA treatment (Fig. 4A); for centriolin, similar results were obtained with two distinct double-strand oligonucleotides that target different centriolin sequences (Gromley, 2003). In all cells with undetectable centriolin at the midbody, exocyst

components were also absent from this structure (Fig 4B). In contrast, when sec8 or sec5 were reduced to undetectable levels at the midbody by siRNA treatments, centriolin localization was unaltered (Fig 4C). These data suggest that centriolin is required for midbody localization of the exocyst, while localization of centriolin appears to be independent of the exocyst.

It has recently been shown that mutants of sec5 in *D melanogaster* disrupt exocyst function (Murthy and Schwarz, 2004) and that RNAi-mediated reduction of sec5 inhibits exocyst-dependent processes in vertebrate cells (Prigent et al., 2003). For these reasons, we targeted sec5 for siRNA reduction in an attempt to disrupt the exocyst complex at the midbody. Under conditions where sec5 levels were reduced globally to below 50%, most cells showed no protein at midbodies. In these cells, other exocyst components were mislocalized from the midbody including sec3, sec8 and sec15 (Fig. 4D). These studies show that reduction of sec5 levels is sufficient to disrupt the exocyst at the midbody.

**Disruption of the exocyst causes failure at the final stages of cytokinesis.**

Localization of the exocyst complex to the midbody and its interaction with a known cytokinesis protein, centriolin, suggests that it may play a role in cytokinesis. In an attempt to identify a cytokinesis function for the exocyst, we performed time-lapse microscopy on cells treated with siRNAs against sec5. Time-lapse imaging showed

that over 40% of mitotic cells had severe cytokinesis defects (Fig. 5C). The vast

majority of these defects occurred late in cytokinesis and appeared to be a failure in

the final abscission step, as the cells remained interconnected by thin cytoplasmic

bridges for extended periods of time (Fig. 5B; supplemental movie #2). In fact, some

cells took two to three times longer to separate as compared to controls, while others

never separated before the end of the time-lapse imaging. We often observed cells

entering a subsequent mitosis while still connected to their partner cell. In some

cases, multiple rounds of mitosis were observed in connected cells. Similar results

were obtained when siRNAs against sec15 and sec8 were used simultaneously (data

not shown). None of these defects were observed when cells were treated with

siRNAs targeting lamin (Fig. 5A; supplemental movie #1). These data show that

disruption of the exocyst produces a phenotype indistinguishable from centriolin

(Gromley et al., 2003) and demonstrates a requirement for the exocyst in the final

stages of cytokinesis.

**Midbody localization of the SNARE complex is centriolin-dependent and**

**disruption of snapin results in cytokinesis defects**

In addition to the exocyst component sec15, our yeast two-hybrid screen for

centriolin interacting partners revealed an association with the SNARE-associated

protein snapin. Snapin was originally thought to be a neuron-specific protein, but

recent studies have demonstrated that it is expressed in neuronal and non-neuronal

cells (Buxton et al., 2003). Although the subcellular localization of this protein was not studied in detail, the authors observed an enrichment of snapin in the perinuclear region of interphase cells. Using antibodies to snapin, we confirmed this localization pattern in interphase cells (data not shown). Additionally, in cells undergoing cytokinesis, we observed localization of snapin to the midbody ring structure with centriolin and exocyst components (Fig 6B).

Previous studies have demonstrated that the SNARE components endobrevin and syntaxin-2 localize to the midbody during cytokinesis, and are enriched in the area of the microtubule-rich intracellular bridge structures on either side of the Flemming body (Low et al., 2003). Using the same antibodies, we confirmed this localization pattern for endobrevin at the earlier stages of cytokinesis (Fig 6A) when the intracellular bridge was rather wide (~ 1μm). However, at later stages of cytokinesis when the intracellular bridge was much thinner (< 0.5μm), this protein formed a ring-like structure similar to that seen for centriolin and the exocyst (Fig 6B). As with the exocyst complex, endobrevin and snapin were mislocalized from the midbody in cells with reduced centriolin (Fig. 6C). In cells treated with siRNAs against snapin, nearly 40% have no detectable snapin signal at the midbody (Fig. 6D). When these cells were observed using time-lapse microscopy, a significant percentage of mitotic cells remained connected by a thin intracellular bridge for significantly longer periods of time than controls (~40% vs 2% for controls). Some cells remained connected for the entire movie (>20hr), while a few eventually separated when one daughter cell re-

entered mitosis (Fig. 6E; supplemental movie #3). These results suggest that centriolin is required for midbody localization of the SNARE complex and that the SNARE-associated protein snapin is necessary for completion of cytokinesis.

## Disruption of the exocyst causes accumulation of secretory vesicles at the midbody.

Since the exocyst is thought to act as a tethering complex to mediate vesicle fusion, we decided to look at localization of the vesicle SNARE (v-SNARE) endobrevin in sec5 siRNA-treated cells, which we have shown lack exocyst components at the midbody. In over 30% of cells that have reduced levels of sec5, we observed a collection of small, spherical structures of less than 500nm in diameter at the midbody (Fig 7A, arrows). In fact, closer examination revealed that these structures are positioned around the phase-dense Flemming body (Fig 7A enlarged inset, large arrowhead). Although these structures were occasionally seen in control lamin A/C siRNA treated cells, they were more prevalent in cells treated with siRNAs against sec5.

In *C. elegans*, disruption of golgi function using brefeldin A (BFA) results in a late stage cytokinesis defect in which cleavage furrow ingression occurs, but abscission does not take place (Skop et al., 2001), suggesting that secretion is important for this

event. Because similar cytokinesis defects were observed in our studies, we decided to look at the effects of BFA in our system. Hela cells were treated with 10 μg/ml BFA and followed by phase contrast time-lapse microscopy. In all mitotic cells observed, abscission was delayed significantly as compared to controls. Most cells remain connected by a thin intracellular bridge for extended periods of time, often twice as long as controls (>6hrs vs 3hr for controls) (Fig 7B; supplemental movie #4). Since an identical phenotype is seen with disruption of centriolin, snapin, and the exocyst, it is likely that the cytokinesis defects we observed are due to inhibition of secretory vesicle delivery from the golgi.

To further demonstrate a role for secretion in this process, we observed cytokinesis by time-lapse microscopy in Hela cells expressing a GFP-tagged construct which contains an amino-terminal signal peptide that targets the protein to the lumen of the ER (lum-GFP), but lacks retrieval or retention motifs. This construct is a useful soluble secretory cargo marker (Blum et al., 2000). In cells expressing this construct, we observed a dramatic and rapid movement of GFP-tagged structures from the cell body of each daughter cell to the midbody. Interestingly, one daughter cell often contributed more of these vesicles than the other. On closer examination, we found that these vesicles accumulate next to the phase-dense Flemming body at the midbody (Fig 7C; arrows, GFP-vesicles; large arrowhead, Flemming body; supplemental movie #5). This accumulation was transient and disappeared within 12 minutes, presumably due to vesicle fusion with the plasma membrane. As this accumulation of

secretory vesicles is both morphologically and temporally similar to the v-SNARE containing structures we see accumulated at the midbody in cells with disrupted exocyst complexes, it is likely that these structures are one in the same. This data suggests a requirement for targeted secretion at the midbody for successful completion of cytokinesis, and that disruption of the exocyst or proteins required for SNARE function inhibits fusion of these vesicles, resulting in their accumulation at the midbody.

**Discussion**

In this paper, we describe the identification of components of the exocyst as well as the SNARE-associated protein snapin as interacting partners with centriolin. These proteins colocalize with centriolin to a ring-like structure at the midbody during cytokinesis. Furthermore, we show that midbody localization of these proteins is dependent on centriolin. Disruption of these proteins, or a block in secretion using BFA, leads to a cytokinesis defect indistinguishable from the centriolin phenotype we reported previously (Gromley et al., 2003). When cells exhibiting cytokinesis defects were studied in more detail, it was found that v-SNARE containing vesicle-like structures accumulated at the midbody. Using a GFP-tagged secretory vesicle marker, it was found that secretory vesicles localize to the midbody in a similar pattern. These results lead us to propose that centriolin-anchoring of the exocyst and

SNARE complex at the midbody produces local fusion of secretory vesicles, which is required for the terminal abscission event of cytokinesis.

To date, localization of the exocyst to the midbody in mammalian cells has not been described. However, members of the *S. cerevisiae* exocyst complex have been shown to localize to the mother-bud neck during cytokinesis (Finger et al., 1998; Mondesert et al., 1997), while the *S. pombe* exocyst components Sec6, Sec8, Sec10, and Exo70 localize to the actomyosin contractile ring late in mitosis (Wang et al., 2002). In addition, a proteomic analysis of the midbody in Chinese Hamster Ovary (CHO) cells found that a component of the exocyst, sec3, is present at the midbody (Skop et al., 2004). These data suggest that exocyst localization to the site of cytokinesis may be conserved.

The accumulation of secretory vesicles in cells disrupted for the exocyst has been described previously in both *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, cells overexpressing the exocyst component Sec15 accumulate vesicles that are positioned toward the mother-bud neck (Salminen and Novick, 1989), the site of cytokinesis in the budding yeast. Similarly, *S. pombe* mutants for the exocyst component Sec8 accumulate 100nm presumptive secretory vesicles in the vicinity of the division septum during cytokinesis (Wang et al., 2002). These results support our observations that disruption of the exocyst leads to an accumulation of secretory vesicles near the site of cytokinesis.

A requirement for the centrosome in cytokinesis has been suggested by several

groups. Laser ablation (Khodjakov and Rieder, 2001) or microsurgical (Piel et al.,

2001) disruption of the entire centrosome, or disruption of single centrosome

components (Gromley et al., 2003; Keryer et al., 2003) result in cytokinesis failure.

These findings suggest that some as yet unidentified component of the centrosome is

important for cytokinesis. Evidence presented here suggests that centriolin links the

centrosome and vesicle fusion machinery, and that this link is needed for successful

completion of cytokinesis.

