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Deconstructing Cell Architecture: Exploring centriole structure, function, and position in the green alga Chlamydomonas reinhardtii

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Deconstructing Cell Architecture:
Exploring centriole structure, function, and positioning in
the green alga Chlamydomonas reinhardtii

by

Jessica L. Feldman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CELL BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
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by
Jessica L. Feldman
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supporting me in more ways than I thought possible.
Abstract

Deconstructing Cell Architecture:
Exploring centriole structure, function, and positioning in the green alga Chlamydomonas reinhardtii

by

Jessica L. Feldman

An enduring question in cell biology is how internal cell geometry is established and maintained through endless rounds of cell division. In many cell types, organelles and sub-cellular networks are partitioned into fine spatial arrangements. Precise cell geometry often plays a functional role in signaling, growth, division, and in establishing cell polarity.

One sub-cellular structure that is precisely localized is the centriole. Centrioles are non–membrane-bound organelles composed of nine triplet microtubule blades arranged around a central cartwheel structure. Centrioles are found as a pair, comprised of a mother and a daughter, which is duplicated during each cell cycle. Mother centrioles are so-called because they were assembled in a previous cell cycle to the daughter centriole. Centrioles have two main functions in the cell. First, centrioles together with pericentriolar material comprise the centrosome, the major microtubule-organizing center of the cell. Second, centrioles serve as basal bodies to nucleate the assembly of cilia.

Although originally named for their centralized location, centrioles are repositioned to more peripheral sites during cell-state transitions such as wound
healing, cell migration, and cell growth. Despite the clear importance of centriole positioning to the cell and the organism, almost nothing is known about this process. *Chlamydomonas* is a unicellular green alga that has served as an ideal model organism in which to study centriole and cilia biology. *Chlamydomonas* cells have centrioles that are structurally and molecularly similar to those of vertebrates. *Chlamydomonas* cells also have robust cell geometry, facilitating the measurement of the positions of intracellular structures.

Here, I use *Chlamydomonas* to understand centriole positioning. Specifically, I developed a novel screen to identify mutants with defects in centriole positioning. From this screen, I identified a number of interesting mutants with defects in cilia and centriole assembly, number, and position. I use these mutants to demonstrate that the mother centriole plays an instructive role in defining cell geometry, directing the positioning of many sub-cellular structures including the daughter centriole and the nucleus. I also use these mutants to understand spindle position, implicating the centrioles as essential for properly orienting the mitotic spindle. Additionally, I demonstrate that one of these mutants, *asq2*, carries a mutation in the conserved gene TBCCd1, which may regulate template-driven centriole assembly.
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CHAPTER 1

Introduction
Cells, like whole organisms have an incredible diversity of form. Far from the amorphous bags depicted in textbooks, cells can adopt a multitude of elaborate patterns and architectures. Much attention over the past century has been focused on unraveling the molecular underpinnings involved in the development of organisms. However, much less focus has been placed on understanding the mechanisms that governs cell architecture.

Cell architecture is crucial for cell function. For example, the rod and cone photoreceptor cells that we use to see and the hair cells that we use to hear would not be able to carry out their function without their stereotypical construction and the partitioning of structures into specialized regions. Interestingly, these two cells types both require the precise positioning of cilia, rod-shaped cellular protrusions composed of microtubules that can be used for sensation or movement. Cilia grow from structures called centrioles. The positioning of cilia requires the precise positioning of centrioles.

Centrioles are cylindrical structures composed of nine microtubule blades arranged around a proximally located central cartwheel structure (Figure 1). Centrioles not only serve as basal bodies to nucleate the assembly of cilia, but also serve together with pericentriolar material as the centrosome, the major microtubule organizing center of the cell.

Centrioles duplicate once per cell cycle (Figure 2). Each centriole will mature and serve as a mother to give rise to a new daughter centriole each cycle. Most cell start with two centrioles. Each centriole matures and bears a daughter, resulting in a total of four centrioles. The cell then divides and one
mother and one daughter centriole segregates to each cell (Figure 2). Therefore, there is always an inherent age difference between the centrioles in each cell. The mother centriole directs the synthesis of a new daughter through a template-driven assembly process. However, centrioles can also arise through a separate \textit{de novo} synthesis pathway (La Terra et al., 2005; Marshall et al., 2001).

Despite being named for their centralized location in the cell, centrioles are often found in more peripheral locations. As discussed above, centrioles need to be specifically positioned on the cell surface when mobilized to assemble cilia. In multi-ciliated tissue, the centrioles that nucleate cilia are distinct from those that serve at the centrosome, arising from a structure known as the deuterosome (Hagiwara et al., 2004; Loots and Nel, 1989). Regardless of their origin, centrioles need to migrate to the cell surface in order to anchor and nucleate the assembly of cilia at the proper position. Following the deposition of centrioles at the cell surface, centrioles also need to become rotationally orientated so that cilia can beat with the proper orientation relative to the cell. This rotational orientation has been shown to require positive feedback from flow created by beating cilia (Mitchell et al., 2007). Perturbation in ciliary positioning has severely deleterious effects in humans. For example, inability of centrioles to properly migrate prior to ciliary assembly has been linked to Meckel-Gruber syndrome (Dawe et al., 2007). Additionally, proper orientation of cilia via centriole positioning towards the posterior of embryonic node cells is critical for establishing left–right asymmetry during mammalian development (Nonaka et al.,
2005). Despite the clear importance of centriole positioning to the cell and the organism, little is known about this process.

When the work presented here was first started, almost nothing was known about the pathway regulating centriole positioning. During the past five years, several studies have been published that have begun to unravel the players in this pathway (Dawe et al., 2007; Park et al., 2006; Park et al., 2008). The planar cell polarity pathway as well as the pathway controlling the actin cytoskeleton have both been implicated in the control of centriole positioning, however, there is still much work to be done to understand this process.

The unicellular alga *Chlamydomonas reinhardtii* provides an ideal genetic system in which to study centriole positioning (Figure 3). Each pair of centrioles, composed of a mother and a daughter, must relocate from the apical cell surface to the spindle poles during mitosis. After division, centrioles return to the apical pole where they nucleate the assembly of two cilia. *Chlamydomonas* centrioles and cilia are structurally and molecularly similar to those of vertebrates, with the vast majority of centriolar and ciliary proteins conserved between humans and *Chlamydomonas*. Unlike flies and worms that have centrioles with doublet and singlet microtubules, respectively, *Chlamydomonas* cells have centrioles with triplet microtubules like those of vertebrates. Additionally, *Chlamydomonas* cells have a haploid genome that has been sequenced and annotated, making it an ideal system in which to study the genetics of centriole and cilia biology. *Chlamydomonas* cells also have robust cell geometry, facilitating measurements between sub-cellular structures (Figure 3).
Chlamydomonas cells have two flagella, located at the apical side of the cell (Figure 3). These flagella are coordinated for directed motility. Chlamydomonas cells respond strongly to light and can positively or negatively phototax. In order to do so, cells must have two flagella that are properly placed with respect to one another and with respect to the eyespot, the site of light transduction for phototaxis (Figure 3). The proper placement of the flagella requires that both the position and rotational orientation of the centrioles be carefully controlled. Cells with impaired flagellar placement, and thus impaired centriolar placement, should have defective phototaxis, as directed motility will be perturbed. In order to identify the molecules involved in directing centrioles for proper flagellar placement, I designed a screen for mutants with impaired phototaxis. Phototaxis screens have been done in the past (Hirschberg and Stavis, 1977; Pazour et al., 1995), but these screens have been on a small scale and none have focused on identifying mutants with centriole defects. A large amount of the work presented here stems from mutants obtained from this phototaxis screen. One class of mutants, the centriole positioning mutants termed askew (asq), will be discussed at length as will the flagella-less or bld mutants.

In this graduate thesis, I will illustrate my findings with respect to centriole and cilia biology. I will begin by describing how the mother centriole acts as a hub around which to organize cell geometry, positioning the daughter centriole and the nucleus. I will then discuss Chlamydomonas mitosis, highlighting the
orchestration of the microtubule cytoskeleton during this process. I will also
discuss insights into centriole duplication gained from observing centrin
localization in living cells. I will then report my discoveries pertaining to spindle
establishment and orientation, highlighting a role for the centriole in spindle
positioning. I will show that one mutant that I generated, asq2, encodes the
conserved protein TBCCd1, which I believe to control template-driven centriole
duplication. I will also relay insights into centriole and flagellar assembly gained
from observing mutants with no flagella, the so-called bld phenotype. Finally, I
will present an assortment of unpublished data on technological advances in
Chlamydomonas and on cilia biology in zebrafish and the ciliate Stentor. I hope
you enjoy the show!
Figure 1. Centriole structure

Cartoon depicting centriole structure. Centrioles are composed of nine triplet microtubule (MT) blades arranged around a proximal cartwheel structure (yellow). The amorphous disc structure (blue ring) appears prior to the assembly of the MT blades. A flagellum grows out of the distal ends of the centriole.
Figure 2. Centriole duplication

Centrioles exist as a pair. The centriole pair duplicates once per cell cycle. Each centriole serves as a mother (gray) to give rise to a new daughter centriole (blue). Centrioles separate and new small daughter pro-centrioles appear. Each daughter elongates and one mother and one daughter centriole is segregated to each cell following mitosis.
**Figure 3. Chlamydomonas reinhardtii**

*Chlamydomonas reinhardtii* is a unicellular green alga. A) Cartoon depicting *Chlamydomonas* cell. Cells have two centrioles (yellow) located at the apical end of the cell. A flagellum (black) grows from each centriole. Four rootlet microtubules emanate from near the centrioles. The centrioles are connected to the nucleus (white) by centrin containing rhizoplasts. The majority of the cell contains chloroplast (green). Embedded in this chloroplast at the basal end of the cell is the pyrenoid (blue), a starch storage structure. The centrioles sense light using an eyespot (red) located laterally on one side of the cell. B) DIC image of a *Chlamydomonas* cell. The flagella, pyrenoid, and eyespot are all visible. C) Chlamydomonas cell labeled with DAPI (blue) and antibodies against acetylated α-tubulin and centrin (green) and Bld10p (red). The nucleus is visible (large blue sphere) as are the smaller plastid genomes (small blue spheres). The two centrioles are present at the apical end of the cell. Two flagella (green) grow from these centrioles. Cells retain their robust cell geometry when fixed, facilitating cellular measurements.
Chapter 2

The mother centiole plays an instructive role in defining cell geometry
Abstract

Centriole positioning is a key step in establishment and propagation of cell geometry, but the mechanism of this positioning is unknown. The ability of pre-existing centrioles to induce formation of new centrioles at a defined angle relative to themselves suggests they may have the capacity to transmit spatial information to their daughters. Using three-dimensional computer-aided analysis of cell morphology in *Chlamydomonas*, we identify six genes required for centriole positioning relative to overall cell polarity, four of which have known sequences. We show that the distal portion of the centriole is critical for positioning, and that the centriole positions the nucleus rather than vice versa. We obtain evidence that the daughter centriole is unable to respond to normal positioning cues and relies on the mother for positional information. Our results represent a clear example of "cytotaxis" as defined by Sonneborn, and suggest that centrioles can play a key function in propagation of cellular geometry from one generation to the next. The genes documented here required for proper centriole positioning may represent a new class of ciliary disease genes, defects in which would be expected to cause disorganized ciliary position and impaired function.
Introduction

A fundamental question in cell biology is how cell geometry is established and maintained (Kirschner et al., 2000; Lwoff, 1950; Shulman and St Johnston, 1999; Sonneborn, 1964). Cell geometry refers to the characteristic positioning of organelles within the cell body in order for a cell to be able to carry out its specified function. Despite the importance of cell geometry in tissue organization and cell function, the mechanistic origins of cell geometry remain a complete mystery. Further compounding the mystery is the fact that, as demonstrated by the classic experiments of Beisson and Sonneborn (Beisson and Sonneborn, 1965), cell organization can be propagated through cell division, alleviating the need for cells to re-establish their infrastructure after each round of mitosis and potentially allowing a coherent organization to be maintained across developing tissue during proliferative growth. Many organelles take part in this elaborate cellular patterning. One organelle that is often found in specific subcellular locations is the centriole.

Centrioles are non-membrane bound organelles composed of nine-triplet microtubule blades arranged around a central cartwheel structure. Each cell has a pair of centrioles, comprised of a mother and a daughter, which is duplicated during each cell cycle. Centrioles have two main functions in the cell. First, centrioles together with pericentriolar material comprise the centrosome, the major microtubule organizing center of the cell. Indeed, centrioles are the highly stable core nucleating centers for the centrosome, providing it with persisting
structural integrity (Abal et al., 2005) and attaching it to cytoplasmic microtubules during G1 (Mogensen et al., 2000). Second, centrioles serve as basal bodies to nucleate the assembly of cilia. In order to carry out these functions in the cell, centrioles often need to be specifically localized.

Although originally named for their centralized location, centrioles are repositioned to more peripheral sites during cell state transitions such as wound healing, cell migration, and cell growth (de Anda et al., 2005; Gomes et al., 2005; Gotlieb et al., 1981). The importance of centriole positioning for development and physiology is perhaps most clearly illustrated in situations involving cilia, which are assembled from centrioles. Proper orientation of cilia via centriole positioning towards the posterior of embryonic node cells is critical for establishing left-right asymmetry during mammalian development (Nonaka et al., 2005). Centriole orientation, and the resulting proper alignment of respiratory cilia, is required for effective mucus clearing in the airway (Biggart et al., 2001). Furthermore, inability of centrioles to properly migrate prior to ciliary assembly has recently been linked to Meckel-Gruber syndrome (Dawe et al., 2007).

As centrioles are anchored to the cytoskeleton during G1, they may act as a set of stable “handles” by which the centrosome can be repositioned to orient the cytoskeleton, cilia, and perhaps other cellular structures as well. Moreover, the process of centriole duplication provides an ideal mechanism to transmit cell geometry across generations. While both planar cell polarity (Montcouquiol et al., 2003; Park et al., 2006) and apical/basal cues (Izumi et al., 2006; Siller et al., 2006) can influence centriole position, the mechanism by which centrioles are
positioned, and the degree to which their positioning is self-propagating, is currently unknown.