**Figure 1. Centriolin localizes to a ring-like structure at the midbody during cytokinesis.**

A) In cells undergoing cytokinesis, centriolin localizes to a distinct ring structure at the midbody that is ~1.5 to 2 μm in diameter and encircles a γ tubulin-containing structure. Insets are enlargements of the boxed area in the full-size image with individual fluorescent channels and a merged image shown. For large image and insets, DNA is in blue, γ tubulin is in red, and centriolin is in green. For all figures, measurement bars in full images equal 10μm and in insets equal 1μm. B) The centriolin ring at the midbody is found inside the plasma membrane and encircles the phase-dense Flemming body. Boxed region of the full sized image is enlarged with insets showing individual images of phase contrast, centriolin (centr), aim1, and merge of all three images (centriolin in green, aim1 in red). Aim1 is used to identify the midbody. Note the ring structure lies inside the plasma membrane and is present before completion of contractile ring constriction. C) The centralspindlin component MKLP-1 also localizes to the midbody ring structure and disruption of this protein using siRNAs causes a mislocalization of centriolin from the midbody. An enlarged image of the midbody reveals localization of MKLP-1 to the midbody ring. MKLP-1 in green, Aim1 in red. Western blot analysis of cells treated with siRNAs against MKLP-1 or Lamin A/C (control) verifies a specific reduction of MKLP-1 protein levels. γ tubulin is used as a loading control. An enlarged image of a midbody from a cell treated with siRNAs against MKLP-1 shows that centriolin is mislocalized from the midbody. Centriolin in green, Aim1 in red.

# Figure 1.



**A**  Centriolin/γ tubulin    γ tubulin    Centriolin    Merge

**B**    Phase  Centr  Aim1  Merge

**C**  MKLP-1/Aim1    Centriolin/Aim1

Lamin siRNA    MKLP-1 siRNA
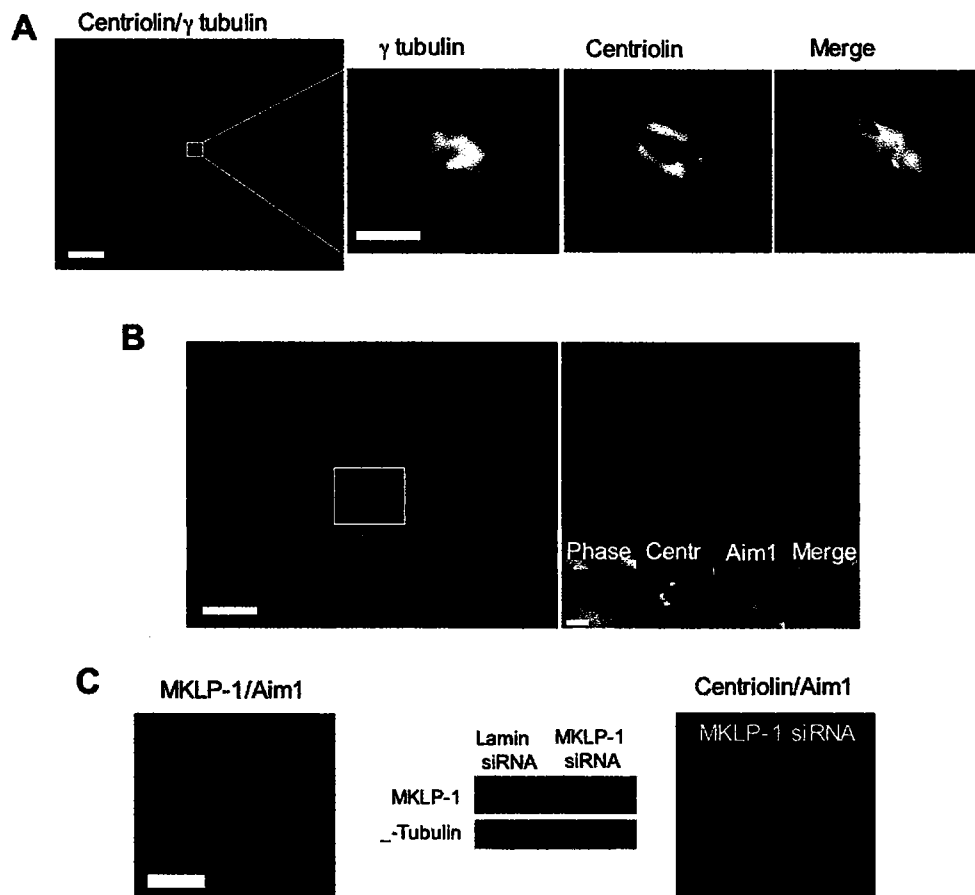
MKLP-1
_-Tubulin

MKLP-1 siRNA

**Figure 2. Centriolin interacts with components of the exocyst complex and the SNARE-associated protein snapin.**

A) Centriolin is found in the peak fractions for the exocyst by density gradient centrifugation. B) The interaction between the Nud1-like domain of centriolin and sec15 revealed in a yeast two hybrid library screen was confirmed by co-overexpressing both constructs in yeast and performing coimmunoprecipitation assays. When the Nud1-DBD construct was immunoprecipitated from lysates using a DBD-specific antibody, sec15-AD was found to coimmunoprecipitate. C) Endogenous centriolin interacts with endogenous exocyst components. Centriolin was immunoprecipitated from RPE lysates and probed for the indicated exocyst components. Both sec8 and sec5 were found to coimmunoprecipitate with centriolin. D) An interaction between the exocyst and centriolin was further confirmed by immunoprecipitating the indicated exocyst components and probing for centriolin. E) Endogenous centriolin interacts with an overexpressed 6 His-tagged snapin.

# Figure 2.

**A**
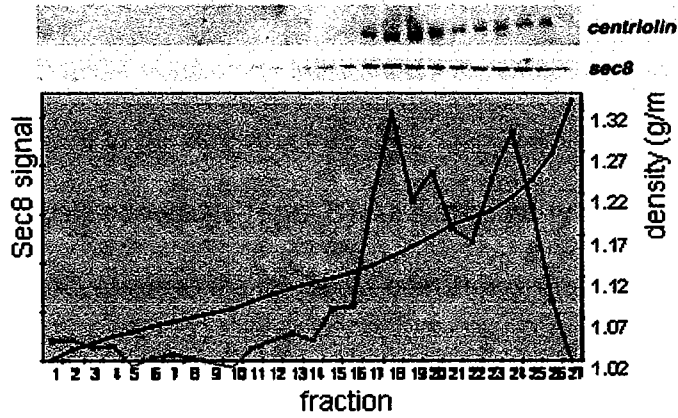


**B**
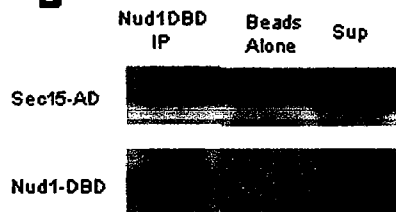


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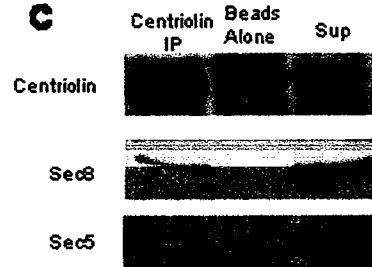


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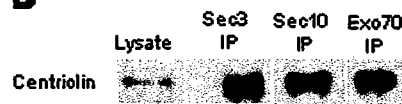


**E**
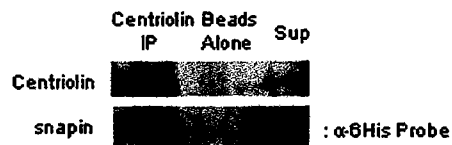
**Figure 3.  Members of the exocyst complex colocalize with centriolin at the midbody ring structure during cytokinesis.**

A) Enlarged immunofluorescence images of the indicated exocyst component (green) costained with anti-α-tubulin antibody (red) to visualize the microtubules of the midbody.  The final panel is a merged image of sec8 (red) and centriolin (green) showing colocalization to the ring structure (Top inset, sec8 stain. Bottom inset, centriolin stain.)  Scale bar equals 1μm.  B) An exogenously expressed myc-tagged sec8 (red in merge) localizes to the midbody, confirming the localization pattern seen by antibody stain.

# Figure 3

**A**

| Exo84/MT | Sec5/MT | Sec3/MT |
|---|---|---|

| Exo70/MT | Sec15/MT | Sec8/Centriolin |
|---|---|---|

**B**

| Sec8-myc | Phase | Overlay |
|---|---|---|

**Figure 4. Midbody localization of the exocyst is centriolin-dependent.**

A) Western blots showing disruption of the indicated proteins with siRNAs. γ

tubulin was used as a loading control for all experiments. B) In cells that lack

centriolin, the exocyst is not present at the midbody. The graph shows quantitation
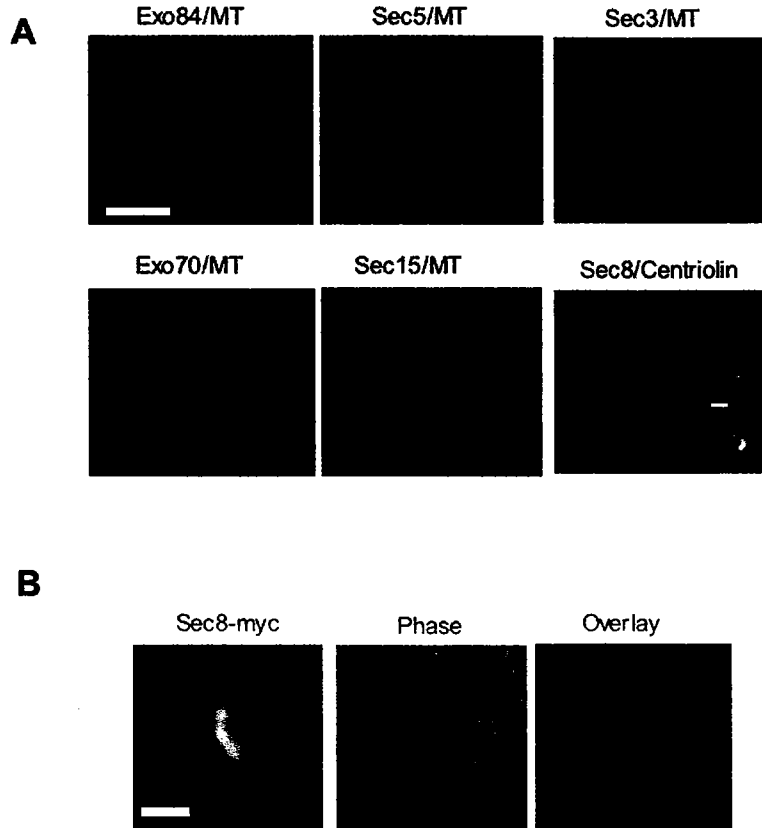
for the percentage of midbodies in telophase cells that lack sec8 stain for both control

lamin A/C siRNA and centriolin siRNA-treated cells. Images are enlargements of

midbodies costained with either centriolin (green) and sec8 (red), first panel, or the

indicated exocyst component (green) and microtubules (red). Inset is a phase contrast

image showing the presence of a Flemming body. C) Disruption of the exocyst does

not affect the midbody localization of centriolin. The graph shows quantitation for

the percentage of midbodies in telophase cells that lack centriolin stain for control

lamin A/C, sec8 siRNA, sec5 siRNA, and centriolin siRNA. Images are

enlargements of midbodies costained with centriolin (green) and either sec8 or Aim1

(red). D) Reduction of sec5 protein levels using siRNA results in disruption of the

exocyst complex from the midbody. The graph shows quantitation for the percentage

of midbodies in telophase cells that lack sec5 stain for both control lamin A/C siRNA

and sec5 siRNA-treated cells. Images are enlargements of midbodies costained with

the indicated exocyst component (green) and microtubules (red), or costained with

two separate exocyst components (last panel, sec15 in green; sec8 in red). Inset is a

phase contrast image showing the presence of a Flemming body. For all panels,

scale bar equals 1μm.
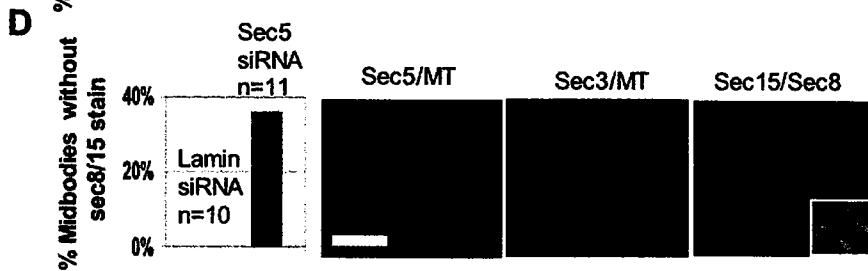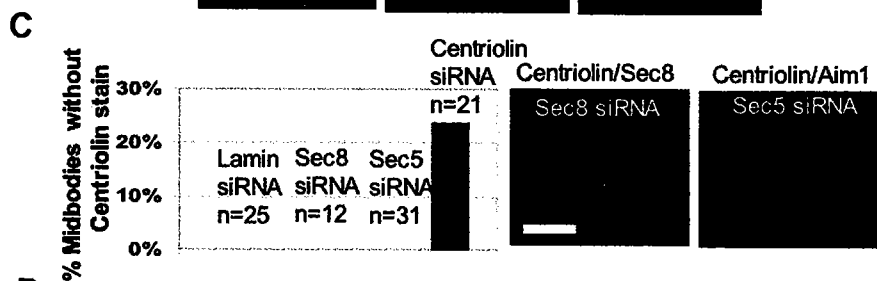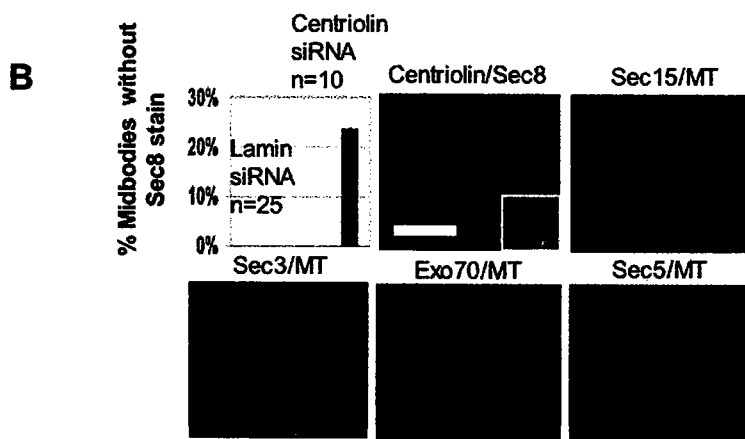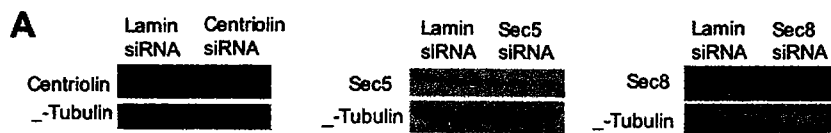
# Figure 4.

**Figure 5. Cells treated with siRNAs against sec5 exhibit cytokinesis defects.**

A) Frames from time-lapse imaging of Hela cells treated with control lamin A/C

siRNAs. A mitotic cell (arrow) enters mitosis, performs cleavage furrow ingression

and eventually completes abscission to form to separate cells in a timeframe of three

hours. Time shown in hours:minutes. B) Cells treated with siRNAs against sec5

remain interconnected for extended periods of time. A mitotic cell (arrow) enters

mitosis and performs cleavage furrow ingression in a timeframe similar to controls

(~50 minutes), but does not complete cytokinesis and remains connected by a thin

intracellular bridge (arrow, panels 1:55 through 17:05) for a period of time

significantly longer than controls (>17 hrs). C) The graph shows quantitation of the

percentage of mitotic cells observed that exhibited cytokinesis failure as determined

by persistent intracellular bridges or abortion of cytokinesis and formation of

binucleate cells.

# Figure 5.

**A**



**B**



**C**

**Figure 6. Midbody localization of the SNARE components endobrevin and snapin is centriolin-dependent and snapin disruption causes cytokinesis defects.**
A) In early stages of cytokinesis, when the midbody is rather wide (>1μm), endobrevin localizes to microtubule structures on either side of the Flemming body. B) Enlarged midbody images of endobrevin (green) and Aim1 (red), and snapin (green) and microtubules (red), showing both SNARE components localize to the ring structure around the Flemming body in later stages of cytokinesis, when the midbody is much thinner (<0.5 μm). C) The graphs show quantitation of the percentage of midbodies in telophase cells that lack either snapin or endobrevin stain for both lamin A/C siRNA and centriolin siRNA-treated cells. Enlarged midbody images of cells costained with either snapin (green) and Aim1, or endobrevin (green) and Aim1 (red) are given as examples. D) The graph shows quantitation of the percentage of midbodies in telophase cells that lack snapin stain for both lamin A/C siRNA and snapin siRNA-treated cells. An enlarged midbody image of cells stained with snapin (green) and Aim1 (red) is given as an example. E) Cells treated with siRNAs against snapin remain connected by a thin intracellular bridge for extended periods of time. At time 0, two daughter cells have progressed through mitosis and the early stages of cytokinesis, but are still connected by an intracellular bridge (arrow). These cells remain connected for over 17 hours before finally separating when one daughter cell appears to enter mitosis (rounded up cell at top of final panel). The graph shows quantitation of the percentage of mitotic cells observed that exhibited cytokinesis

failure as determined by persistent intracellular bridges or abortion of cytokinesis and formation of binucleate cells.
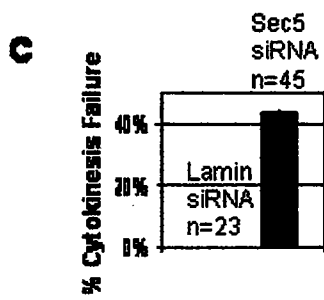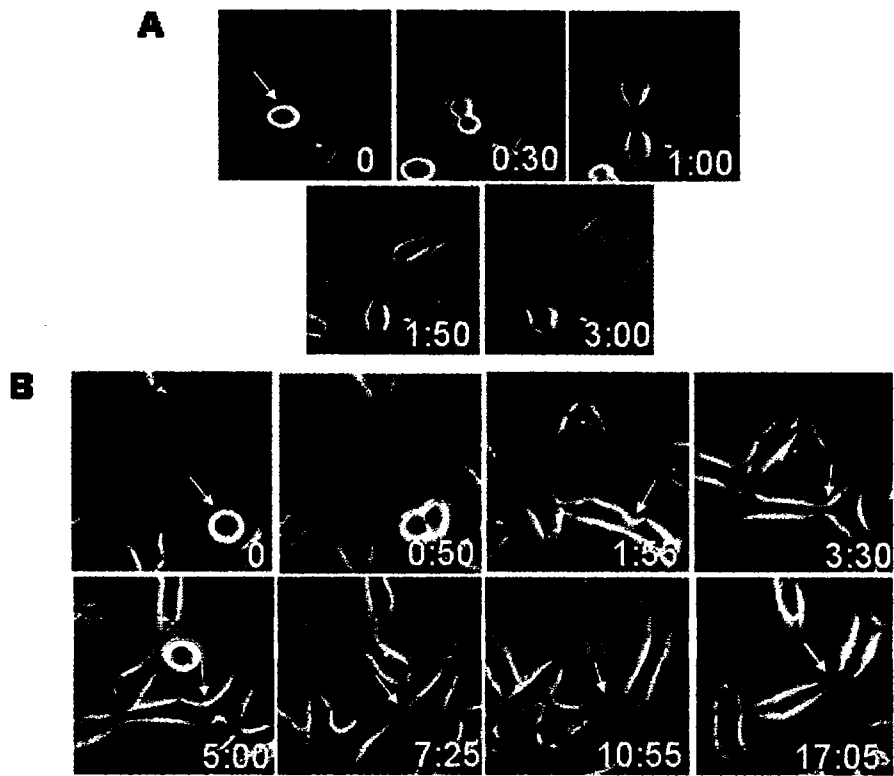
# Figure 6.