The unicellular alga *Chlamydomonas reinhardtii* provides an ideal genetic system in which to study centriole positioning. Each pair of centrioles, composed of a mother and a daughter, must relocate from the apical cell surface to the spindle poles during mitosis. After division, centrioles return to the apical pole where they nucleate the assembly of two cilia (called flagella in this organism). *Chlamydomonas* centrioles and cilia are structurally similar to those of vertebrates, with the vast majority of centriolar and ciliary proteins conserved between humans and *Chlamydomonas*. *Chlamydomonas* cells also have reproducible chiral cell geometry with many characteristically positioned structures (Holmes and Dutcher, 1989) (illustrated in Figure 1A and 1B), facilitating quantification of geometric relationships within the cell.

Using *Chlamydomonas* cells, we identified mutants with defects in centriole positioning. Combining genetic analysis, three-dimensional imaging, and a novel algorithm for quantifying cellular geometry, we demonstrate that the mother centriole guides the daughter centriole to the proper subcellular location. Specifically, in mutants where mother and daughter centrioles are separated, only mother centrioles localize properly. We further show that in mutants in which the centrioles are detached from the nucleus, the nucleus becomes randomly positioned while the mother centrioles retain correct positioning, indicating that normally the mother centriole plays a role in properly positioning
the nucleus and not vice-versa. These data indicate that the mother centriole may act as a node to coordinate the positioning of many subcellular structures.
Results

Phototaxis screen uncovers mutants with defects in centriole positioning

To initiate a genetic analysis of the mechanism of centriole positioning and its impact on cell geometry, we began with a screen based on Chlamydomonas phototaxis. Chlamydomonas cells phototax using a light sensing organelle called the eyespot. Cells rotate while swimming, sweeping out a 360° path looking for light. When the eyespot detects light, it signals to the flagella via calcium signaling, inducing the cell to turn towards the light (Witman, 1993). We predicted that cells with aberrantly placed centrioles, and therefore aberrantly placed flagella, would lack the necessary geometrical relationship between the eyespot and the flagella that is required for phototaxis, and would be revealed in a screen for phototaxis defects. We screened 10,000 insertionally mutagenized lines for defects in phototaxis using an assay similar to previously described techniques (Hirschberg and Stavis, 1977; Horst and Witman, 1993; Pazour et al., 1995). Phototaxis defective lines were visually rescreened by Differential Interference Contrast (DIC) microscopy to identify mutants with defective cell morphology. Screen details are listed in Supplementary Figure S1.

Centriole positioning mutants were identified as those whose flagella are displaced from the apical pole of the cell (the usual position of centrioles in G1 in Chlamydomonas) and were verified using a three-dimensional computer-aided image analysis strategy as follows. We defined the long axis of the cell using the center of mass of the pyrenoid (Figure 1E, yellow circle), a starch storage structure that is located basally, and the cellular centre of mass (Figure 1E,
purple circle). We then marked the centrioles (Figure 1E, white cylinders) and using the long axis to construct a spherical coordinate system, we determined the angle by which each centriole was displaced off the long axis of the cell \( \theta_{\text{centriole}} \), Figure 1E). We identified thirteen mutants, which we termed askew \( (asq) \), in which centrioles are mispositioned as judged by \( \theta_{\text{centriole}} \). For example, \( asq1 \) cells have a mean \( \theta_{\text{centriole}} \) of \( 42.3 \pm 21.3^\circ \) (Figure 1G, \( n = 54 \), all reported angles are the mean \( \pm \) standard deviation). \( asq2 \) cells have a mean \( \theta_{\text{centriole}} \) of \( 61.7 \pm 32.3^\circ \) (Figure 1H, \( n = 71 \)). These values differ significantly (one-tailed t-test, \( asq1: p < 5.4e^{-10} \), \( asq2: p < 9.8e^{-17} \)) from wild-type (wt) cells, which have a mean \( \theta_{\text{centriole}} \) of \( 20.5 \pm 9.0^\circ \) (Figure 1F, \( n = 62 \)). The angle in wt is non-zero because the two centrioles are on either side of the apical most point, and hence displaced off the long axis.

In \( asq \) cells, the angles tend to be restricted to the apical half of the cell due to the occlusion of the basal portion by other cellular structures. The basal portion and some of the apical portion of \( Chlamydomonas \) cells contain chloroplast. We measured the position of the chloroplast by using the same long axis assignment described above. We then marked each plastid nucleoid (Figure 1I, green circles, visualized using DAPI, Figure 2A, left) and determined the angle each nucleoid was displaced off the long axis of the cell. Wild-type cells have a mean \( \theta_{\text{chloroplast}} \) of \( 112.1 \pm 36.0^\circ \) (Figure 1J, \( n = 181 \)). The pyrenoid center of mass is defined as \( 180^\circ \) in all of our \( \theta \) measurements as it is used as one of the points to define the long axis. The outer bounds of the pyrenoid span the basal part of the cell. The mean pyrenoid boundary in wild-type cells is 139.0
± 14.4° (Figure 1J, yellow shaded region, n = 90). The region of the cell that is occupied by the chloroplast and pyrenoid is thus complimentary to the region in which \textit{asq} centrioles can be found, consistent with the notion that in \textit{asq} mutants, centrioles are randomly distributed over the accessible part of the cell cortex.

\textit{asq} mutants can be subdivided into two classes based on the pairwise association of centrioles. Normally, mother and daughter centrioles are held together by a system of connecting fibers. The \textit{asq1} mutant represents a class of mutants (containing 9/13 \textit{asq} mutants) in which mother and daughter centrioles are attached to each other as in wild-type, but are randomly localized together on the cell surface (Figure 1C and Figure 2B and C). The \textit{asq2} mutant represents a second class (containing 4/13 \textit{asq} mutants) in which the mother and daughter centrioles are independently positioned on the cell surface (Figure 1D and Figure 2D and E). In \textit{asq2} cells some centrioles appear at the correct apical location (Figure 2E and Supplemental Figure 5B), while other centrioles can occupy atypical positions (Figure 2D and E).

\textit{Centriole segregation mutants have centriole positioning defects}

In addition to centriole positioning defects, \textit{asq2} cells also have variable numbers of centrioles, and therefore make variable numbers of flagella (Figure 3B and 3C). In contrast to wild type cells, which always have two flagella (Figure 3A and 3D, black bars), \textit{asq2} cells can have from zero to seven centrioles per cell (Figure 3D and Supplementary Table 1). Other \textit{Chlamydomonas} mutants with a similar variability in centriole number have been previously identified.
(Adams et al., 1985; Wright et al., 1989; Wright et al., 1983) and are referred to as vfl (variable flagellar number) mutants because the variable number of centrioles nucleates the assembly of variable numbers of flagella (Figure 3D) when the centrioles become basal bodies. These mutant phenotypes are thought to result from defective centriole segregation (Wright et al., 1989) and from defects in centriole mother-daughter cohesion (Adams et al., 1985; Wright et al., 1983).

The similarity between the variable flagellar number phenotypes of asq2 and the vfl mutants raised the possibility that the vfl mutants might also share the centriole positioning phenotype. We therefore tested vfl2 and vfl3 for defects in centriole positioning and found that these mutants have centriole positioning defects comparable with those of asq2 when analyzed using our computational strategy. vfl2 cells have a mean $\theta_{\text{centriole}}$ of $55.2 \pm 28.8^\circ$ (Figure 3E, n = 64) and vfl3 cells have a mean $\theta_{\text{centriole}}$ of $59.4 \pm 35.2^\circ$ (Figure 3F, n = 90). Genetic mapping studies show that asq2 is not an allele of any of the previously described VFL genes (data not shown).

**The mother centriole instructs the daughter centriole to the proper subcellular location**

Using these mutants, we can begin to ask which component of the centrosome responds to polarity cues during positioning. The centrosome is composed of a mother centriole, a daughter centriole, pericentriolar material, and is attached to the nucleus. In *Chlamydomonas*, these structures are spatially
distinct, but connected by fibers. Mother-daughter pairs are linked by striated fibers and connected to the nucleus by rhizoplasts (Kater, 1929; Wright et al., 1989) in *Chlamydomonas* and Hook/Sun domain proteins in other organisms (Malone et al., 2003; Tzur et al., 2006). In principle, any of these components (the mother centriole, the daughter centriole, or the nucleus) could localize the others in response to polarity cues.

We first tested whether the mother centriole can localize the daughter or vice-versa. Previous studies have demonstrated that the *vfl* mutants result in dissociation of mothers from daughters and/or centrioles from the nucleus (Wright et al., 1989; Wright et al., 1983). We verified that mother and daughter centrioles are likewise disconnected in *asq2* cells using electron microscopy (Figure 4B). In wt cells, electron dense fibers connect mother and daughter centrioles (Figure 4A, arrow). In contrast, *asq2* cells lack these connecting fibers (Figure 4B, arrow), confirming a loss of mother-daughter connections. These mutants therefore allow us to test which of these structures is able to localize properly when detached from the others.

Visual examination of *asq2* and *vfl* mutants suggested to us that the centriole distribution can be interpreted as a mixture of two populations: a population of correctly positioned centrioles (Figure 2E, Supplemental Figure S5B) and a population of randomly positioned centrioles (Figure 2D and 2E). Based on these observations and the known inherent disparity in maturation state between centrioles in each cell, we propose a model in which centriole maturity affects positioning. We considered a model in which the mother
centriole is necessary for positioning the daughter centriole (Figure 4C). In accordance with this model, in the asq1 class of mutants, the mother centriole can no longer respond to the cell polarity cue and the mother-daughter pairs end up randomly localized. In the asq2 class, the mother and daughter centrioles would be detached from each other, resulting in a population of properly positioned mother centrioles and a population of misplaced daughter centrioles. Because mother and daughter centrioles are no longer connected, centrioles will not segregate properly following mitosis, resulting in cells with variable numbers of centrioles. The key prediction of this model is that the mother centrioles in asq2 cells should be properly localized, while the daughter centrioles should be improperly localized (Figure 4C).

To test the prediction that mother centrioles are correctly positioned while daughters are mislocalized, we must be able to differentiate mother and daughter centrioles in 3D microscopy images. Mother centrioles have ultrastructural modifications that are lacking on daughter centrioles and are visible by electron microscopy, but serial section EM is not suitable for analyzing large numbers of cells. In order to be able to distinguish mothers and daughters in a more high-throughput manner, we employed a genetic strategy to render mother and daughter centrioles distinguishable by light microscopy. To do this, we took advantage of the uni1 mutant in which flagella are formed predominantly by mother centrioles (Huang et al., 1982) (see flagellar distribution in Supplemental Table 1). We then tested if mother centrioles localize to the proper position at the apical pole by measuring the \( \theta_{\text{centriole}} \) (Figure 1E) for all flagellated (mother)
centrioles in \textit{asq2uni1} double mutant cells. If mother centrioles can respond to polarity cues, they should account for the properly positioned centrioles sometimes seen in \textit{asq2} mutants, hence the mean $\theta_{\text{centriole}}$ of flagellated centrioles in \textit{asq2uni1} cells should be smaller and less variable than that of \textit{asq2} cells (Figure 5C). Indeed, we find that \textit{asq2uni1} cells have a mean $\theta_{\text{centriole}}$ of $32.4 \pm 13.1^\circ$ (Figure 5D, green lines, n = 60), which is significantly (one-tailed t-test, $p < 2.02e^{-10}$) smaller than the mean $\theta_{\text{centriole}}$ for \textit{asq2} cells (Figure 1H and Figure 5D, grey lines). The mean $\theta_{\text{centriole}}$ for flagellated centrioles in \textit{asq2uni1} cells is slightly higher than wt (Figure 1F, mean $\theta_{\text{centriole}} = 20.5 \pm 9.0^\circ$) and \textit{uni1} (Supplemental Figure S2A, mean $\theta_{\text{centriole}} = 20.4 \pm 8.5^\circ$), but this is expected because the \textit{uni1} phenotype is incompletely penetrant, such that some daughter centrioles still bear flagella in \textit{uni1} mutants (Supplemental Table 1).  

So as not to rely solely on the pyrenoid and cellular center of mass measurements, we employed an alternative measure of geometry based on distance measurements. We measured the three-dimensional through-space distance between flagellated centrioles in \textit{asq2uni1} cells. If mother centrioles localize to the same subcellular site, then the distance between flagellated centrioles should be relatively low in the double mutant especially when compared to that of \textit{asq2} cells in which both mother and daughter centrioles have flagella (Figure 5A, right). In contrast, if mother centrioles are randomly localized, then the interflagellar distance in \textit{asq2uni1} double mutant cells should be at least as large as in \textit{asq2} cells and just as variable (Figure 5A, left). We find that in \textit{asq2uni1} double mutants, the interflagellar distance is significantly smaller.
(Figure 5B, blue bars, mean = .89 \( \mu \text{m} \) \( \pm \) .04 S.E.M., n = 85) than that of \textit{asq2} cells (Figure 5B, yellow bars, mean = 1.48 \( \mu \text{m} \) \( \pm \) .09 S.E.M., n= 88) and less variable, confirming that mother centrioles cluster in the same subcellular location.

**The \textit{uni1} mutation does not suppress the \textit{asq2} phenotype**

An alternative explanation for these data is that the \textit{uni1} mutation acts as a suppressor of the centriole segregation and/or positioning phenotype in \textit{asq2} cells. Centriole number in \textit{asq2uni1} cells (Supplemental Figure S3A, mean centriole number = 1.67 \( \pm \) 1.25, n = 317) is indistinguishable (one tailed t-test p < .3) from that of \textit{asq2} cells (Supplemental Figure S4, \textit{asq2} mean centriole number = 1.72 \( \pm \) 1.27, n = 440), indicating that \textit{uni1} does not suppress the centriole segregation defect.