**A** Endobrevin/Aim1

**B** Endobrevin/Aim1    Snapin/MT

**C** % Midbodies without snapin stain

Centriolin siRNA n=37

Snapin/Aim1

Centriolin siRNA

Lamin siRNA n=36

% Midbodies without endobrevin stain

Centriolin siRNA n=51

Endobrevin/Aim1

Centriolin siRNA

Lamin siRNA n=52

**D** % Midbodies without snapin stain

Snapin siRNA n=23

Snapin/Aim1

Snapin siRNA

Lamin siRNA n=36

**E**

0    3:20    7:30

15:50    17:30    20:25

% of Mitotics with cytokinesis failure
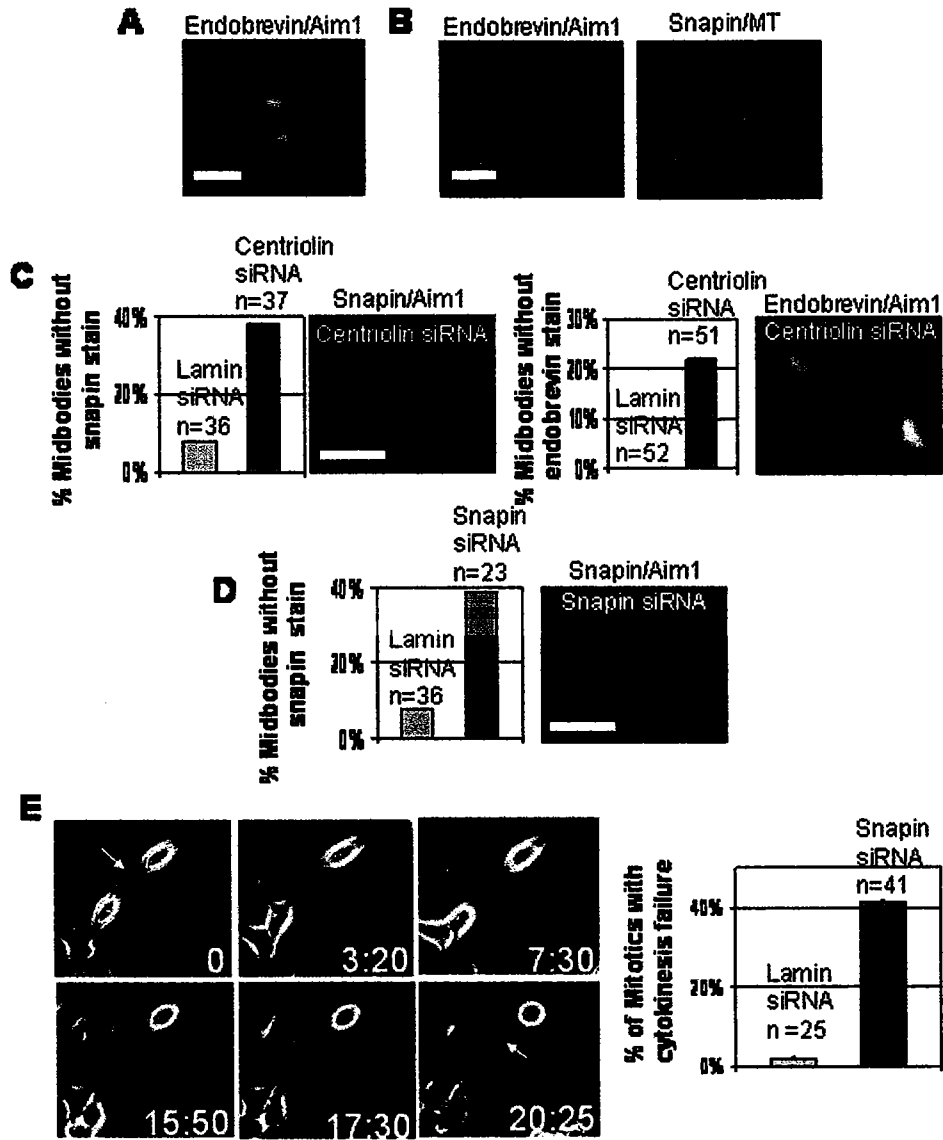
Snapin siRNA n=41

Lamin siRNA n =25

**Figure 7. Disruption of the exocyst causes accumulation of secretory vesicles at the midbody.**

A) In cells treated with siRNAs against sec5, an accumulation of endobrevin-containing vesicles is seen at the midbody. Enlarged image of the midbody is a merged image of endobrevin (green) and aim1 (red) to show colocalization to small vesicles (arrows, < 500nm) at the midbody. Merged image is an overlay of endobrevin, aim1, and phase contrast images (arrows, small vesicles; large arrowhead, Flemming body). Dotted lines are included to help visualize the outline of the plasma membrane at the midbody. The graph shows the quantitation of the percentage of telophase cells that have vesicles at the midbody in both control lamin A/C siRNA and sec5 siRNA treated cells. B) Frames from time-lapse imaging of cells treated with BFA. A mitotic cell (arrow; time 0) exits mitosis and performs cleavage furrow ingression with normal timing. However, the daughter cells remain connected by a thin intracellular bridge (arrow, panels 2:35 through 6:45) for a significantly longer period of time than controls (>6hrs vs 3hrs for controls) before finally separating. C) During cytokinesis, cells expressing a luminal-GFP construct accumulate a large number of secretory vesicles at the Flemming body that quickly disappear, presumably due to fusion with the plasma membrane. Images are frames from time-lapse imaging with phase and GFP signals overlayed. Arrows, GFP-vesicles, large arrowhead, Flemming body. For all time-lapse panels, time is in hours:minutes. . D) A telophase cell expressing luminal-GFP was stained with

antibodies against endobrevin. Full size image is a merge of endobrevin (red) and luminal-GFP (green). Insets are enlargements of separate channels and merge for the boxed area in the full size image. Note colocalization of endobrevin and luminal-GFP in the midbody region. Scale bar for insets equals 1μm.

# Figure 7.

**A**

| Endobrevin/Aim1 | Merge |
|---|---|

Sec5 siRNA



**B**



**C**



**D**
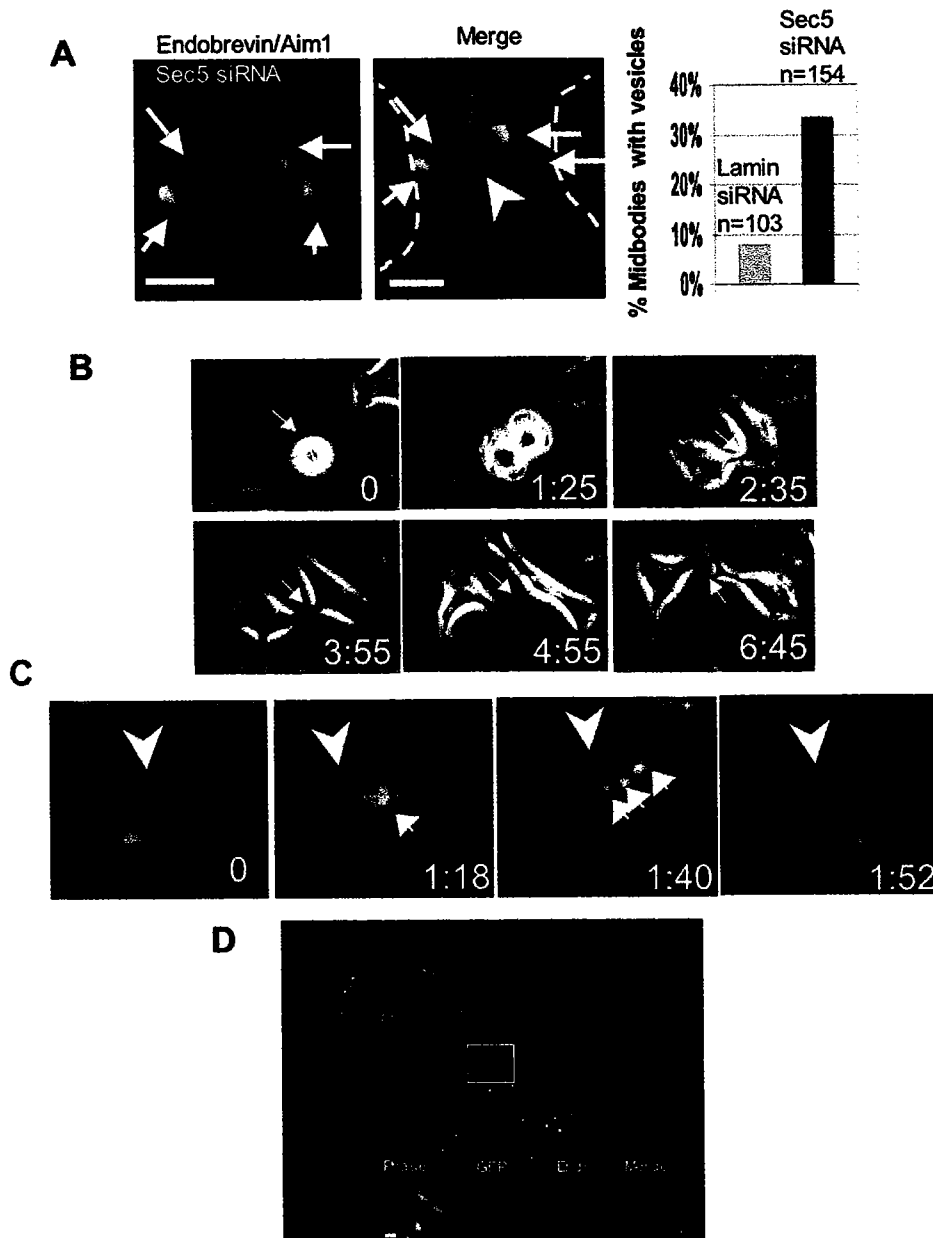
# CHAPTER III

# CLONING AND CHARACTERIZATION OF A NOVEL GTPASE-ACTIVATING PROTEIN

The final chapter of my dissertation describes my attempts to clone and characterize a novel protein identified as an interacting partner with centriolin. Although the material presented here certainly does not represent the whole story, and there are many more experiments to perform, I believe this is sufficient data to serve as the basis for a future project. This chapter is written and formatted as the typical results section that one would find in a major cell biology journal.