Furthermore, \textit{uni1} does not act as a suppressor of centriole positioning defects as intercentriolar distance is similar in \textit{asq2} (mean = 1.39 \( \pm \) .94, n = 168) and \textit{asq2uni1} (mean = 1.42 \( \pm \) 1.12 , n= 174) cells (Supplemental Figure S3B, one-tailed t-test, p >.39). Three-dimensional immunofluorescence imaging of \textit{asq2uni1} cells demonstrates that the mother and daughter centrioles remain detached in the double mutant just as in the \textit{asq2} single mutant, demonstrating that the \textit{uni1} mutation does not simply behave as a suppressor, either of the mother-daughter detachment phenotype or of the centriole mispositioning phenotype of the \textit{asq2} mutation. Indeed, mother centrioles properly localize to the apical pole (Figure 5E, flagellated centrioles, white arrow), while
disconnected daughter centrioles can wander to atypical sites (Figure 5E, unflagellated centriole, blue arrow). These observations confirm that mother centrioles are competent to be properly positioned and normally play an instructive role in leading the daughter centriole to the correct subcellular location. We therefore conclude that in asq2 cells centriole positioning is intact as mothers can find the proper subcellular location, but daughters are mispositioned because they are detached from their mother.

**Centrioles position the nucleus**

Mother centrioles guide daughters to the correct subcellular position, but does the mother centriole play a role in instructing the position of other organelles? In a wt *Chlamydomonas* cell, the centrioles sit atop the nucleus and are attached to it by centrin-containing fibers called rhizoplasts (Kater, 1929) (Figure 6A). This juxtaposition suggests that centriole and nuclear positioning could be intimately linked. In most cell types, there tends to be a correlation between nuclear and centrosomal position. In asq mutant cells, the nucleus seems to be mispositioned along with the centrioles (Figure 6B), suggesting that centrioles position the nucleus or vice-versa. A recent study has suggested that nuclear reorientation affects the position of the centrosome during cell migration in mammalian cells (Gomes et al., 2005). However, it has also been demonstrated that centrosomes are able to reach the cell cortex during Drosophila development without the aid of the nucleus (Raff and Glover, 1989). To help address the controversy over who positions whom, nucleus or
centrosome, we wanted to determine whether the nucleus could be impacting the localization of the mother centriole.

To test directly whether nuclear positioning has a causal impact on centriole position, we made use of the vfl2 mutant in *Chlamydomonas* that has a mutation in centrin (Taillon et al., 1992), a protein component of the rhizoplast. vfl2 cells lack the centrin-based rhizoplast structure that connects the centrioles to the nucleus (Wright et al., 1989). As shown in Figure 6D, vfl2 centrioles have increased variability in positioning, but, like asq2, the mother centrioles remain properly localized at the apical pole as determined in vfl2uni mutants. We quantified nuclear position (θ_{nucleus}) in vfl2uni1, uni1 and wt cells in a manner similar to the determination of θ_{centriole}. We determined the long axis of the cell using the same method described above, but instead of marking each centriole, we obtained the nuclear center of mass and measured how much this point was shifted off the long axis of the cell. In wt cells, the mean angle θ_{nucleus} is 15.5 ± 8.1° (Figure 6E, n = 62). This value is similar to that of uni1 cells (Supplemental Figure S2B, θ_{nucleus} = 14.3 ± 5.6°, n = 40). In vfl2uni1 cells where the nucleus has been uncoupled from the centrioles, the θ_{nucleus} is much more variable and the mean θ_{nucleus} (mean θ_{nucleus} = 25.0 ± 11.8°, Figure 6F, n = 49) is significantly higher (one-tailed t-test, p < 2.9e^{-6}), indicating that the nucleus is free to visit a wider range of positions once detached from the centrioles (Figure 6C). In contrast to the variable nuclear position, we find that as in asq2uni1, in vfl2uni1 cells flagellated mother centrioles are properly localized, while the position of daughters is randomized (Figure 6D, vfl2uni1 θ_{centriole} (orange lines), vfl2 θ_{centriole}
vfl2uni1 cells have a mean $\theta_{\text{centriole}}$ that is statistically similar (one tailed t-test, $p > .03$) to wt and uni1, indicating that the mother centrioles can be correctly positioned despite the variable position of the nucleus.

We further tested whether the nucleus dictates centriole position by measuring the correlation of nuclear position to that of centriole position on a cell-by-cell basis. In vfl2uni cells, $\theta_{\text{centriole}}$ for flagellated centrioles does not correlate with $\theta_{\text{nucleus}}$ (Figure 6H, $n = 49$, correlation coefficient of .10). When we compare the mean $\theta_{\text{centriole}}$ of cells with a correctly positioned nucleus ($\theta_{\text{nucleus}}$ is less than one standard deviation from the mean $\theta_{\text{nucleus}}$ for wt cells) to the mean $\theta_{\text{centriole}}$ of the cells with an incorrectly positioned nucleus ($\theta_{\text{nucleus}}$ is more than one standard deviation from the wt mean), the values do not differ significantly (one-tailed t-test, $p > .33$, Figure 6H, inset). These data indicate that the position of the nucleus has no obligatory impact on the position of centrioles in the cell and that correct centriole positioning does not require the nucleus. Conversely, because the nucleus is mispositioned with the centrioles in asq mutant cells (Figure 6B), we wondered whether centrioles are involved in positioning the nucleus. In a population of wt cells, the $\theta_{\text{centriole}}$ correlates with $\theta_{\text{nucleus}}$ (correlation coefficient $= .63$, Figure 6G, $n = 62$). The fact that centriole position is unaltered and nuclear position randomized in a mutant that detaches centrioles from the nucleus, together with the fact that centriole position and nuclear position are correlated with each other when the centrioles are attached to the nucleus by the rhizoplast, indicates that centrioles appear to dictate the position of the nucleus rather than vice versa.
Recent studies in migrating cell lines demonstrated that nuclear reorientation is important in positioning the centrosome towards the leading edge of the cell (Gomes et al., 2005). However, these studies only measured translational position of the centrosome and therefore cannot rule out a model in which rotation of the centrosome drives nuclear movement rather than vice-versa. It would be interesting to repeat those experiments in cells lacking the nucleus-centrosome connections.

In addition to the nucleus, we also found that the rootlet microtubules (acetylated microtubule bundles involved in cleavage furrow placement in *Chlamydomonas* cells) are mispositioned with centrioles in *asq* mutants (Supplementary Figure S4), demonstrating that the centrioles, particularly the mother, play a role in positioning multiple cellular structures. We have also observed that the contractile vacuoles are positioned along with the centrioles (Figure 3B and 3C).

**The distal ends of centrioles may play a role in positioning**

To begin to analyze which part of the mother centriole is responsible for positioning, we took advantage of known *Chlamydomonas* mutants with defects in centriole assembly, *bld2* and *bld10*. *bld2* cells have a mutation in epsilon tubulin (Dutcher et al., 2002) and are missing the B- and C-tubule of each of the nine triplet microtubule blades that normally comprise the centriole (compare Figure 7A and 7B). As a result, *bld2* centrioles have nine short singlet microtubules and are lacking portions of the distal end. *bld10* cells, which are
defective in the production of the centriole cartwheel localized protein Bld10p, are
missing all centriole microtubules, and have at most just the most proximal
portions of the centriolar structure (Matsuura et al., 2004).

Because bld2 and bld10 cells both lack flagella, we first determined the
centriole positioning phenotype of bld1 cells, which also lacks flagella but has a
structurally normal centriole. bld1 cells have a mutation in the gene that
encodes IFT52 (Brazelton et al., 2001). These cells have centrioles that are
structurally identical to wt cells, but due to a defect in a component of
intraflagellar transport, they are unable to make flagella (Figure 7A). We found
that bld1 cells have a mean $\theta_{\text{centriole}}$ of $19.8 \pm 8.0^\circ$ (Figure 7G), similar to wild-type
and demonstrating that assembly of flagella is not necessary for proper centriole
positioning.

To determine if the distal portion of the centriole is necessary for
positioning, we measured the $\theta_{\text{centriole}}$ for bld2 and bld10 cells and compared it to
$\theta_{\text{centriole}}$ for bld1 cells. bld2 cells have a mean $\theta_{\text{centriole}}$ of $45.9 \pm 26.9^\circ$ (Figure 7H)
and bld10 cells have a mean $\theta_{\text{centriole}}$ of $40.2 \pm 30.8^\circ$ (Figure 7I). These values
differ significantly from that of bld1 cells (bld2: one tailed t-test, $p < 5.4 \times 10^{-8}$, bld10:
one-tailed t-test, $p < 3.1 \times 10^{-5}$), which indicates that the distal portion of the
centriole may be necessary for positioning. One potential explanation for the
mispositioning of centrioles in bld2 and bld10 cells is that the centrioles are not
actually attached to the cell surface. In many bld2 and bld10 cells (Figure 7E and
7F, respectively) centrioles appear in the cell interior and not at the apical
membrane as in bld1 cells (Figure 6D) and wt cells (Figure 2A). Therefore,
structures at the distal ends of centrioles such as the transition fibers (Figure 7A) may be responsible for properly positioning the mother centriole by docking the centriole onto the cell surface.
Discussion

Towards a pathway of centriole positioning

These data reveal a set of gene products required for proper centriole positioning (Table 1) which will serve as a starting point for a molecular dissection of the centriole positioning pathway. Moreover, the data support a model in which the mother centriole plays a role in establishing cell geometry. Particularly, the mother centriole leads the daughter to the proper location. Additionally, the centrioles position the nucleus and may position the rootlet microtubules and contractile vacuoles.

Using the uni1 mutation, we were able to distinguish between mature and immature centrioles in asq2 and vfl2 cells and determine their subcellular locations. One intriguing possibility is that at least some of the mispositioned unflagellated centrioles in asq2uni1 and vfl2uni1 cells are de novo assembled centrioles, which are known to form in vfl mutants (Marshall et al., 2001). As de novo assemble centrioles are perhaps the most immature form of centrioles, this possibility would not invalidate our model that centriole maturity affects positioning. In fact our model only presumes that mature centrioles can find their way to the proper subcellular site while immature centrioles (which could include both templated daughter and de novo assembled centrioles) cannot.

Loss of positioning versus saturation of docking sites

An alternative model to explain centriole positioning is that there are only two slots for centrioles to dock into at the correct apical location, such that any
cell with more than two centrioles would have more centrioles than could dock into these slots and the extra centrioles would be mispositioned by default (an equivalent model for the case of ciliates was proposed (Lwoff, 1950)). Although cells with three or more centrioles per cell occur in vfl2 and asq2 populations (e.g. Supplemental Figure S3A), those cells represent a small fraction of the population and hence would not account for the large increase in $\theta_{\text{centriole}}$ on average. Furthermore, a strong prediction of this model is that any cell with only one or two centrioles should have properly positioned centrioles because the two slots could accommodate these centrioles. However, we often observe cells with one or two centrioles that are clearly not at the correct position (Figure 2D and Supplemental Figure S5A) and conversely we also see cells with more than two centrioles that have all of their centrioles clustered near the apical pole. Competition for a limited number of docking sites cannot explain these data. Therefore, while there may be specific docking sites on the cell surface, these sites alone are not sufficient to drive correct centriole positioning.

How does the mother centriole become properly positioned and in turn establish other aspects of cell geometry? One possibility is that the mother centriole could be interpreting a global polarity cue and tracking to the correct position where it would be able to read out aspects of cell polarity to the position of other cellular structures. Alternatively, the mother centriole could itself be the mark to establish aspects of cell polarity after each round of division, exerting its effect through the orientation of the cytoskeleton. This possibility is supported by the observation that $bld2$ and $bld10$ cells are often more round than are wt cells
(compare cell shape in Figure 7E and 7F to 2A), perhaps indicating a perturbation in global cell polarity. As centrioles do not appear docked onto the cell surface in \textit{bld2} and \textit{bld10} cells, it would follow that the centriole requires its distal portion not only for positioning but also for exerting its effect on cell polarity. The mother centriole has structural appendages in the subdistal region that may couple centriole position and orientation with cell geometry through the cytoskeletal network.

\textit{Comparison to previous studies with particular regard to the experiments of Beisson and Sonneborn}

A model in which the mother centriole can impact and propagate local cell geometry is appealing as experiments in ciliates (Beisson and Sonneborn, 1965; Grimes and L'Hernault, 1979; Ng and Frankel, 1977) and vertebrate ciliated tissues (Boisvieux-Ulrich and Sandoz, 1991) demonstrate that ciliary orientation is dictated and propagated by a heritable local mark. These prior experiments demonstrated that a heritable mark exists, but were not able to reveal the identity of this mark because they could not dissociate the cellular components from one another. For instance in Paramecium, experiments of Beisson and Sonneborn (Beisson and Sonneborn, 1965) showed that if rows of cilia are inverted from their normal orientation, the inverted orientation can propagate during cell division. However, each hexagonal cortical unit on the surface of Paramecium contains not only a cilium and centrioles but also kinetodesmal fibers, trichocysts, striated bands, infraciliary lattice fibers, the "fork/bone node" (Iftode and Fleury-
Aubusson, 2003), and an apparently self-duplicating oriented structure called the "post" (Allen, 1971). Because inversion of rows simultaneously inverts the orientation of all of these other structures (Aufderheide, 1986) it is not possible to determine which of the sub-structures within the cortical unit serves as a coordinating local signal to direct the orientation of the other structures during formation of new cortical units in cell division, and it has been suggested that the centrioles may take their orientation from some other elements of the cortical unit, rather than vice versa (Ehret and Powers, 1959; Klein, 1932). Radioactive pulse-labeling experiments have indicated that many components of the cortical unit are stable and persist for multiple generations (Ehret et al., 1964), consistent with a possible role in transmission of orientational information. The fact that supernumerary cortical rows of cilia reform following encystment and excystment in \textit{Oxytricha}, during which process centrioles appear to be degraded and then re-assembled, cast further doubt on whether the centrioles in the ciliate cortex are active participants in the propagation of spatial information (Hammersmith and Grimes, 1981).

The difficulty in interpreting the results of ciliate micromanipulation studies arises because such procedures leave the interactions between centrioles and other cortical structures intact, making it impossible to say who is positioning whom. In contrast, genetic manipulation using \textit{Chlamydomonas} mutants allowed us to separate mother and daughter centrioles from each other and from other oriented structures, allowing us to determine that the local signal responsible for inheritance of orientation appears to be the mother centriole. That the mother
centriole has differential potential from the daughter to affect aspects of cell geometry is interesting as recent studies in Drosophila male germ line (Yamashita et al., 2007) have shown that the mother centrosome behaves differently from the daughter centrosome during asymmetric cell division. Specifically, the mother centrosome is always inherited by the stem cell, while the daughter centrosome is inherited by the differentiating cell.

The centriole is unique among cellular structures in its complexity, chirality, stability, and templated replication, and these features make it an ideal hub around which to organize and propagate the architecture of the cell. In particular, the fact that a mother centriole can not only produce a daughter but instruct the daughter centriole concerning the correct positioning within the cell provides a potential basis for the phenomenon of "cytotaxis", defined by Sonneborn (Sonneborn, 1964) as the ability of a pre-existing cellular structure to determine the position or organization of newly formed cellular structure during cell replication. Our results have implications for the general problem of organelle positioning and cell geometry. The ability of the mother centriole to position the daughter and to orient the nucleus suggests that a complete understanding of organelle positioning will require analysis not only of individual organelles, but also of the pairwise mechanical linkages that may exist among distinct organelles.
Experimental Procedures

Strains and Culture Conditions

*C. reinhardtii* cells were grown and maintained in Tris-acetate-phosphate (TAP) media (Harris, 1989). To generate insertional mutants to screen for phototaxis defects, the cell wall-less strain CC-849, *cw10* was electroporated (Shimogawara et al., 1998) with linearized plasmid DNA containing the *aph7* gene, which confers resistance to hygromycin (Berthold et al., 2002). Strains were backcrossed to a wt strain of the opposite mating type (CC-125) and tetrads were dissected as previously described (Harris, 1989). Double mutant strains were constructed by crossing the pertinent single mutants to wild-type and choosing spores from NPD tetrads that showed a non-wild type phenotype.

Immunofluorescence and Microscopy

Cells were fixed with Lugol’s iodine solution to maintain robust cell geometry and prevent flagellar shearing and allowed to adhere to polylysine-coated coverslips. Cells were permeabilized with Methanol and blocked with 5% BSA, 1% cold water fish gelatin and 10% normal goat serum in PBS. Cells were then incubated in primary followed by secondary antibodies (Jackson Immunological) diluted in 20% block, with six washes of 20% block in between. Cells were incubated with DAPI (diluted 1µg/mL in water) and mounted in Vectashield mounting media on microscope slides. Slides were imaged using a 100x lens (N.A. = 1.4) on a Deltavision deconvolution microscope with an air
condenser for DIC imaging. Images were processed and manipulated using Softworx image processing software.

**asq Measurements**

Cells were fixed and stained as described above. For asq analysis, cells were labeled with DAPI and antibodies against centrin (diluted 1:100, generous gift from J. Salisbury), acetylated tubulin (diluted 1:100, Sigma), and Bld10p (diluted 1:100, generous gift from M. Hirono), which together allow unambiguous identification of centrioles. A three-dimensional stack through each cell was generated and used in the asq analysis. Using Softworx software, the centre of mass of the nucleus, pyrenoid, and cell was defined. The center of mass was determined by obtaining the centroid, approximated by the midpoint of the three orthogonal edges of a bounding box containing the structure of interest and whose edges were parallel to the x-, y-, and z-axes of the 3D image. The appropriate structure for each specific θ measurement (e.g. the centrioles for θ_{centriole} ) were also marked. These coordinates were entered into a PERL script to calculate θ.

**Statistical Analysis**

Comparison of means was performed using a one-tailed Student’s t-test in Excel. Unless indicated, error is shown as the standard deviation of the mean. For measuring correlation of datasets, the Pearson correlation coefficient was used.
Acknowledgements

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Table 1. Genes shown in this study to be required for centriole positioning.

Genbank accession numbers are given for genes whose products are known. For genes with unknown products, the genomic localization (linkage group) as determined by genetic mapping is given.

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Figure Legends

Figure 1. Identification and quantification of defects in asq mutants.

A) *Chlamydomonas* cell geometry. Flagella ("f") extend from the centrioles (white), which are located apically and are attached to the nucleus (yellow) by centrin containing fibers. The pyrenoid ("p", blue), a starch-containing structure, is located basally and is embedded in a cup-like mass of chloroplast (green). The eyespot ("e", red), the light sensing organelle, is located laterally at a reproducible angle relative to the centrioles.

B) DIC image of a wt *Chlamydomonas* cell. The pyrenoid (P), eyespot (E), and flagella (F) are indicated. All DIC images are sections through full three-dimensional data sets.

C) In asq1 cells, mother–daughter centriole pairs are randomly localized on the cell surface.

D) In asq2 cells, centrioles are independently positioned on the cell surface and no longer found in pairs.

E) Defining $\theta_{\text{centriole}}$. A three-dimensional vector reflecting the long axis of the cell is drawn from the centre of mass (yellow circle) of the pyrenoid (blue) to the cellular centre of mass (purple circle). $\theta_{\text{centriole}}$ is the angle between the vector defining the long axis of the cell and the vector from the cellular centre of mass to each centriole (white). All angle measurements are made in three dimensions using three dimensional image datasets.

F) The mean $\theta_{\text{centriole}}$ (black line) for wt cells is $20.5 \pm 9.0^\circ$ (n = 59),
G) $\theta_{\text{centriole}}$ increases to $42.3 \pm 21.3^\circ$ for $asq1$ cells ($n = 54$). Angles are biased toward the top half of the cell, presumably because the lower half of the cell is occluded by the chloroplast which is localized in the basal half of the cell body (J) and closely apposed to the plasma membrane, thus reducing access of basal bodies to the cell surface.

H) $\theta_{\text{centriole}}$ increases to $61.7 \pm 32.3^\circ$ for $asq2$ cells ($n = 71$).

I) Defining $\theta_{\text{chloroplast}}$. The cell center-pyrenoid axis is defined as described in E). $\theta_{\text{chloroplast}}$ is defined as the angle between the vector defining long axis of the cell and the vector from the cellular centre of mass to each plastid genome (large green circle).

J) The $\theta_{\text{chloroplast}}$ for wt cells is shown in green ($\text{mean} = 112.1 \pm 36.0^\circ$, $n = 181$). Each line represents the position of one plastid genome. The yellow shaded area represents the area of the cell occupied by the pyrenoid. The non-$180^\circ$ edge of this shaded region indicates the mean position of the pyrenoid boundary ($\text{mean} = 139.0 \pm 14.4^\circ$, $n = 90$).
Figure 2. *asq* mutants can be divided into two classes based on the pairwise distribution of centrioles.

Images of fixed cells stained with DAPI and antibodies against centrin and acetylated tubulin (green) and Bld10p (red). DIC images are shown in the left panels and fluorescence images of the same cells are shown on the right. All images are positioned so that the pyrenoid is located at the bottom.

**A)** wt cells have two centrioles located together at the apical side of the cell. One pro-centriole can be seen in the foreground of this images (red, marked by antibodies recognizing Bld10p). The other pro-centriole is occluded from view by the centriolar pair.

**B and C)** *asq1* cells have two centrioles that are positioned together at random locations on the cell surface.

**D)** In *asq2* cells, centrioles can found at independently locations from one another. In this cell, both centrioles appear to be mispositioned.

**E)** In this *asq2* cell, one centriole along with its pro-centriole (marked by Bld10p staining in red) is found at the correct apical location. Another mispositioned centriole is found on the left shifted off the long axis.
Figure 3. Mutants with variable numbers of centrioles also have centriole positioning defects

A) DIC image of a wt cell. wt cells have two flagella located at the apical side of the cells.

(B and C) DIC images of asq2 cells. asq2 cells have variable numbers of centrioles and therefore make variable numbers of flagella. Some centrioles are randomly localized, while some are in the correct apical position.

D) Distribution of flagellar number is asq2 cells is reminiscent of the vfl (variable flagellar number) phenotype. asq2 cells (white) have a mean of 1.46 ± 1.1 flagella per cell (n = 1274). This distribution is similar to that of vfl2 cells (green, mean = 1.33 ± 1.05, n = 593) and vfl3 cells (purple, mean = 1.12 ± 1.8, n = 466), but is in contrast to wt cells, which make two flagella (black, mean = 1.94 ± .34, n = 1005).

E) vfl2 cells, previously identified as defective in centriole segregation, have a mean $\theta_{centriole}$ of 55.2 ± 28.8° (n = 64).

F) vfl3 cells, defective in mother-daughter centriole cohesion, have a mean $\theta_{centriole}$ of 59.4 ± 35.2° (n = 90).
Figure 4. Using *asq2* cells to test the role of the mother centriole.

A) Electron micrograph showing electron dense connecting fibers (distal striated fiber, denoted by arrow) joining mother and daughter centrioles.

B) Electron micrographs of *asq2* cell showing that centriole connecting fibers are missing.

D) Model for centriole positioning by mother centriole. In wt cells (left box), two centrioles are localized to the apical pole. These centrioles are connected by electron dense connecting fibers (see panel A, arrow). During duplication, each centriole will serve as a mother (white) to give rise to a daughter centriole (blue). New connections will form between each new mother-daughter pair. One mother-daughter centriole pair will be segregated to each cell following cell division. Each centriole will give rise to a flagellum, resulting in two cells with two centrioles and two flagella. In *asq2* cells (right box), centrioles are no longer connected (see panel B, arrow). As in wt cells, each centriole will serve as a mother (white) to give rise to a daughter centriole (blue). However, because mother and daughter centrioles are no longer connected, centrioles will not segregate properly following mitosis, resulting in cells with variable numbers of centrioles. Amongst the centrioles that are distributed between cells, there will be a mix of mother and daughter centrioles. If the mother centrioles contains the necessary mark (purple) that allows them to find their proper sub-cellular location, while daughter centrioles are naïve and unable to track to the correct place in the cell, then cells will have a population of properly positioned mother centrioles and a population of randomly localized daughter centrioles.
Figure A shows a wild-type (wt) condition with an arrow indicating a specific feature. Figure B displays an asq2 condition with a similar arrow. Figure C compares the wt and asq2 conditions in a more detailed diagram, highlighting differences in specific structures.
Figure 5. In asq2 cells mother centrioles are properly localized while daughters are not.

A) If mother centrioles (white) are properly positioned, then the distance between flagellated centrioles in asq2uni1 mutant cells should be much smaller and less variable than that of single mutant cells.

B) The distance between flagellated centrioles in biflagellate cells is much smaller and less variable in asq2uni1 cells (mean = .89 ± .36 µm, n = 85) than in asq2 cells (mean = 1.48 ± .85 µm, n = 88). This difference is highly significant (one-tailed t test, p < 1.23 e^{-8})

C) If mother centrioles are properly localized, then the $\theta_{\text{centriole}}$ should be much smaller and less variable for flagellated centrioles in asq2uni1 cells than for asq2 cells.

D) $\theta_{\text{centriole}}$ for flagellated centrioles in asq2uni1 cells is significantly (one-tailed t test p < 2.02e^{-10}) smaller (green lines, mean $\theta_{\text{centriole}} = 32.4 \pm 13.1^\circ$, n = 60) and less variable than in asq2 cells (grey lines, mean $\theta_{\text{centriole}} = 61.7 \pm 32.4^\circ$, n = 71).

E) Flagellated mother centrioles (m, white arrow) are properly localized in asq2uni1 cells, while unflagellated daughter centrioles (d, blue arrow) are not. Cells are labeled with anti-acetylated tubulin and centrin antibody (green), anti-Bld10p antibody specific for centrioles (red) and DAPI (blue). Misplacement of non-flagellated daughter centrioles in vfl2uni1 indicates that uni1 does not simply suppress the centriole positioning phenotype of vfl2.
Random mother position
Correct mother position

\[ \theta_{asq2uni1} > \theta_{asq2}, \theta_{asq2uni1} < \theta_{asq2} \]

Interflagellar Distance (microns)

\[ n = 88 \]

\[ n = 85 \]

\[ 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 \]

Percent of Cells

\[ asq2 \]

\[ asq2uni1 \]

\[ wt \]

\[ m, d \]
Figure 6. Centrioles are not positioned by the nucleus, but may position the nucleus.

A) In wt cells, centrioles (red) are attached to the nucleus (blue) via centrin fibers (red). Both the centrioles and nucleus are properly localized near the apical part of the cell. Other plastid genomes are visible with DAPI staining (smaller blue dots).

B) asq1 cell showing centrioles (red) and nucleus (blue) mis-localize together.

C) When centrioles are uncoupled from the nucleus in vfl2uni1 cells, flagellated (green) mother centrioles (red) are properly localized to the apical side of the cell, while the nucleus (blue) can visit variable positions.

D) Mean $\theta_{\text{centriole}}$ for mother centrioles in vfl2uni1 cells is $24.9 \pm 14.7^\circ$ (n = 49), statistically similar to wt.

E) wt cells have a mean $\theta_{\text{nucleus}}$ of $15.5 \pm 8.1^\circ$ (n = 58). $\theta_{\text{nucleus}}$ was determined by measuring the angle between the vector defining the long axis of the cell and a vector from the nuclear centre of mass to the pyrenoid centre of mass.

F) vfl2uni1 cells have a significantly higher (one-tailed t-test, $p < 2.9e^{-6}$) mean $\theta_{\text{nucleus}}$ of $25.0 \pm 11.8^\circ$ (n = 49) compared to wild type.

G) $\theta_{\text{nucleus}}$ and $\theta_{\text{centriole}}$ are correlated in wt cells indicating the position of the two organelles is coupled.