*The Nud1 domain of centriolin interacts with a novel protein*

To determine the molecular function of centriolin in cytokinesis, a yeast two-hybrid

screen was performed. We used a domain of centriolin homologous to genes

involved in mitotic exit and cytokinesis in budding and fission yeasts (Nud1/Cdc11),

which is required for the cytokinesis function of centriolin (Gromley et al., 2003).

This domain was used as bait to screen approximately 12 million clones in a human

testis cDNA library (Fig.1). After eliminating false positives using several control

parameters (see Materials and Methods), we identified three interacting proteins of

interest. Two of these proteins were found to be components of protein complexes

involved in vesicle fusion events and these results were reported previously (Gromley

et al., 2004). The third protein was a novel regulatory protein, which I attempted to

clone and characterize. This protein will hence be referred to as agGAP for reasons

discussed later.

In order to rule out the possibility that the interactions found in the yeast two hybrid

system were due to an artificial activation of the reporter gene (false positive), the

yeast strain expressing the agGAP-Activation Domain (AD) construct was mated to

the appropriate partner strain containing either the DNA-Binding Domain (DBD)

alone or an unrelated protein (Lamin C) fused to the DBD (Fig 2A). An additional

control was performed to ensure that the centriolin Nud1-DBD construct alone did

not induce expression of the reporter gene. To further confirm the interaction between

agGAP and the Nud1 domain of centriolin that was revealed in the yeast two hybrid system, a myc-tagged agGAP construct was overexpressed in the human cultured cell line HEK 293, followed by immunoprecipitation of endogenous centriolin (Fig. 2B). It was found that the myc-tagged agGAP coimmunoprecipitated with endogenous centriolin, further supporting the possibility that this is a true interaction.

### The novel protein agGAP contains a RhoGAP domain

Since the sequence contained within this positive clone represented a continuous open reading frame, lacking both a start and stop codon, it was evident that this clone was only a partial sequence (~1kb) of the full length transcript. In order to determine the full length sequence, a bioinformatics approach combined with PCR techniques and 5' RACE was used. First, the sequence was BLASTed against the human genome (Fig. 3A). This revealed a segment of chromosome 1 with 100% identity (Fig. 3B). In an attempt to identify the gene contained within this region, approximately 250kb of genomic sequence upstream and downstream of this region was submitted to the gene prediction program Genscan (Fig. 3C). Genscan predicted a transcript within this genomic sequence of 4,176 bases, encoding a protein of 1,391 amino acids with a predicted molecular weight of ~150kDa . In order to determine if this predicted sequence represented an expressed sequence, the sequence was BLASTed against the human EST database at the NCBI website. The results of this search revealed

overlapping ESTs spanning approximately 80% of the predicted sequence, confirming that this predicted sequence was expressed (Fig. 3D). The predicted agGAP sequence was then used as a template for primer design in an attempt to PCR the full length sequence out of a cDNA library or RTPCR from Poly A+ mRNA (Fig. 3E). Multiple sets of primers were made, targeting both the GAP domain alone and the full length predicted sequence. The largest clone obtained using this approach was approximately 3kb in size. This clone contains a continuous open reading frame running from nucleotide 1187 to 4132 of the Genscan predicted full length sequence. Initial attempts at retrieving the 5' end using 5' RACE produced a product of the predicted size. However, upon sequencing it was found that this product was a nonspecific contamination band.

Unfortunately, subsequent attempts to amplify the missing 5' end using 5' RACE techniques were unsuccessful, and, thus, the full coding sequence of this gene remains unknown. Recently, a gene predicted by the program GNOMON was submitted to the NCBI database (accession # XM_086186) that is 100% identical to nucleotides 592-3138 of the sequence predicted by Genscan. This suggests that the furthest 5' sequence predicted by Genscan (nucleotides 1-592) may not be accurate, and, therefore, this region may not be a useful template for designing primers to search for the 5' end.

Analysis of the predicted amino acid sequence using the Simple Modular

Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/) (Letunic et

al., 2004; Schultz et al., 1998)revealed a GTPase Activating Protein (GAP) domain,

as well as a short coiled-coil (CC) domain (Fig. 4A). Based on the presence of this

GAP domain, we have tentatively named this novel protein agGAP. Upon further

examination, it was found that this GAP domain is highly homologous to those found

in GAPs that activate the Rho-like family of GTPases (Rho/Rac/Cdc42) (Fig. 4B).

Additionally, this protein possesses a key conserved arginine residue (yellow

highlight, Fig. 4B), which is common among Rho GAPs (Leonard et al., 1998; Nassar

et al., 1998). The arginine residue is inserted into the active site of the Rho GTPases

and stabilizes the conformation needed for hydrolysis of the bound GTP (Nassar et

al., 1998; Rittinger et al., 1997a; Rittinger et al., 1997b). Thus, based on sequence

characteristics, this novel protein appears to be a GAP against the Rho-like family of

GTPases.

An additional feature of agGAP is the presence of a C2 domain. These domains were

first described in Protein Kinase C (PKC) (Nishizuka, 1988), and are modules within

proteins that bind phospholipids in a Ca2+-dependent manner. C2 domain-containing

proteins typically function in either signal transduction pathways or membrane

trafficking events. Since other centriolin-interacting partners identified in the same

yeast two hybrid screen were found to be members of membrane-associated

complexes (Gromley et al., 2004), it seems likely that agGAP may function in membrane trafficking.

*agGAP localizes to the midbody during cytokinesis*

A segment of agGAP that included the GAP domain (amino acids 959-1275) was cloned into pDL2 vector in frame with the 6 His tag. This construct was expressed in E.coli strain BL2. The expressed protein was purified using a nickel column and the purified protein was then used to immunize rabbits to make polyclonal antibodies. The polyclonal antibodies generated were used for indirect immunofluorescence studies in the cultured cell line hTERT-RPE, which are telomerase-immortalized human Retinal Pigment Epithelial cells. In interphase, agGAP localized to punctuate structures which appeared to be near or on the surface of the cell (Fig 5A). Further studies revealed that these punctuate structures do not colocalize with antibodies against clarthrins (Fig 5B), suggesting that these structures are not endocytic vesicles. In mitosis, this protein is found diffusely throughout the cytoplasm, with weak staining of the spindle microtubules (data not shown). However, upon entering telophase, agGAP antibodies strongly stain the intracellular bridge on either side of the phase dense Flemming body in the early stages of cytokinesis, when the midbody is wide (~1µm) (Fig 5C). Later, when the midbody is much narrower (<0.5µm), this protein localizes to the ring-like structure that forms around the Flemming body (Fig

5D). This midbody localization pattern is similar to the localization of the v-SNARE component endobrevin, which we reported previously (Gromley et al., 2004).

### *Disruption of agGAP leads to defects in cytokinesis*

Localization to the midbody in telophase, as well as the presence of a Rho GAP domain, suggested that this protein may have a function in cytokinesis. In an attempt to identify the function of agGAP, Hela cells were treated with siRNAs against agGAP for 48 hrs, followed by timelapse microscopy. Cells treated with agGAP siRNAs proceeded through early stages of cytokinesis (cleavage furrow formation and ingression), but remained connected by a thin intracellular bridge for an extended period of time as compared to control lamin siRNA treated cells. In fact, many of these cells remained connected throughout the entire timelapse imaging (24hr), never physically separating. In some cases, the interconnected cells entered a subsequent mitosis while still remaining connected, while other cells eventually aborted the attempt at abscission and fused back together to form a binucleate cell (Fig 6A). This cytokinesis defect was observed in approximately 40% of mitotic cells (Fig 6B).

### *agGAP interacts with the centralspindlin component MKLP-1*

In an attempt to identify the GTPase which this GAP activates, a yeast two hybrid

screen was performed. A domain of agGAP spanning amino acids 1039-1282, which

included the GAP domain as well as a portion of the centriolin-binding domain, were

used as bait in a yeast two hybrid screen against a human testis cDNA library.

Approximately 12.5 million clones screened. After eliminating false positives, five

interacting proteins were identified. Following sequencing of these positive clones, it

was found that one of these clones represented the centralspindlin component MKLP-

1. MKLP-1, along with MgcRacGAP, comprise a complex termed centralspindlin

which localizes to the central spindle, the microtubule bundle formed from the

spindle microtubules, and has been shown to have a function in cytokinesis (Mishima,

et al, 2002). Thus, not only does agGAP appear to function in cytokinesis, but it also

interacts with a known cytokinesis protein. Future experiments will be required to

identify the exact role these proteins have in cell division.

**Figure 1. Yeast two-hybrid screen for centriolin interacting partners identifies a novel protein.** The Nud1 domain of centriolin was used as bait in a yeast two hybrid screen against a human testis cDNA library. Approximately 12 million clones were screen, and after multiple rounds of selection followed by testing against control constructs, five true positive clones were identified. Upon sequence analysis, it was found that one of these clones was a novel protein, which is referred to here as agGAP.