H) When the nucleus is detached from the centrioles in vfl2uni1 cells, the nuclear position no longer correlates to centriolar position. Scatter plot visually shows loss of correlation between $\theta_{\text{centriole}}$ and $\theta_{\text{nucleus}}$. Points are color coded into two groups of cells, those with a nucleus whose position is within the correct wild-
type range (defined as $\theta_{\text{nucleus}}$ is less than one standard deviation from wt mean and plotted in orange) and those with a nucleus whose position is incorrect (defined as $\theta_{\text{nucleus}}$ is more than one standard deviation from wt mean and plotted in gray). The two groups of points classified in this manner span the same range of values for $\theta_{\text{centriole}}$, further supporting a lack of correlation between nuclear and centriolar position when the nucleus is detached from the centriole. (Inset) The mean $\theta_{\text{centriole}}$ (mean $\theta_{\text{centriole}} = 25.7 \pm 11.3^\circ$, gray bar, n = 29) of cells with an improperly positioned nucleus (NI) is indistinguishable from the mean $\theta_{\text{centriole}}$ (mean $\theta_{\text{centriole}} = 23.7 \pm 18.8^\circ$, orange bar, n = 20) of cells with a correctly positioned nucleus (NC). This shows that the mother centrioles can still attain the correct localization regardless of nuclear position.
Figure 7. Mutant centrioles with defective distal ends are mis-positioned.

A) Centrioles contain nine-triplet microtubule blades (yellow) arranged around a central cartwheel, which sits on an amorphous disc structure (blue). At the most distal ends of centrioles in the region just proximal to the site of flagellar assembly, transition fibers are assembled (black ellipses) near the apical membrane. *bld1* mutant cells have normal centrioles and transition fibers but are defective in flagellar assembly due to a loss of intraflagellar transport.

B) *bld2* cells are defective in centriole assembly and lack the B- and C- tubule of the triplet microtubule blades. As a result, the distal portion of *bld2* centrioles is missing.

C) *bld10* cells lack centriolar microtubules and have just the very proximal portion of the centriolar structure.

D) *bld1* centrioles (green represents centrin/acetylated tubulin labeling) localize to the apical membrane.

E and F) *bld2* and *bld10* cells have mispositioned centrioles (green) that appear in the cell interior. They are still found closely apposed to the nucleus (blue).

G) *bld1* cells have normally positioned centrioles (mean $\theta_{\text{centriole}} = 19.8 \pm 8.0^\circ$, n = 52) despite their lack of flagella. This demonstrates that neither flagella themselves, nor the intraflagellar transport machinery, is required for centriole positioning.

H) *bld2* cells which lack the distal region of the centriole have mispositioned centrioles (mean $\theta_{\text{centriole}} = 45.9 \pm 26.9^\circ$, n = 44).

I) *bld10* centrioles are also mispositioned (mean $\theta_{\text{centriole}} = 40.2 \pm 30.8^\circ$, n = 46).
Supplemental Material

**Supplemental Table 1.** Distribution of Flagellar Number in Mutants (%)

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Supplemental Figure Legends

Figure S1. Results from cell geometry screen.

A) Phototaxis was assayed using an opaque tube rack with a horizontal slit that permits light to strike the centre of each test tube in the rack. When the door is closed (inset), light only enters the rack from through the slit. Light from a 25 W fluorescent bulb with an intensity of approximately 8000 lux was used.  B) Cells that phototax (ptx +) form a band at the level of a light source in about ten minutes.  C) Cells that are defective in phototaxis (ptx -) are uniformly present throughout the tube. Mutant lines that were defective in phototaxis were retained and re-screened by DIC microscopy to identify defects in cellular morphology.  D) 252 phototaxis defective mutants are categorized into 16 phenotypic classes: askew (asq), no flagella (bld), uniflagellate (uni), stumpy flagella (stumpy), short flagella (shf), long flagella (lf), unequal length flagella within a cell (ulf), variable length flagella within population (vlf), clumpy groups of cells (clumpy), cell size, cell shape, cells look unhealthy (sick), defective eyespot (eyespot), normal morphology (norm). Cells with variable flagellar numbers are contained with the asq class of mutants.
Figure S2. Centriole and nuclear positioning in uni1 is similar to wt.

A) uni1 cells have a mean $\theta_{\text{centriole}}$ of $20.4 \pm 8.5^\circ$ (n = 40), which is statistically similar to that of wt.

B) uni1 cells have a mean $\theta_{\text{nucleus}}$ of $14.3 \pm 5.6^\circ$ (n = 40).

C) Centriole and nuclear position is highly correlated in uni1 (correlation coefficient = .79)
A

\[ \theta_{\text{centriole}} \]

B

\[ \theta_{\text{nucleus}} \]

C

\[ \theta_{\text{Centriole}} \]

\[ \theta_{\text{Nucleus}} \]
Figure S3. The *uni1* mutation does not suppress centriole number or position defects in *asq2uni1* cells.

**A)** *asq2uni1* cells have a mean of 1.67 ± 1.25 (blue bars) centrioles per cell. This number is statistically similar (one tailed t-test, p > .30) to that of *asq2* cells (yellow bars), which have a mean centriole number of 1.72 ± 1.27.

**B)** Intercentriolar distance in *asq2* (mean = 1.39 ± .94, n = 168, yellow bars) cells is similar to that of *asq2uni1* cells (mean = 1.42 ± 1.12, n= 174, blue bars, one tailed t-test: p > .39)
Figure S4. Rootlet microtubules are misplaced in asq cells.

A) In a wt cell, acetylated microtubule bundles emanate from near the centrioles. Normally, these rootlets are draped over the apical pole of the cell.  

B) When centrioles are misplaced in asq cells, the rootlet microtubules are also misplaced, suggesting that either centrioles position the rootlet microtubules or vice-versa.
**Figure S5.** Single centrioles are found in correct and incorrect locations in *asq* cells.

DIC (left panels) and fluorescence images (right panels) of *asq2* cells with one centriole. Cells are labeled with DAPI (blue) and antibodies against acetylated tubulin and centrin (green) and Bld10p (red). Images are oriented with the pyrenoid on the bottom.

**A)** *asq2* cell with one incorrectly positioned centriole

**B)** *asq2* cell with one correctly positioned centriole.
CHAPTER 3

Mitosis in *Chlamydomonas*
Mitosis has been studied extensively in *Chlamydomonas* (Johnson and Porter, 1968; Kater, 1929), however these studies largely used electron microscopy to observe these events. The images are beautiful, but the data are fragmented due to small sample sizes. Some studies have been published with immunofluorescence images (Doonan and Grief, 1987), however, from these data, it is difficult to glean the subtleties of mitosis. Using the iodine fixation protocol that I developed for looking at cell geometry, I have been able to gain further insight into mitosis in *Chlamydomonas*. This analysis is facilitated by the use of an antibody against phosphorylated histone H3, which I can use to identify cells in the earliest and the latest stages of mitosis. I focused on the arrangement of the microtubule cytoskeleton, the rootlet microtubules, the nuclear-centriole connections known as the rhizoplasts, and the centrioles. Based on these data, I have divided the stages in mitosis with respect to the status of the cytoskeleton. These stages are: microtubule rearrangement, centriole separation, MTOC, and spindle formation. Each stage is described in detail below. *Chlamydomonas* cells can go through several successive mitoses without entering back into the cell cycle. For the purposes of simplicity, I restricted the bulk of my analysis to cells that are in their first mitosis and unattached to surrounding cells.

*Microtubule Rearrangement*

During the earliest stages of prophase, the microtubules begin to dramatically reorganize (Figure 1). During interphase, microtubules are
normally diffuse and line the cortex of the cell, running from the apical to the basal end of the cell (Figure 1A and C). At the earliest stages of mitosis, the microtubules begin to sweep across the surface of the cell and gather in bundles around the rootlet microtubules (Figure 2A). The microtubule bundles, which resemble ropes, are then gathered up and retracted towards the nucleus, leaving what looks like hair draped over the nuclear envelope (Figure 1B and D). Mitosis in *Chlamydomonas* is semi-closed, so at some point the microtubules must penetrate the two large fenestrae in the nuclear envelope. This nuclear penetration appears to occur after the centrioles separate (see below). During the microtubule rearrangement, the rhizoplasts begin to retract (compare Figure 1C and D), potentially pulling the nucleus closer to the centrioles, as has been previously described (Salisbury et al., 1988). The rhizoplasts will be discussed further below.

**Centriole separation**  

Following microtubules rearrangement, the centrioles begin to separate (Figure 2A-F). At this point, both centrioles of each pair appear to have elongated/matured as four clear acetylated microtubule spots are visible. During interphase, only two of the centrioles are identifiable by acetylated tubulin localization. As the centrioles are separating, two pairs of centrioles are observed attached to a pair of rootlet microtubules (Figure 2A-C). During interphase, four rootlet microtubules are connected to the centrioles. These are two four-membered rootlets and two two-membered rootlets (Dutcher, 2003).
One two- and one four-membered rootlet appears attached to each pair of centrioles during mitosis. The attachment is only on one of the two centrioles (Figure 2B). This centriole is presumably the mother centrioles, which can be inferred on the basis of the rootlet status during interphase. During interphase, the original centriole pair is attached to the rootlets. As this pair will mature into a mother-grandmother pair, each with its own daughter, the unattached centrioles are likely the new daughters. As the centrioles separate, the centriole pairs appear to push past each other on their respective rootlet pairs (Figure 2A-C).

The rootlets form hook-like structures over the nucleus, giving the appearance that the rootlets are orchestrating the movement of the centrioles. Alternatively, the centrioles could be towing the rootlet microtubules to their final destination. To distinguish these two possibilities, we need to perturb the rootlets and ask what happens. These possibilities will be discussed below.

MTOC appearance

After the centrioles have separated, microtubules form radial arrays surrounding the centrioles, which begin to look like proper microtubules organizing centers (MTOC) (Figure 2C and F). The microtubules appear to radiate from around the centriolar pair in all directions. These microtubules then penetrate the nuclear fenestrae to contact the kinetochores. A subset of these microtubules will form the astral microtubules, which are dim, but can be observed between the centrioles and the cell cortex.
Spindle Formation

After microtubules have reached through the nuclear fenestrae, the metaphase spindle is established (Figure 3). The *Chlamydomonas* metaphase spindle is quite striking, especially in its precise positioning with respect to other aspects of cell geometry. Spindle positioning will be further discussed in Chapter 4. The metaphase spindle is positioned orthogonal to the long axis of the cell (Holmes and Dutcher, 1989; Kater, 1929), so the spindle axis is at 90º to the central cell axis. A pair of centrioles can be found at each pole. Each centriole pair is connected to a pair of rootlet microtubules. The centrioles are at a distance of about 1 μm from the poles (Figure 3A). The rhizoplasts are more closely apposed to the poles (Salisbury et al., 1988) (Figure 3C), but are not necessary for spindle attachment (Chapter 4). The rhizoplast attachment is probably to the nuclear envelope and not to the pole per se. Thus, rhizoplast attachment might facilitate centriole connection to the poles, but is not necessary for it.

The DNA is then separated and, at some point the nuclear envelope must divide. Internuclear microtubules are present during telophase (Figure 3B). As the two new nuclei are separated, the spindle microtubules begin to become unbundled. Some return to a cortical location, while others begin to reach between the two cells where they will help establish the cleavage furrow (Holmes and Dutcher, 1989).

*Chlamydomonas* cells undergo rapid successive mitoses
Although the analysis described so far was restricted to cells undergoing their first mitosis, I occasionally photographed cells that were undergoing multiple rounds of mitosis. It is interesting to observe the appearance of new centrioles in these cases. Because cells bypass most stages of the cell cycle to re-enter mitosis in these cases, centrioles must still reduplicate at potentially abnormal times. This system puts pressure on the centrioles to duplicate during mitosis. Some groups have reportedly observed centriole duplication during mitosis (Piasecki et al., 2008). This type of observation might be the exception rather than the rule and actually represent cells preparing to go into an immediate round of mitosis. Live observation of centriole duplication and mitosis would address many of these outstanding questions.

*Rootlet microtubules: Cause or consequence of spindle establishment and orientation*

As described above and shown in Figure 4, rootlet microtubules appear to be aptly poised to be driving events during mitosis such as centriole separation and spindle establishment and positioning. Some experiments have been done to identify the role of rootlet microtubules (Ehler and Dutcher, 1998), however, the specific impact on mitosis was not identified. To determine the role of the rootlet microtubules during mitosis, I made use of a mutant from the screen described in Chapter 2. This mutant, *lfunk1* (long-flagellar mutant, unknown 1) not only has long flagella, but also has long and aberrant rootlet microtubules (Kim Wemmer, unpublished data). The rootlet microtubules are incredibly long,
wrapping around the cell cortex (Figure 5). The ends of the rootlets appear frayed in some cases.

To determine the involvement of the rootlet microtubules in mitosis, I looked at spindle establishment and positioning in *lfunk1* cells (Figure 5). Despite clearly aberrant rootlets microtubules, spindles appear to be correctly established in *lfunk1*. No gross defects in spindle morphology are observed. Furthermore, the spindles appear to be correctly positioned. These data suggest that the rootlets are not involved in spindle establishment and positioning and instead are cargo being pulled by their centriolar tugboats.

There are, of course, several caveats to these experiments. While the rootlets are long and frayed in *lfunk1* cells, it is not clear whether the core rootlet structure is perturbed. Although the long rootlets might be cumbersome to the point of delaying mitosis, they may not interfere with the choreography of the main structure of the rootlets with regard to the centrioles and spindle. The complete abolition of rootlets and analysis of mitosis will be necessary to fully understand the impact of the rootlets microtubules on mitosis.

*Chlamydomonas cells retain their flagella during mitosis*

The general dogma in the field has been that prior to mitosis, *Chlamydomonas* cells resorb their flagella. This step was thought of as a switch that allowed for the basal bodies to stay anchored until mitosis. The resorption of flagella was thought to provide temporal control over entry into mitosis. In addition to being resorbed, flagella are thought to be severed from the basal
body by katanin (Lohret et al., 1998). This two-step deflagellation was thought to control the timely entry into mitosis.

Using the iodine fixation method described earlier, I was able to observe many cells that had retained their flagella during mitosis (Figure 6). During all stages of mitosis, cells are visible with flagella of variable lengths. Mitotic cells retain flagella during all stages of mitosis. Mitotic cells reorganize their cytoskeleton (Figure 6A, CR), centrioles separate (Figure 6B, CS), and finally the spindle is established (Figure 6C, SP). In all three stages, flagella are present (Figure 6E). I observed 24 cells that had established their mitotic spindle. Of these cells, 15 (63%) still had flagella, but these flagella were detached from the basal bodies. Similar results have been seen before (Johnson and Porter, 1968) and since this discovery (Piasecki et al., 2008).

These results have interesting implications for control over entry into mitosis. Namely, cells must rely on the severing of centrioles from flagella in order to enter into mitosis. Flagellar resorption seems to be more of a consequence, rather than a cause of entry into mitosis. The necessary signal is apparently katanin severing at the base. This hypothesis is supported by the fact that katanin is essential in *Chlamydomonas* (Q. Rasi, J. Parker, and L. Quarmby, unpublished data). Knockdown of katanin by RNAi is not a sustainable phenotype unless in a background lacking flagella (*bld* cells). Alleviating the attachment of basal bodies to flagella, as in *bld* mutants, is permissive for katanin knock down. Katanin is an essential gene in cells that have flagella; inactivation
of katanin leads to the inability of flagella to detach from centrioles, and the failure of mitotic execution.

**Appearance of pro-centriole-like structure on the nucleus during mitosis**

All of the experiments described so far to analyze mitosis in *Chlamydomonas* have been done in fixed cells. While we have learned a wealth of information from these disparate frames in our mitosis movie, observation of live cells will answer a lot of our outstanding questions. I have used overexpression of centrin labeled with GFP in order to observe centrioles in live cells during mitosis (Ruiz-Binder et al., 2002). In doing so, I have discovered that cells have feet-like structure on or near the nucleus (Figure 7-10). It was previously shown that the centrin-GFP construct may perturb the rhizoplast structure (Koblenz et al., 2003; Ruiz-Binder et al., 2002). The rhizoplast is composed of elastic centrin fibers that connect the centrioles to the nucleus. Thus, it is possible that these feet-like structures are the collapsed rhizoplasts at the site of nuclear attachment. Regardless of their origin, the foci of fluorescence have revealed that the points of nuclear attachment normally duplicate along with the centrioles. These feet-like structure are highly motile during interphase and mitosis.

In fixed images of wild-type mitotic cells, the rhizoplasts clearly retract up to near the centrioles (compare Figure 7 and Figure 3C and D). There is no indication that feet-like structures exist in these cells. However, centrin staining on the nuclear envelope is present (Figure 7A). Thus, the collapsed rhizoplasts
are elucidating an underlying mechanism of rhizoplast duplication. In fact, this duplication in some cases appears before centriole duplication (Figure 8-10). The nuclear feet are also quite dynamic, transiting all over the cell prior to and during mitosis (Figure 8-10). Nuclear attachment site duplication may be an important step in centriole duplication. Because centrioles are not themselves attached to the spindle, these feet-like structures could represent an attachment point to the spindle. Further investigation into feet dynamics is necessary.

*Insight into mitosis from mutants*

Most of the analysis that I have described so far has focused on understanding mitosis in wild-type cells. This analysis has laid the groundwork to be able to dissect the complex mitotic phenotypes that can arise in *Chlamydomonas* mutants. I have looked at mitosis in *bld2* mutants that have severely perturbed centriolar structure. I have also explored mitosis in the variable flagellar number (*vfl*) mutants and *asq2*. Much of these data are presented in Chapter 4. From analysis in these mutants, centrioles appear to be necessary for spindle positioning. In contrast, spindle morphology defects do not appear to correlate with the presence or absence of centrioles at the poles in the *vfl/asq2* class of mutants. Furthermore, *bld2* mutants have a range of spindle defects similar to those of *vfl/asq2* mutants, but a large percentage of cells form bipolar spindles. These data may be indicative of dynamic spindle morphology changes that will only be observable in live cells.
**Figure Legends**

**Figure 1. Microtubule rearrangement during mitosis**

Cells were labeled with DAPI (blue) and antibodies against α-tubulin (red) and phosphorylated histone H3 (CY5, not shown). A and B are labeled with an antibody against acetylated α-tubulin (green) and C and D are labeled with an antibody against centrin (green). As shown in A and B, microtubules begin to form bundles around the rootlet microtubules. The rhizoplasts begin to retract as the cells proceed through mitosis (compare C and D). Centrin localization can also be seen on the nuclear envelope at this time.
Figure 2. Centriole separation and MTOC formation

Cells were labeled with DAPI (blue) and antibodies against $\alpha$-tubulin (red) and phosphorylated histone H3 (CY5, not shown). A-C are labeled with an antibody against acetylated $\alpha$-tubulin (green) and D-F are labeled with an antibody against centrin (green). The green channel alone is shown in A'-F'. At this stage of mitosis, four clear centrioles are visible by acetylated $\alpha$-tubulin localization (A-C). Each pair of centrioles is attached to two rootlet microtubules (A'-C'). The centriole pair is obscured by the rhizoplast structure that has been retracted (D-F). Following centriole separation, microtubules emanate from around the centrioles, forming what resembles a proper MTOC (C and F).
Figure 3. Spindle formation

Cells were labeled with DAPI (blue) and antibodies against α-tubulin (red) and phosphorylated histone H3 (CY5, not shown). A and B are labeled with an antibody against acetylated α-tubulin (green) and C and D are labeled with an antibody against centrin (green). The green channel alone is shown in A’-D’. The mitotic spindle forms orthogonal to the long-axis of the cell. A) Rootlet microtubules form a hook-like structure over the nucleus. The centrioles are at a distance from the spindle poles. B) During telophase, microtubules are present between the two forming nuclei and around the rootlet microtubules. (C and D) Centrin fibers form a buckle-like structure at the spindle poles. A cell labeled with DAPI (blue) and antibodies against α-tubulin (red and E’), centrin (orange and E’’), and acetylated α-tubulin (green and E’’’) is shown in E.
Figure 4. Rootlet microtubule placement during spindle formation

Cells were labeled with DAPI (blue) and antibodies against α-tubulin (red), acetylated α-tubulin (green), and phosphorylated histone H3 (CY5, not shown). A’ and B’ show acetylated α-tubulin localization alone. The rootlet microtubules appear to form hook-like structures that drape over the nucleus. Two rootlets are attached to each centriolar pair. Each pair of rootlets passes by the other as the centrioles separate.
Figure 5. Perturbation in rootlet morphology does not impact spindle formation or positioning

*Ifunk1* cells were labeled with DAPI (blue) and antibodies against α-tubulin (red), acetylated α-tubulin (green), and phosphorylated histone H3 (CY5, not shown). A’-D’ show acetylated α-tubulin localization alone. A) Interphase cells have elongated and frayed rootlet microtubules that wrap around the cell. (B-D) Mitotic *Ifunk1* cells have normal spindle establishment and positioning, despite the severely perturbed rootlet microtubules (B’-D’).
Figure 6. Chlamydomonas cells retain their flagella during mitosis

Mitotic cells were defined as those cells expressing phosphorylated histone H3 (pH3). Flagella are visible in the middle and bottom panels. Scale bars = 5µm. (A-C) Cells are labeled with four different markers, pH3 (top panels), acetylated-tubulin (middle and bottom panels (green)), alpha-tubulin (bottom panels (red)), and DAPI (bottom panels (blue)). (A) During the first stages of mitosis, cytoskeletal rearrangement (CR) is visible. (B) Centrioles then begin to separate (CS). (C) Finally, the mitotic spindle is established (SP). (D) This cell is labeled with pH3 (top panel), alpha-tubulin (middle and bottom panel (red), centrin (bottom panel (green), and DAPI (bottom panel (blue)). (E) Quantification of the number of cells that retain flagella during mitosis. Flagellar status for each class (CR (green, n=14, +: 12 (85.7%), -: 2 (14.3%)), CS (blue, n=10, +: 7 (70%), -: 3 (30%)), SP (purple, n=24, +: 15 (62.5%), -: 9 (37.5%)), is shown, as is the total number of cells for all three classes (TOTAL (black, n=48, +: 34(70.8%), -: 14(29.2%)). F) Cartoon of a Chlamydomonas spindle. A pair of centrioles (green) is found at the end of two rootlet microtubules (one pair shown in grey and one in black). Centrioles localize to the spindle (red) poles, while flagella can be retained in the cell wall.
Figure 7. Centrin localization in fixed and live cells

A) Wild-type cell labeled with DAPI (blue) and antibodies against α-tubulin (red), and centrin (green). Centrin localizes to the centrioles and to the rhizoplast structure that connects the centrioles to the nucleus. B) Wild-type cell expressing centrin-GFP. Centriole localization (white arrow) and rhizoplast structure (red arrow) are visible. The rhizoplast structure appears collapsed back onto the nucleus.
Figure 8. *In vivo* localization of centrin reveals insight into nuclear attachment dynamics and duplication

A wild-type cell expressing centrin-GFP was visualized. Images are time points taken every 40 minutes. Successive time points are shown from left to right.
Figure 9. *In vivo* localization of centrin reveals insight into nuclear attachment dynamics and duplication

A wild-type cell expressing centrin-GFP was visualized. Images are time points taken every 20 minutes. Successive time points are shown from left to right.
Figure 10. *In vivo* localization of centrin reveals insight into nuclear attachment dynamics and duplication

A wild-type cell expressing centrin-GFP was visualized. Images are time points taken every 4 minutes. Successive time points are shown from left to right.
Chapter 4

The ASQ2 gene required for centriole cohesion and mitotic spindle orientation encodes a conserved TBCC-like protein
Summary

Background: Centriole biology has captivated scientists for over a century. Part of this intrigue is due to the fact that these highly complicated structures duplicate once per cell cycle and the cell has precise control over their number.

Results: We have identified a mutant, \textit{asq2}, with defects in mother-daughter centriole cohesion, resulting in cells with variable numbers of centrioles. Here, we characterize mitosis in \textit{asq2} cells. We show that the majority of cells establish a bipolar spindle, but that cells have defects in spindle positioning. A small subset of \textit{asq2} cells have centrioles at both poles, and these cells have properly positioned spindles, indicating that centrioles at the poles may be important for spindle positioning. We additionally show that \textit{ASQ2} encodes the novel conserved protein, TBCCd1, a member of a protein family that includes a tubulin folding co-chaperone and the retinitis pigmentosa protein, RP2, involved in tubulin quality control during ciliogenesis.

Conclusions: We postulate that TBCCd1 may regulate template-driven centriole assembly, normally suppressing the \textit{de novo} assembly pathway either directly or indirectly.
Introduction

Centrioles are cylindrical structures composed of nine microtubule blades arranged around a proximal central cartwheel. Together with pericentriolar material (PCM), centrioles comprise the centrosome, the major microtubule organizing center of the cell. Normally, centrioles duplicate once per cell cycle. Most cells have a pair of centrioles with one older mother centriole and one less mature daughter centriole. Each centriole will mature and serve as a mother centriole to give rise to a new daughter each cell cycle through a template-driven synthesis process. A number of studies have demonstrated that centrioles can arise through a separate de novo synthesis pathway (La Terra et al., 2005; Marshall et al., 2001), but this pathway is normally suppressed by template-driven assembly.

Proteomic and comparative genomic studies have begun to reveal the molecular composition of centrioles, but the mechanistic questions of how exactly these structures duplicate as well as how their number is precisely controlled remain unanswered. Centriole number control constitutes an important question for development and disease as aberrant numbers of centrioles have been linked to cancerous cell states, although it is unclear whether this is a cause or effect (Nigg, 2002). Moreover, spindle defects resulting from improper numbers of centrosomes can have catastrophic effects during cell development and differentiation (Rodrigues-Martins et al., 2008; Stevens et al., 2007). Defects in spindle organization, particularly in the orientation of the spindle have also been proposed to play a role in development of polycystic kidney disease (Fischer et
al., 2006; Simons and Walz, 2006). Because many cystic kidney disease genes encode proteins linked to centrioles, it is interesting to consider whether these diseases arise from defects in centriole number or position.

Several models exist to explain the manner by which centriole duplication is controlled. For example, recent studies suggest that the amount of PCM around the duplicating centriole provides a nest for the birth of new centrioles, suggesting that the number of daughters is proportional to the amount of PCM (Loncarek et al., 2008). Other work has suggested that the centriolar pair remains engaged until it is ready to duplicate. The process of disengagement is followed by a licensing event that allows for new centriolar growth. This model suggests that the site of centriole duplication/growth is normally occluded by the engagement of the centrioles and that the disengagement allows for duplication (Tsou and Stearns, 2006). These models predict that mother-daughter cohesion plays a central role in controlling centriole number.

The unicellular green alga Chlamydomonas reinhardtii has been an important model for the study of centriole number control, as a class of mutants exists in which cells have variable numbers of centrioles and therefore make variable numbers of flagella (vfl) (Adams et al., 1985; Silflow et al., 2001; Taillon et al., 1992; Wright et al., 1989; Wright et al., 1983). This phenotype results from a mis-segregation of centrioles following mitosis. This apparently random segregation arises from centriolar detachment, which could be due to a lack of mother-daughter cohesion, however, it is also clear that centrioles can arise by the normally suppressed de novo synthesis pathway (Marshall et al., 2001). As a
result, some *vfl* cells can inherit zero centrioles while other cells can inherit many more than two centrioles. We have identified a new member of this class of mutants, *asq2*, from a screen for centriole positioning mutants (Feldman et al., 2007). We previously demonstrated that mature mother centrioles are properly positioned in interphase cells, while daughter or immature centrioles are found in uncharacteristic positions (Feldman et al., 2007).