# Figure 1
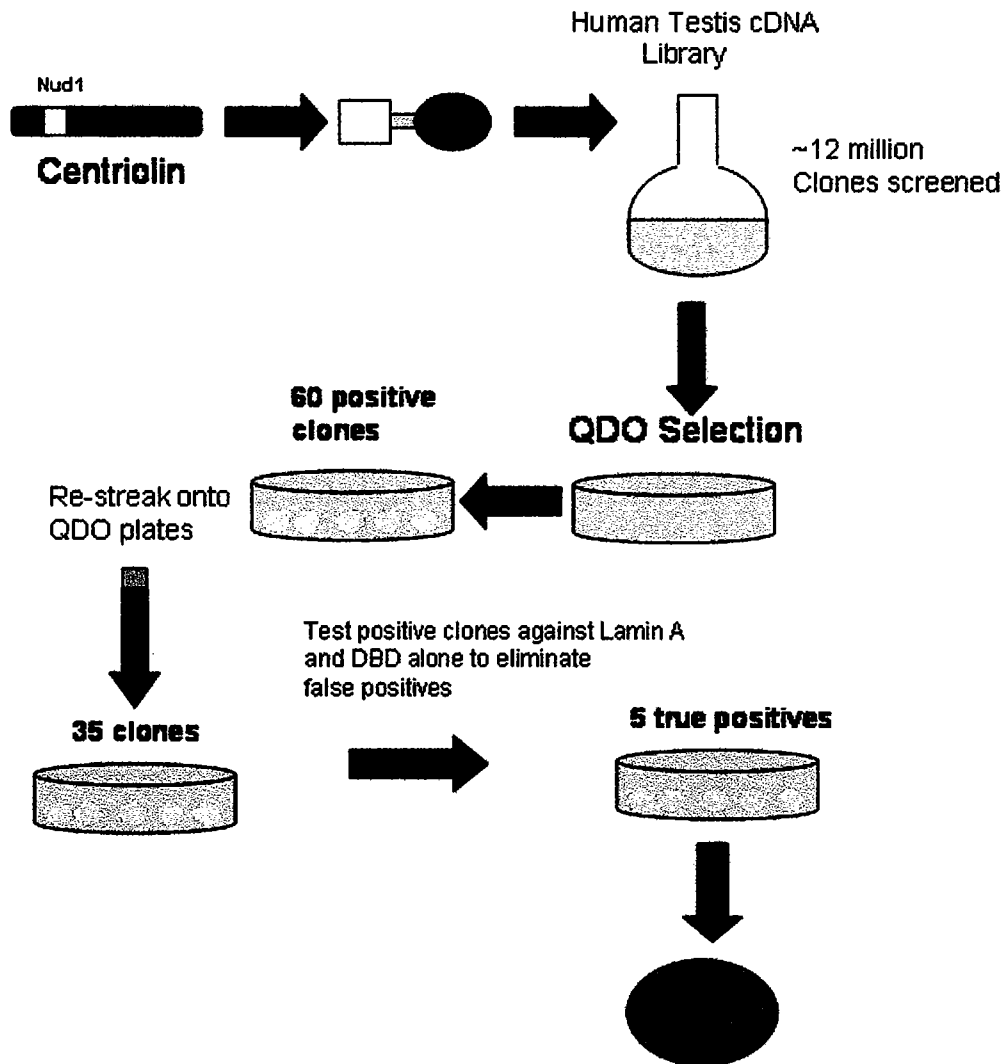
**Figure2. The novel protein agGAP interacts with the Nud1 domain of centriolin.**

A) Diploids produced from the mating of centriolin Nud1-DBD expressing cells with

agGAP-AD expressing cells are able to grow under restrictive conditions, while the

negative controls centriolin Nud1-DBD alone, lamin-DBD x agGAP-AD, or DBD-

Alone x agGAP-AD are unable to grow, suggesting a specific interaction between

centriolin Nud1 and agGAP. B) Myc-tagged agGAP coimmunoprecipitates with

endogenous centriolin in HEK 293 cells, confirming the interaction seen in the yeast

two hybrid system.

# Figure 2.



**A**

Centriolin Nud1-DBD × agGAP-AD    Centriolin Nud1-DBD Alone    Lamin-DBD × agGAP-AD    DBD-Alone × agGAP-AD

**B**

|  | Centriolin IP | Beads Alone | Sup |  |
|---|---|---|---|---|
| Centriolin Probe: | | | | ← Centriolin |
| Myc Probe: | | | | ← agGAP-Myc |
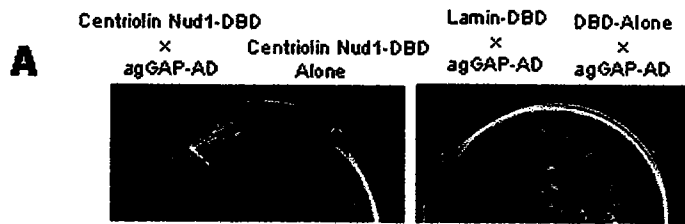
**Figure 3. Scheme for identifying the full length sequence of the novel protein agGAP.** A) The positive clone from the yeast two hybrid library screen was sequenced. It was found that this clone did not contain the full open reading frame for this protein, so the partial sequence was BLASTed against the human genome at the NCBI website. B) The BLAST results demonstrated that the gene corresponding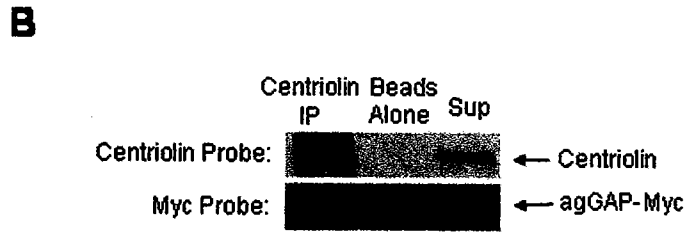 to this partial sequence was contained within a region of chromosome 1. C) To predict the full sequence of this gene, a large portion of the genomic sequence (~500 kb) from either side of the aligned partial sequence was submitted to the gene prediction program Genscan. D) This predicted sequence was then BLASTed against the human EST database at NCBI to validate that this predicted sequence was indeed expressed. Several overlapping ESTs were found that spanned ~80% of the predicted sequence, verifying that this was likely an expressed transcript. E) The sequence was then used as a template for designing primers against the unknown regions. These primers will be used in future PCR and 5' RACE reactions to identify the endogenous full length transcript.

# Figure 3.



**A**

Partial sequence
from Y2H screen
(~ 1kb)

BLAST against
Human Genome

**B** **Chromosome 1**

~500kb
of Genomic
Sequence

Genscan
Gene Prediction
Program

**C**

Predicted Sequence

**E**

Design primers based on
predicted sequences present
in ESTs and perform 5'
and 3' RACE

**D** BLAST against
Human EST Library

Full Length agGAP sequence ?,
including kozak consensus sequence
ATG start codon, and poly A tail

Overlapping ESTs

**Figure 4. The novel protein agGAP contains a RhoGAP domain.**

A) Schematic representation of agGAP primary amino acid sequence. Amino acids 878-978 contain a C2 domain (Ca2+-dependent membrane binding domain). A GAP domain is found between amino acids 1026 and 1225, and amino acids 1359-1386 contain a small coiled coil domain. The centriolin-binding domain that was identified in a yeast two hybrid screen comprises amino acids 1213-1391. B) Conserved domain analysis of the GAP domain reveals high identity with GAPs against the Rho-like family of GTPases. The conserved arginine residue that is characteristic of this family of GAPs is highlighted in yellow.

# Figure 4.

## A



```
1                                    (878-978) (1026-1225)   1,391

      agGAP      ███      GAP    ███

                                 (1359-1386 )

                    1213            1391
                    Centriolin-binding
                    Domain
```

## B
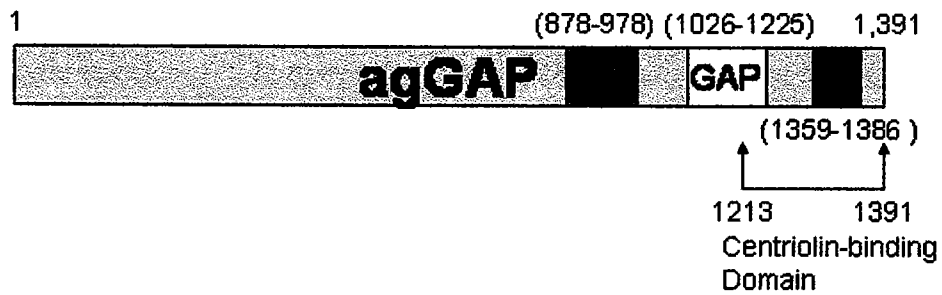
• gi|CDD:24251, smart00324, RhoGAP, GTPase-activator protein for Rho-like GTPases, GTPase activator proteins towards Rho/Rac/Cdc42-like small GTPases etc.; domain limits and others.

```
          CD-Length = 175 residues,  97.7% aligned
          Score =  156 bits (396), Expect = 4e-38

Query:  1027  EVPLLTQPCIREIERRGCQVVGL1?LCGSAAVERILREAIERDSKAVGLCENQYPDINVI  1086
Sbjct:  2     PIPIIVERCIEYLEKRGLDTEGI?+CGGSASRVREILREAYDSGPDPDLDLSEY--DVNDV  59

Query:  1087  TOVLKDTLRELPSPLITKQLYEAVLDAKARSPLRECSNOCEKDPGOSKYYVDLLDCLPEI  1146
Sbjct:  60    AGLLKLFLRELPEPLITFELYEEYIEARKLEPEKERLRALR----------ELLSLLPPA  109

Query:  1147  EKATLRILLDRLKLVASYNEVREKTCONLAVCFGPVLLSQRQEPSTIREKRVFTDSEELAS  1206
Sbjct:  110   NRATLRYLLARLRRVAERSEENKNTARNLAIVFGPTLLBPPD------------GESAR  156

Query:  1207  ALDFRKHIEVLNYLLQ  1222
Sbjct:  157   LKDIRNQNKVVEFLIK  172
```

**Figure 5. agGAP localizes to punctuate structures in interphase cells and to the midbody in telophase.** A) Interphase hTERT-RPE1 cell stained for agGAP (red), $\gamma$-tubulin (Green), and DNA (blue). agGAP is prominent on dispursed punctuate structures which appear to be on or just below the surface of the cell. B) Costain of interphase cells with agGAP (red) and clathrin (green) antibodies reveals no apparent colocalization (inset, arrows), suggesting that the punctuate agGAP structures are not endocytic vesicles. C) In early telophase, agGAP localizes to the midbody bridge (inset, arrows) on either side of the phase dense Flemming body. D) At late stages of telophase, agGAP accumulates around a ring-like structure at the site of the Flemming body (inset, arrow). For panels C and D: agGAP (green), MT (red), and DNA (blue).