Here, we characterize mitosis in *asq2* cells. We show that the majority of cells establish a bipolar spindle, but that cells have defects in spindle positioning, potentially because of the differential potential of mature and immature centrioles to interact with the spindle poles. We therefore hypothesize that mother-daughter centriole interactions are necessary for regulating spindle positioning. We cloned the ASQ2 gene and found that it encodes the novel conserved protein, TBCCd1. We show that new centrioles can form at a distance from existing centrioles and therefore are potentially arising *de novo*. Based on the existence of these apparent *de novo* assembled centrioles, we postulate that TBCCd1 may regulate template-driven centriole assembly, normally suppressing the *de novo* assembly pathway either directly or indirectly.
Results

\textit{asq2 cells have defects in centriole cohesion and have variable numbers of centrioles.}

The \textit{asq2} mutant was identified in a phototaxis screen for centriole positioning mutants (Feldman et al., 2007). \textit{asq2} cells have defects in centriole cohesion which results in the dissolution of centriole pairs, leading to cells that have variable numbers of centrioles (Figure 1B-D). This phenotype is reminiscent of the \textit{vfl1}, \textit{vfl2}, and \textit{vfl3} mutants (Adams et al., 1985; Silflow et al., 2001; Taillon et al., 1992; Wright et al., 1989; Wright et al., 1983), but \textit{asq2} is not allelic to these mutations (data not shown). We have previously shown that \textit{asq2} cells have defects in centriole positioning due to an inability of daughter centrioles to find the correct position on the cell surface (Feldman et al., 2007).

Using cells that express an HA-tagged copy of Vfl1p (Silflow et al., 2001), we investigated the location of newly forming centrioles in \textit{asq2} cells. Wild-type cells that have duplicated their centrioles have four Vfl1p spots (Figure 1E). Two of these spots represent the original mother-daughter pair and two represent the newly formed pro-centrioles that have not begun to mature. Only the more mature centrioles (the original mother-daughter pair) localize acetylated $\alpha$-tubulin. The other two pro-centrioles only localize Vfl1p. We used this pattern of localization to distinguish newly formed pro-centrioles from the older structures that were assembled in a previous cell cycle.

In \textit{asq2} cells, we find instances of pairs of centrioles (Figure 1F) as well as lone centrioles. In the case of paired centrioles, pro-centrioles may have formed
by the template-driven assembly process (Figure 1F, arrow). Similarly, some of the lone centrioles also localize acetylated α-tubulin, suggesting that they may represent older centrioles that are not being duplicated. We frequently find lone centrioles that only localize Vfl1p. These centrioles may represent newly born daughters that, untethered to their mothers, have wandered to uncharacteristic locations in the cell (Figure 1G, arrow). Alternatively, these lone pro-centrioles may represent centrioles that have been assembled through the de novo synthesis pathway. We also find these pro-centrioles in cells that lack existing centrioles, demonstrating that they can arise through the de novo synthesis pathway (Figure 1H, arrow) as has been shown for other members of the vfl class of mutants (Marshall et al., 2001).

Despite defects in centriole cohesion, asq2 centrioles appear to be molecularly normal, recruiting acetylated α-tubulin, centrin, Bld10p (Figure 1B-D), and Vfl1p (Figure 1F-H). As VFL1 and centrin are mutated in vfl1 and vfl2 (Silflow et al., 2001; Taillon et al., 1992), respectively, asq2 appears to be downstream of these genes if their products are recruited to centrioles in a hierarchical assembly pathway. Alternatively, these proteins may be recruited by independent pathways. Previous ultrastructural analysis (Feldman et al., 2007) has shown that the asq2 centrioles appear normal apart from a loss of mother-daughter cohesion, confirming the notion that the asq2 mutation does not grossly perturb centriole composition. We next asked whether the asq2 centrioles, while structurally normal, might be functionally aberrant.
asq2 cells have some defects in spindle morphology, but the majority of spindles are bipolar

We examined spindle establishment in asq2 cells to test the requirement of centriole cohesion during spindle formation and to observe how and if centrioles interact with the spindle poles. In Chlamydomonas cells, mitosis is semi-closed, with spindle microtubules penetrating the nucleus through large fenestrae on either side of the nuclear envelope (Johnson and Porter, 1968). Each pole has a pair of centrioles, which is attached to a two-membered rootlet structure composed of acetylated α-tubulin (Figure 2A and 3D, green, Supplemental Figure 1) that forms a hook-like structure over the nucleus.

In asq2 mutants, some centrioles are found at the poles (Figure 2B-D), while others are unassociated with the spindle apparatus during mitosis (Figure 2B). In some instances, asq2 mutants form irregular spindles (35.5%, 2H), however, in most cells (64.5%, 2H), a bipolar spindle is established. Among the aberrant spindles in asq2 cells, are cells with monopolar spindles (Figure 2E), most of which have one centriole, which is found at the pole. In rare cases, tripolar spindles can be observed with centrioles at each of the three poles (Figure 2F) or cells can form end-on-end spindles (Figure 2G). The general irregularity of the spindle does not seem to correlate with the presence or absence of centrioles at the poles.

The majority of asq2 cells form bipolar spindles (Figure 2B-D). A subset of this population has slightly irregular spindles, either with asymmetric pole brightness/focusing (Figure 2D) or stray microtubules that do not appear
anchored at the chromosomes (Figure 2C). A similar set of spindle defects has been seen in other vfl mutants (Koblenz et al., 2003; Wright et al., 1989) (J.L.F, L.C.K., W.F.M., unpublished data). Because most asq2 cells form a bipolar spindle, but show clear defects in centriole position, we next asked if asq2 cells would show spindle orientation defects.

*Centriole cohesion is required for spindle orientation*

We observed a severe defect in spindle orientation in asq2 cells (Figure 3G and H). To measure this defect, we used a spherical coordinate system as described previously (Feldman et al., 2007). Specifically, we used the pyrenoid (a starch storage structure that marks the basal end of the cell) center of mass and the cell center of mass to define a central cell axis (Figure 3B). We then marked each end of the spindle to define the spindle axis and asked at what angle the spindle axis intersected the central cell axis ($\theta_{\text{spindle}}$, Figure 3B). In wild-type cells, the average $\theta_{\text{spindle}}$ is $94.6 \pm 17.2^\circ$ (Figure 3E), consistent with previous reports (Holmes and Dutcher, 1989; Kater, 1929) that *Chlamydomonas* cells divide perpendicular to the long axis of the cell (Figure 3C-E). The deviation from $90^\circ$ presumably comes from the noise introduced by the duplicating pyrenoid structure. However, it is interesting to postulate that spindle oscillations might also contribute to this noise in the measurement. Because we cannot differentiate the two poles during our analysis of $\theta_{\text{spindle}}$, we cannot judge these angles as absolute. Therefore, for all statistical analysis, we compare the deviation from $90^\circ$ of the angular measurement. In asq2 cells, $\theta_{\text{spindle}}$ is $78.9 \pm$
36.4° with a mean deviation of 29.0 ± 24.2° (Figure 3H, n=36), which differs significantly from wild-type (one-tailed t-test, p < 2.5e-3; F-test on the variance, p < 4.6e-4). This defect in spindle positioning is observed in other vfl mutants as well (Figure 3I-K). In vfl2 cells, θ_{spindle} is 80.5 ± 41.3° with a mean deviation of 33.4 ± 25.6° (Figure 3K, n=54). Similarly, vfl1 cells have a mean θ_{spindle} is 90.3 ± 38.1° with a mean deviation of 32.7± 20.3° (Figure 3N, n=33). These data suggest that centriole cohesion is generally required for proper spindle positioning.

**Centrioles may be required at the poles for proper spindle positioning**

In most asq2 cells, centrioles are missing from one or both poles (distance is > 1 standard deviation from the wild-type mean distance). The distance from each centriole to the nearest pole is significantly increased in asq2 cells (mean = 1.51 ± .96 µm, n= 96, one-tailed t-test: p < 1.9e-10) as compared to wild-type cells (mean = .80 ± .19 µm, n= 40). This trend is also seen in vfl2 cells (mean = 1.68 ± 1.07 µm, n= 193, one-tailed t-test: p < 4.7e-22). These data are plotted in Figure 4A. The majority of asq2 cells as well as vfl2 cells do not have centrioles at both poles (Figure 4B and D, respectively). These cells have severe defects in spindle positioning (asq2: Figure 4F, mean = 77.1 ± 33.4°, n=31, and vfl2: 4H, mean = 78.5 ± 45.3°, n=44).

In a small subset of asq2 and vfl2 cells, centrioles can be found at both poles (Figure 4C and E, respectively, distance is < 1 standard deviation from the wild-type mean distance). In these cases, the spindles are correctly positioned.
(asq2: Figure 4G, mean = 90.4 ± 11.2°, n=5, and vfl2: 4I, mean = 89.5 ± 9.7°, n=10). These data suggest that centrioles are required at the pole in order to promote proper spindle positioning. bld2 cells which have truncated centrioles (Goodenough and StClair, 1975) that fail to attach to the cortex (Feldman et al., 2007) also have a defect in spindle positioning (Ehler et al., 1995) (Supplemental Figure 2), suggesting that full-length centrioles able to attach to the membrane are important for spindle orientation.

**asq2 maps to linkage group IX**

The original asq2 mutant was generated in an insertional screen (Feldman et al., 2007) by transforming cells with a cassette carrying the aphVII gene, which confers resistance to hygromycin (Berthold et al., 2002). Using a degenerate PCR strategy (Gonzalez-Ballester et al., 2005), we determined that this cassette inserted at position 1487009 on scaffold 22 of the *Chlamydomonas* genome sequence (Merchant et al., 2007) between two predicted genes (version 3.0, gene models 79905 and 191060) on linkage group IX (Supplemental Figure 3). Tetrad analysis indicated that the aphVII insertion was linked to the asq2 mutation at a distance of approximately 16.8 cM (96/572 recombinants). Because the aphVII cassette does not strictly co-segregate with the asq2 phenotype, this insertion is not the causative mutation.

Physical markers for mapping were generated by trolling the genome sequence surrounding the cassette insertion site (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html, primers for new fragment length
polymorphisms are listed in Experimental Procedures). asq2 cells were outcrossed to a highly polymorphic strain (cc-1952) and the mutation was mapped to a <.3 cM region on scaffold 23 of linkage group IX between polymorphic markers near gene models 148927 and 174289 (Figure 5A). As a large stretch of poor sequencing data resides in this interval of the genome, we used synteny with Volvox carteri (http://genome.jgi-psf.org/Volca1) to pursue genes in this region. We identified sequence homologous to the Volvox gene model 121354, which is predicted to encode a homologue of the conserved protein TBCCd1 in the vicinity of the poorly sequenced region (Figure 5A). A polymorphism near the corresponding Chlamydomonas gene (sequence listed in Experimental Procedures) indicates that it is tightly linked to the asq2 mutation (0/750 recombinants).

*asq2 cells have an insertion in the conserved gene TBCCd1*

To determine if asq2 cells have a mutation in the previously un-annotated gene homologous to the Volvox TBCCd1 gene found in the corresponding genomic position, we first identified the open reading frame in Chlamydomonas. Using primers in regions homologous to the Volvox open reading frame, we sequenced the corresponding genomic region in Chlamydomonas as well as the cDNA and identified a 2043 base pair (bp) open reading frame for the Chlamydomonas homologue (Figure 5A, GenBank accession number EU816954). Because a correctly predicted gene model does not exist in
*Chlamydomonas*, TBCCd1 had escaped prior proteomic and comparative genomic analysis.

We identified a 513 bp insertion in *asq2* genomic DNA (Figure 5B). The insertion sequence shares homology with fish retroviral genes. Primers to the inserted DNA amplify the same sequence from the salmon sperm DNA (Sigma) used for transformation (data not shown), implying that the insertion resulted from integration of the carrier DNA. This genomic insertion lies in a splice junction between exons 8 and 9 of the predicted cDNA (Figure 5A, arrow). This insertion is in the middle of the TBCC domain, the only predicted functional domain of the protein. By sequencing RT-PCR products, we found that the *asq2* mutant cDNA has a 168 bp insertion, but other splice products are visible (Figure 5C).

To determine whether the insertion in the TBCCd1 gene causes the *asq2* phenotype, we transformed mutant cells with a wild-type version of *Chlamydomonas* TBCCd1 (Figure 6A). The *asq2* mutation is recessive (data not shown) and introduction of the wild-type version of the gene rescues the phenotype. Rescued cells appear morphologically normal (Figure 6B), have wild-type centriole positioning as measured by our previously described (Feldman et al., 2007) metric (mean $\theta_{\text{centriole}} = 21.0 \pm 9.6^\circ$, n= 60, compare Figure 6D-F), $\theta_{\text{spindle}}$ (Figure 6G-I, mean = $94.2 \pm 14.2$ (n=23)), and flagellar number distribution (Figure 6J). The rescue construct co-segregates with a wild-type phenotype in 30 full tetrads/octetts (62/124 PAR resistant rescued progeny and 62/124 PAR sensitive mutant progeny). Backcrossing the rescued line to wild-
type cells (cc-125) yields both wild-type and mutant lines (9/40 mutant progeny and 31/40 wild-type progeny). Together, these data indicate that ASQ2 encodes TBCCd1, a 681 amino acid protein. This protein has a predicted TBCC domain, named for the tubulin binding cofactor C protein. The insertion in asq2 cells is in the TBCC domain, presumably perturbing its function.

Three clades of protein families with TBCC domains have been previously identified (Stephan et al., 2007). The first is the canonical tubulin binding cofactor C (TBCC) protein after which the domain is named. TBCC catalyzes the last step in a long pathway for tubulin folding, acting as a GTPase activating protein (GAP) for β-tubulin (Tian et al., 1999; Tian et al., 1996; Tian et al., 1997).

*Chlamydomonas* has one mutual best hit for TBCC (Figure 5D, blue box, gene model 147601). The second class of TBCC proteins contains homologues of the human gene RP2, which is mutated in patients with the hereditary X-linked retinal degeneration disease retinitis pigmentosa. The trypanosome homologue of this gene has been implicated in quality control for tubulin heterodimers that are transported into cilia (Stephan et al., 2007). *Chlamydomonas* has one mutual best hit homologue of this gene (Figure 5D, green box, gene model 179569) and one paralogue (gene model 172780). Both genes, as well as the sole canonical TBCC family member in *Chlamydomonas* (gene model 147601) are distinct from ASQ2.