**Figure 5.**



A — Interphase

B

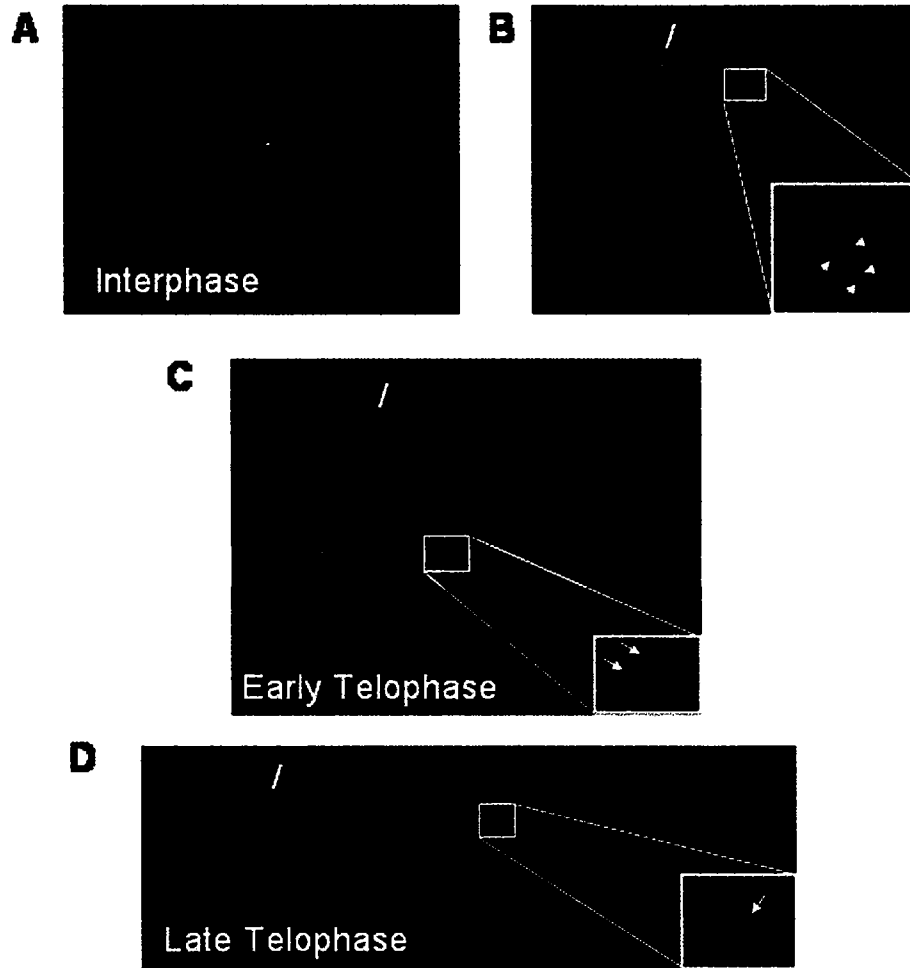C — Early Telophase

D — Late Telophase

**Figure 6. Disruption of agGAP using siRNAs results in cytokinesis defects.**

A) Images from phase-contrast time-lapse studies of Hela cells treated with siRNAs against agGAP for 48hr. At time 0, two daughter cells are connected by a thin intracellular bridge (first panel, arrowhead). These cells remain connected for an extended period of time (over 4 hours; arrowhead, panel 4:30) before finally fusing back together to form a binucleate cell (final panel; 8 hours 20 minutes, nuclei are circled in white). For clarity, the cells of interest are outlined in white. B) Quantification of cytokinesis defects observed during time-lapse imaging. The percentage of mitotic cells exhibiting either failure of abscission, as measured by remaining connect for the entire length of the time-lapse movie, or cleavage furrow regression resulting in binucleate cells is graphed for both agGAP siRNA and control lamin A/C siRNA treated cells.

# Figure 6.
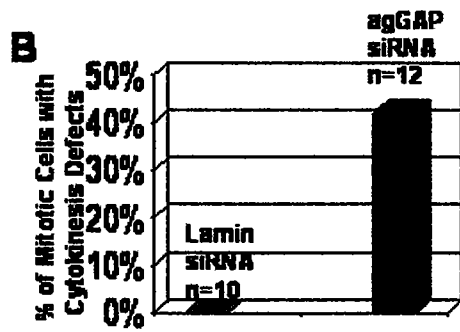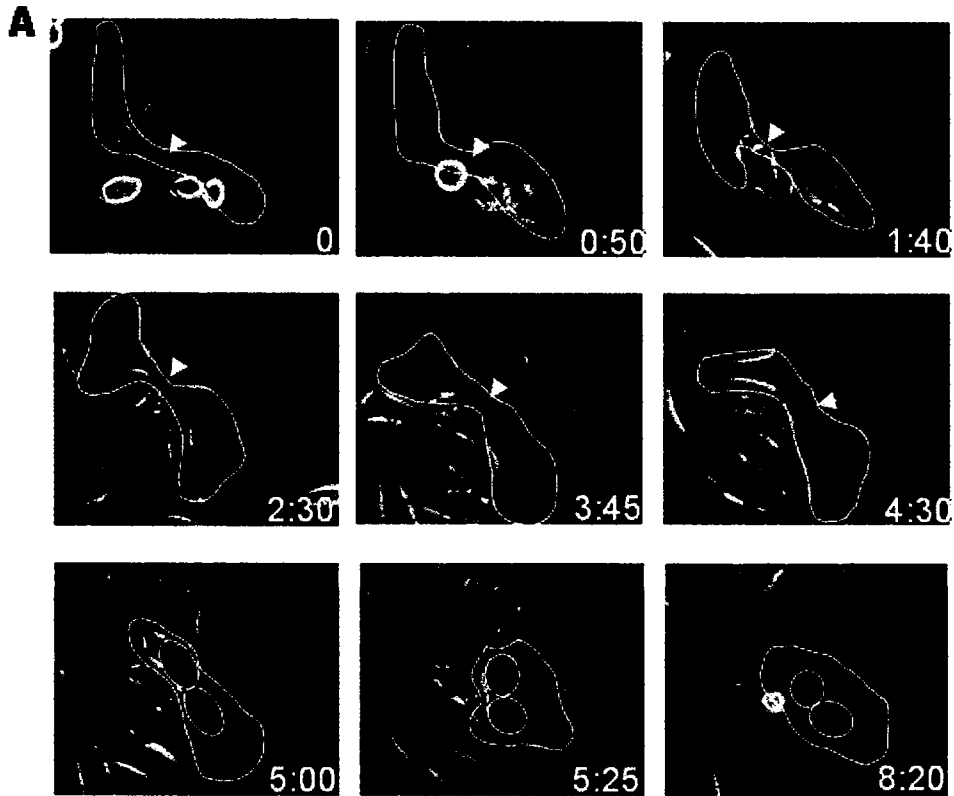
# Discussion:

In this dissertation, I describe the identification and characterization of a novel centrosome protein, termed centriolin. Centriolin was shown to localize to the subdistal appendages of the maternal centriole in interphase, and to the midbody during cytokinesis. Disruption of centriolin levels in cultured cells revealed a requirement for this protein in successful completion of cytokinesis. Specifically, centriolin appears to function at a specific time in cytokinesis, during the final abscission event. Further studies identified components of the exocyst and SNARE complexes as centriolin-interacting partners, and demonstrated that localized secretion to the midbody during cytokinesis is necessary for successful abscission. Additionally, I describe the discovery of a novel GAP protein, agGAP, as a centriolin interacting partner. The phenotype seen upon disruption of this protein is similar to that seen for centriolin, the exocyst, and the SNARE complex, suggesting that this protein may function in the same process.

The fact that centriolin interacts with the yeast GAP Bub2p (Gromley et al., 2003) as well as the novel human GAP, agGAP, suggests that centriolin may represent a mammalian homologue of the yeast MEN/SIN component Nud1p/Cdc11p. Future experiments will be required to determine which GTPase agGAP activates, although several candidates exist. Rho1 is a GTPase which has been shown to have a function in cytokinesis in *D melanogaster* (Prokopenko et al., 1999). Since agGAP contains a

domain with high homology to GAPs against the Rho-like family of GTPases, the mammalian homologue of this Rho GTPase, RhoA, is a likely candidate. Conversely, centriolin also interacts with the exocyst complex, which means it is possible that agGAP may in fact act on Rab GTPases, as sec4, a Rab GTPase, is involved in exocyst function (Guo et al., 1999).

The observation that centriolin levels decrease at the spindle poles in late anaphase while, at about the same time, it becomes visible at the phase dense Flemming body presents an interesting question: How does centriolin get to the midbody? There are two intriguing possibilities. First, centriolin may be actively transported from the spindle pole towards the midzone via a molecular motor. The obvious candidate for this would be a member of the kinesin family, as this spindle pole-to-midzone movement would be directed towards the plus ends of the spindle microtubules. In fact, we have found that midbody localization of centriolin is dependent on the mitotic kinesin-like protein MKLP-1 (Gromley et al., 2004). Furthermore, centriolin interacts with the novel protein agGAP, which, in turn, interacts with MKLP-1 in a yeast two hybrid system (our unpublished results). Another possibility that we cannot rule out is that centriolin may be degraded specifically at the spindle poles at this stage of mitosis, and the centriolin that we see accumulating at the midbody may originate from an as yet unidentified cytoplasmic pool.

One requirement for the successful completion of abscission is the disassembly of the densely packed microtubule bundle present at the midbody. This disassembly would presumably involve some type of microtubule destabilization reaction. Interestingly, centriolin appears to have several domains that share homology with proteins known to have a function in microtubule stability. The TACC (Transforming Acidic Coiled-Coil) proteins are a family of microtubule-binding proteins which function in stabilizing microtubules. Centriolin appears to have two domains with homology to the TACC proteins TACC1, TACC2, and TACC3, suggesting that centriolin may possess a microtubule-binding capacity mediated through these domains. Furthermore, a small domain of centriolin shares homology with the microtubule destabilizing protein stathmin. Due to the presence of these microtubule-association domains, it is intriguing to think that centriolin not only functions as a scaffold for signaling molecules necessary for mitotic exit as well as vesicle fusion machinery required for abscission, but also contains the microtubule destabilizing activity needed to remove the densely packed microtubule structure at the midbody. Future studies of these domains will be necessary to uncover what, if any, microtubule function is present.

Although the cell culture system is a convenient way to study cellular events due to the amount of manipulation that can be performed, ultimately one would like to relate the results obtained to the *in vivo* situation. In fact, the event studied here, abscission, may be more relevant to an *in vivo* situation as cultured cells may have a mechanism

to bypass this process in the event of problems by crawling far enough apart to physically break the connection, a process known as traction mediated cytofission, which has been observed in some cell types (Burton and Taylor, 1997; Spudich, 1989). This may explain why the phenotype we see has been described by some as "low penetrance." Perhaps in our assays a subset of affected cells are able to separate by this traction mediated cytofission mechanism. Unfortunately, it would be very difficult to study this process in a confluent monolayer, a situation closer to *in vivo*, as their close proximity would likely inhibit visualization of the abscission event.

These results represent several new findings. To our knowledge, this is the first evidence for localization of the exocyst to the midbody in mammalian cells. Furthermore, this is the first report of a cytokinesis function for the mammalian exocyst. Our report of localized secretion to the midbody during cytokinesis is the first of its kind, although a similar mechanism has been suggested in *C. elegans* (Skop et al., 2001). Additionally, this is the first detailed description of a novel ring-like structure around the phase-dense Flemming body. Lastly, this work includes the discovery of a novel GTPase Activating Protein. While many questions remain to be answered, I believe this work makes a significant contribution to the understanding of the events leading to physical separation of the two daughter cells at the end of the cell cycle in vertebrate cells.

## Materials and methods

### Cell culture and transfections

The cells used primarily in this study were diploid, telomerase-immortalized human RPE-1 cells (hTERT–RPE-1s; CLONTECH Laboratories, Inc.) (Morales et al., 1999) and Hela cells. Other cells included COS-7, hTERT–HME-1 (human mammary epithelia), U2OS, and *Xenopus* tissue culture (XTC) cells. All were grown as previously described (American Type Culture Collection). COS-7 cells were transfected as previously described (Lipofectamine; Invitrogen).

### Antibodies

Amino acids 268–903 or centriolin were fused with GST (CLONTECH Laboratories, Inc.), overexpressed in *Escherichia coli*, and purified as previously described (Doxsey et al., 1994). Antibodies raised in rabbits were affinity purified by passing sera over a GST column to remove anti-GST antibodies and then over a GST–centriolin column. Antibodies to the following proteins were also used in this study: lamin A/C (Cell Signaling Technology), $\alpha$ – and $\gamma$-tubulins, monoclonal $\alpha$-6His, and monoclonal $\alpha$-myc (Sigma-Aldrich), LexA and polyclonal MKLP-1 (Santa Cruz Biotechnology, Inc.), monoclonal GAL4 transactivation domain (TAD) and

monoclonal GAL4 DNA binding domain (DBD) (CLONTECH Laboratories, Inc.),

Ki-67, Aim1/Aurora B (Transduction Laboratories) and HA (BD Biosciences).

Antibodies against the exocyst components sec3, sec5, sec15, sec8, exo70, and exo84

were a gift of Dr. Charles Yeaman (University of Iowa). Snapin antibodies were a

gift of Dr. Zu-Hang Sheng (NIH). Endobrevin antibodies were a gift of Dr. Thomas

Weimbs (Cleveland Clinic).


**Immunofluorescence and electron microscopy**


Cells were prepared for immunofluorescence, imaged, and deconvolved (Metamorph;

Universal Imaging Corp.), and centrosomes were quantified 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. Immunogold electron microscopy was

performed as previously described (Doxsey et al., 1994) using centrosome fractions

from HeLa cells (Blomberg-Wirschell and Doxsey, 1998) and antibodies to centriolin

followed by antibodies coupled to 5-nm gold particles (Amersham Biosciences).

Pillar cells were prepared and stained as previously described (Mogensen et al.,

1997).

## Centriolin and agGAP cloning

A cDNA of ~1.7 kb was identified by screening a human placenta expression library

with serum from individuals with scleroderma (Doxsey et al., 1994). The nucleotide

sequence was compared with others (blastn) in the human genome database (National

Center for Biotechnology Information) and revealed a sequence with 99% identity on

chromosome 9 q34.11–34.13. Genscan predicted an ~7-kb gene comprising 40 exons.

PCR primers were used to obtain an ~7-kb cDNA in a human testes cDNA library.

The 5' end, obtained by rapid amplification of cDNA ends (RACE), was identical to

the predicted sequence. Cloning of agGAP was performed in a similar manner, except

the 5' RACE reactions proved unsuccessful. A full-length HA-tagged centriolin was

obtained by inserting an HA tag (YPYDVPDYASL) 5' to the RACE fragment and

ligating the HA–centriolin cDNA to the original fragment. The fulllength centriolin

cDNA contained 6,975 nucleotides with an ORF of 2,325 amino acids and predicted a

molecular mass of 269 kD, consistent with the molecular mass of endogenous

centriolin (Fig. 1). Regions flanking the ORF had a translational start (Kozak

sequence), polyadenylation sequence, poly-A tail, and multiple upstream and

downstream stop codons. The construct was inserted into pcDNA 3.1 Zeo (+)

(Invitrogen) using BamHI and NotI restriction sites. Centriolin was translated in vitro

(TNT; Promega) and expressed in cultured cells using conventional procedures

(Lipofectamine). Centriolin amino acids 435–623 and 1385–1658 were 24%

dentical/47% similar and 20% identical/41% similar to the COOH-terminal half of

TACCs, respectively. Amino acids 879–913 were 40% identical/51% similar to amino acids 72–106 of human stathmin. Amino acids 126–234 were 31–35% identical/47–50% similar to Nud1p and Cdc11p.

**siRNAs and morpholino antisense**

siRNAs targeting centriolin, sec8, sec5, snapin, lamin A/C, and GFP mRNAs were made as complimentary single-stranded 19-mer siRNAs with 3' dTdT overhangs (Dharmacon Research), deprotected, annealed, and delivered into cells using a 400 µM stock (Oligofectamine; Invitrogen). The nucleotides targeted were as follows: in centriolin, 117–136 and 145–163; in lamin A/C, 608–630; in MKLP-1, 189-207; in sec5, 260-278; in sec8, 609-627; in snapin, 312-330 and in pEGFP-C1 (CLONTECH Laboratories, Inc.), 233–252. Fluorescein-conjugated morpholino antisense DNA oligonucleotides (Gene Tools) targeting the start codon of centriolin were introduced into cells using the EPEI agent (Gene Tools). The inverse sequence was used as control.

**Time-lapse imaging and brefeldin A treatment**

HeLa cells plated on coverslips (25 mm diameter) were treated with si-RNAs targeting the indicated protein for 48 h. They were placed in a chamber (PDMI-2; Harvard Apparatus) in complete medium with $CO_2$ exchange (0.5 liters/min) at 37°C.

Cells were imaged every 10 min for 12–20 h using a 20x or 40x phase contrast lens with a green interference filter on an inverted microscope (Olympus IX-70). Images were captured on a CoolSnap HQ CCD camera (Roper Scientific) and concatenated using Metamorph software (Universal Imaging Corp.). For BFA experiments, Hela cells were treated with 10μg/ml brefeldin A (Sigma-Aldrich) and immediately subjected to phase contrast time-lapse microscopy. For lumenal-GFP expressing cells, two concurrent time-lapse programs were used, one for the GFP signal and one for phase constrast. For each, images were taken every 2 minutes over a period of 3 to 4 hours.

**Primary cilium formation and microtubule nucleation**

Primary cilia were induced by culturing hTERT–RPE-1 cells in medium with 0.25% serum for 48 h and identified using the GT335 antibody raised to polyglutamylated α- and β-tubulins (Bobinnec et al., 1998). Microtubule nucleation was performed as previously described (Purohit et al., 1999) by treatment with nocodazole (1 mg/ml) for 1 h at 37°C, fixing cells at various times after washing out drug, and then staining for microtubules.

**RT-PCR**

Centriolin mRNA levels were assayed by RT-PCR using 10 µl mRNA (OneStep RT-PCR; QIAGEN); α-tubulin served as an internal control in the same reaction. All products were sequenced.

**Yeast two-hybrid analysis**

A fragment containing amino acids 127–233 of centriolin was cloned into EcoRI and SalI sites of pGADT7 (CLONTECH Laboratories, Inc.) to produce a fusion with the GAL4 TAD. This construct was used for the yeast two hybrid library screen and the Bub2p interaction assays. Constructs pEG202 (LexA), pGP69 (LexA-*BUB2*), and pGP122 (LexA-*BFA1*) and the yeast strain SGY37 were from Elmar Schiebel (Paterson Institute for Cancer Research, Manchester,(UK). SGY37, which contains a *LacZ* reporter gene under the control of a LexA operator, was transformed with plasmid DNA using LiAc (Ito et al.,1983), and transformants were selected for on dropout medium. We used semiquantitative β-galactosidase assays (Schramm et al., 2001) and more quantitative β-galactosidase assays with CPRG (chlorophenol red-β-Dgalactopyranoside; Roche) as a substrate per the manufacturer's instructions. Yeast two-hybrid library screens were performed following the manufacturer's 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 positive clone with strains expressing either lamin C or the

GAL4 DNA Binding Domain alone and plating on quadruple drop-out media.

**Immunoprecipitations**

Antibodies to centriolin or exocyst components were added to freshly prepared

hTERT-RPE cell extracts and incubated at 4° C overnight. The lysis buffer consisted

of 50mM Tris HCl (pH 7.5), 10 mM $Na_2HPO_4$ (pH 7.2), 1 mM EDTA, 150 mM

NaCl, 1% IGEPAL CA-1630 and protease inhibitors (Complete Mini tablets from

Roche Diagnostics GmbH, Mannheim Germany). These samples were bound to pre-

washed protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4° C for 2 hours.

SDS-PAGE and immunoblotting were performed as previously described (Harlow

and Lane, 1988). Coimmunoprecipitation of LexA, GAL4 AD, and GAL4 TAD

fusion proteins was performed as previously described (Schramm et al., 2001).

**Flow cytometry**

Cells treated with siRNAs for 50–70 h were treated with 100 ng/ml for12 h, removed

from plates, and fixed in methanol. Cells stained with propidium iodide were

analyzed by flow cytometry (FACSCAN®; Becton Dickinson) using Flojo software

(Tree Star, Inc.).

**Exocyst purification, centrosome purification, and *in vitro* transcription/translations**

Exocyst complex purification using an Opti-prepgradient was performed as described (Yeaman, 2003). Purification of centrosomes from Hela cells was performed as described (Blomberg-Wirschell and Doxsey, 1998). In vitro transcription/translation reactions were performed following the manufacturer's protocols (TNT Reticulocyte Lysate System, Promega Corp.).

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