ASQ2 falls into the third class of TBCC proteins, named TBCCd1 for TBCC-domain containing, which have a TBCC domain, but which reportedly (Stephan et al., 2007) lack the conserved catalytic residues shared by members
of the canonical class of proteins (Bartolini et al., 2002). This arginine residue constitutes the arginine finger that is responsible for GAP activity (Bartolini et al., 2002; Scheffzek et al., 1998). We have identified an arginine residue (Supplemental Figure 4) two positions away from the functional residue in TBCC and RP2 proteins that may suffice for GAP activity. The gene in which asq2 cells have a mutation encodes a mutual best hit for TBCCd1 (Figure 5D, yellow box, partial gene model 148930).

**ASQ2/TBCCd1 localizes to centrioles**

To identify the localization pattern of ASQ2/TBCCd1, we generated an antibody to a 200 amino acid fragment at the N-terminus of the protein (Figure 7A). This region contains a predicted glycine-rich domain, but does not include the TBCC domain, which is located closer to the C-terminus of the protein. In wild-type cells, TBCCd1 localizes to the centrioles (Figure 7B and C). In many cells, the localization pattern is not only at the centrioles, but also appears in a pericentriolar localization pattern. In asq2 cells, TBCCd1 localization is absent or significantly reduced (Figure 7D and E).
Discussion

ASQ2 encodes TBCCd1

Here, we present a new mutant, asq2, with defects in centriole number control. We demonstrate that ASQ2 encodes TBCCd1, a novel conserved protein that defines a new class of TBCC domain containing proteins. As TBCC proteins are known to interact with tubulin, it is an interesting possibility that TBCCd1 might help control centriole duplication through interactions with tubulin. Because TBCCd1 proteins reportedly lack the catalytic residue necessary to catalyze canonical tubulin folding, an interesting alternative is that the TBCCd1 family of proteins regulates folding/assembly of the atypical tubulins such as δ- and ε- tubulin, which are important for centriole duplication and assembly (Dutcher et al., 2002; Chang, 2000 #47; Dutcher and Trabuco, 1998). However, as we have also identified a conserved arginine near the conserved arginine finger (Supplemental Figure 4) that functions in the TBCC and RP2 families of proteins, this residue may function in GAP activity in TBCCd1 proteins.

Spindle positioning in Chlamydomonas: Contributions of mothers vs. daughters

Asymmetric cell divisions and tissue morphogenesis require precise control over the orientation of the spindle so as to control the plane of division during development (Ciruna et al., 2006; Fischer et al., 2006; Gong et al., 2004; Simons and Walz, 2006), but the mechanism regulating spindle position remains unclear. Here, we demonstrate that proper spindle positioning in Chlamydomonas requires centriole cohesion. In asq2 and vfl2 cells, as centriole
cohesion is perturbed, mother and daughter centrioles do not retain their proper connection to each other or the spindle apparatus. These results suggest that correct spindle positioning might require either mature or immature centrioles at the poles, or both. Membrane attachment is potentially necessary for spindle positioning, as *bld2* cells in which cortical attachment of centrioles is lost (Feldman et al., 2007) have defects in orienting their spindles (Ehler et al., 1995) (Supplemental Figure 2). As spindle position in *asq2* and *vfl2* cells appears to be correct when centrioles are found at both poles, one possibility is that the mother centriole coordinates spindle position through its interaction with the cortex and spindle apparatus. Alternatively, Lechtreck et al. reported that the daughter centrioles are attached to the spindle (Lechtreck and Grunow, 1999), thus daughters may regulate spindle positioning or may need to be attached to mothers on the proper place on the cortex for proper spindle position.

*Function of VFL/ASQ2 genes: Emergence of the vfl phenotype*

Ultimately, the *vfl/asq2* phenotype emerges from a mis-segregation of centrioles following mitosis. However, this mis-segregation arises due to a defect in centriole cohesion. How then might defects in cohesion arise? The simplest possibility is that the proteins encoded by this class of genes could be directly involved in forming the fibers that connect mothers to daughters. Few protein components of these joining fibers have been identified. Centrin, which is encoded by *VFL2*, localizes to the connecting fibers (Geimer and Melkonian, 2005; Lechtreck and Grunow, 1999), but the major fiber components are
unknown. An alternative possibility is that TBCCd1, like other VFL proteins, could be involved in the rotational orientation of the centriole (Silflow et al., 2001), such that improper centriole rotational alignment in the mutants could prevent mother-daughter cohesion. This hypothesis is supported by the fact that Vfl1p localizes asymmetrically on the distal end of the centriole (Silflow et al., 2001).

A final possibility is that the lone centrioles in vfl and asq2 cells are formed by de novo synthesis and therefore lack associated mother centrioles. It is clear that de novo centrioles can be assembled in vfl cells when cells have no centrioles present (Marshall et al., 2001). Here, we demonstrate that new pro-centrioles in asq2 cells can form at a distance from existing centrioles (Figure 1F-H), suggesting that they from de novo.

TBCCd1 may regulate templated vs. de novo assembly

Why might loss of TBCCd1 function result in the dissolution of mother-daughter centriole pairs? To address this question, we considered the location of newly forming centrioles in asq2 cells. If new centrioles form near existing centrioles, this suggests that in asq2 cells, daughters are born from existing mothers, but are not connected to them. If newly forming centrioles are found at a distance from existing centrioles, this suggests that daughters are formed de novo, unassociated with mothers. As shown in Figure 1F-H, newly forming centrioles can be found at a distance from existing centrioles, suggesting that they may have formed de novo.
In normal cells, *de novo* centriole assembly does not occur when pre-existing centrioles are present (La Terra et al., 2005; Marshall et al., 2001). Because centrioles assemble *de novo* in *asq2* cells containing pre-existing centrioles (Figure 1F-H), we hypothesize that TBCCd1 may be involved in suppressing the *de novo* synthesis pathway. TBCC proteins can interact with tubulin, thus an intriguing possibility is that TBCCd1 enhances the kinetics of template-driven centriole assembly (Figure 8, A), thereby out-competing *de novo* synthesis. With TBCCd1 function perturbed in *asq2* cells, *de novo* centrioles are able to form. Because the *asq2* mutation is unlikely to be a null mutation, some templated assembly may still exist in these cells, explaining why we occasionally see paired centrioles. This model is supported by a computational analysis (Supplemental Figure 5) showing that loss of templated duplication is predicted to result in a similar centriole number distribution seen in *asq2* and *vfl* mutants. Furthermore, the localization of TBCCd1 at centriolar and pericentriolar sites suggests that it may be positionally restricted to control templated duplication. Pericentriolar material has previously been suggested to regulate templated duplication, controlling the number of daughter centrioles born per mother (Loncarek et al., 2008).

Alternatively, TBCCd1 could impact centriole cohesion directly. The appearance of *de novo* centrioles in this case could be due to the aberrant licensing of duplication created by the breaking of cohesion (Figure 8, B). As suggested by Tsou et al., duplication might normally be limited by cohesion (Tsou and Stearns, 2006). Although our data do not allow us to distinguish these
possibilities, the fact that *asq2* provides a link between centriole cohesion and templated assembly should serve as a starting point for an extensive future dissection of these two important processes.
Experimental Procedures

**Strains and Culture conditions**

*Chlamydomonas* cells were grown and maintained in Tris-acetate-phosphate (TAP) media (Harris, 1989). The original *asq2* mutant (Feldman et al., 2007) was generated by transforming *cw10* cells (cc-849) with a linearized plasmid containing the aphVII gene, which confers resistance to hygromycin (Berthold et al., 2002). *asq2* was backcrossed to wild-type stain cc-125. Tetrad analysis was performed by previously described standard methods (Harris, 1989). For dominant/recessive and complementation tests, diploid cells were constructed as previously described (Harris, 1989). Specifically, *asq2* cells were mated to cells expressing bleGFP (Fuhrmann et al., 1999), which confers resistance to zeocine. Diploidy was assessed by double selection on hygromycin and zeocine and mating type was scored by PCR (Zamora et al., 2004) to ensure that diploids had both mating types present in their genomes.

**Immunohistochemistry**

Cells were pre-stained with Lugol’s iodine solution (Harris, 1989) and allowed to adhere to poly-lysine coated coverslips. Cells were then fixed and permeabilized in cold methanol at -20°C. Cells were rehydrated in PBS and blocked with 5% BSA, 1% cold-water fish gelatin, and 10% goat serum. Cells were incubated with the appropriate primary antibodies (acetylated α-tubulin, α-
tubulin, phosphorylated histone H3, centrin, Bld10p) followed by secondary antibodies (Jackson). Cells were then stained with DAPI at a final concentration of 1 µg/mL. Coverslips were mounted onto slides using Vectashield (Vector Laboratories).

**Linkage Analysis**

To map the physical position of the asq2 mutation the original asq2 mutant was backcrossed to wild-type cells as described previously (Harris, 1989). asq2 mutants carry one mutation (asq2 x wt: 286/572 are mutant) and one insertion (asq2 x wt: 286/572 are HYG resistant). The resulting backcrossed asq2 line was then crossed to the polymorphic stain cc-1952 (S1-C5) (Silflow et al., 1995). Tetrads were dissected and either full tetrads or one random progeny from each tetrad was scored for phenotype and genotype. Fragment length polymorphisms were identified by designing primers to intergenic regions of the *Chlamydomonas* genome (Merchant et al., 2007) and separating the PCR products in 4% MetaPhor agarose (Cambrex) dissolved in 1x TAE. Primers for polymorphic regions on scaffold 22 and 23 are:

**Intergenic:**

174261 5' F: TGCAGTCAAGTGGTTGAACGTG
174261 5' R: GTGGGACAACGGCTTCTAAAC

148916 3' F: ACAGTCGTATGGCTCCCTGTT
148916 3’ R: CACATGCCTCGAATACATTGC

174289 3’ F: GCGCTCTATGCGGACTATAC
174289 3’ R: GCCTATCTCTCTGCACCTGCT

TTL 5’ F: ACGATAGTACGCTCCGGCTA
TTL 5’ R: GGGATCTTCCATTCTGGACA

174299 3’ F: GCGTGATGAGGAATGAACGTA
174299 3’ R: TCGTGAGCGTTAATTTCG

174284 3’ F: CAGGCCTATTCCGTCTGAT
174284 3’ R: TACCGTATGCAGTGGGTCAA

191420 3’ F: AGAAGGGGATCATGGCTACC
191420 3’ R: AGCATGTATGGGCGTGTGTA

TBCCd1 5’ F: CGCGTACAACTGCCACAG
TBCCd1 5’ R: GGCGATGAGAAGCATGA

Intragenic:
CEP131 F: GCCATAAACAGCAGCAGCA
CEP131 R: ACTCACCTGCAACTCCACCT
Construction of rescue construct

*Chlamydomonas* TBCCd1 cDNA was amplified and cloned into vector PCR2.1 (TA cloning system, Invitrogen). A genomic fragment containing the 507 bp of upstream sequence, the first exon, the first intron, and part of the second exon was joined to the rest of the cDNA at a unique *Bsa*I site. The genomic-cDNA hybrid fragment was subcloned into pBluescript SKII+ between *EcoRV* and *SpeI* sites. The aphVIII construct was subcloned into this vector at the *SpeI* site. The plasmid was linearized with *VspI* for transformation.

Analysis of swimming behavior

Following transformation with the rescue construct, mutant lines were plated into .35% washed Bacto-agar (Sigma) dissolved in TAP media. After 7-10 days, mutant lines formed compact colonies, while normally swimming lines formed diffuse patches.

Swimming behavior was also analyzed using phototaxis as described previously (Hirschberg and Stavis, 1977). Cells were grown in 18mm x 15 cm culture tubes with Kaput closures. TAP was added to near the top of the tube. Tubes were hung by their caps in an opaque rack. Light entered through a small slit in the rack. Phototaxis was measured as the ability to swim towards the light.
entering through the slit in the rack. Cells were stained and fixed with Lugol's iodine solution to immobilize and visualize flagellar number.

Isolation of nucleic acids

*Chlamydomonas* DNA was extracted by two methods. For mapping, a crude extraction protocol was used. A loop-full of cells was added to 100 µL of water and frozen at -80°C. Cells were then boiled for 10 minutes. 5 µL of this mixture was used for subsequent amplification reactions.

For clean genomic DNA isolation, cells were grown in TAP media with shaking under continuous light, concentrated by centrifugation, frozen and lyophilized. Dried cells were pulverized with glass beads and treated with CTAB extraction buffer (100mM Tris HCl, pH 7.5, .7M NaCl, 10mM EDTA, 1% CTAB, 1% β-mercaptoethanol) for at least 2 hrs at 65°C. The cell lysates were mixed with chloroform and nucleic acids from the aqueous phase were precipitated with isopropanol. The precipitate was concentrated by centrifugation, washed, and resuspended in TE. This solution was treated with .05 mg/mL of RNase A and incubated at 37°C, followed by treatment with .5 mg of proteinase K and incubated at 55°C. This solution was extracted with phenol: chloroform. Nucleic acid from the aqueous phase was precipitated using 1/10 volume of 3M sodium acetate and ethanol. The precipitated DNA was concentrated, washed and resuspended in water.

*Chlamydomonas* RNA was extracted from cells grown in TAP media with shaking and continuous light using Trizol (Invitrogen) per the manufacturer’s
specifications. RNA was treated with RNase-free DNase (New England BioLabs) prior to subsequent manipulation.

*Reverse transcription and PCR*

Reverse transcription was performed using Superscript III First Strand Synthesis System (Invitrogen). *Chlamydomonas* total RNA was primed using random hexamers per the manufacturer’s specifications.

PCR of cDNA and crude *Chlamydomonas* genomic DNA was performed using Advantage GC 2 Polymerase Mix (Clontech). PCR of *Chlamydomonas* genomic DNA was performed using AmpliTaq (Applied BioSystems) and 6 µL of 5M Betaine per 25 µL reaction.

*Chlamydomonas Transformation*

Cell wall-less *Chlamydomonas* lines were grown to a cell density of $10^6$ to $10^7$ cells per mL in TAP media with shaking under continuous light. $10^7$ to $10^8$ cells were mixed with 1-3 µg of linearized plasmid DNA and 50 µg salmon sperm DNA. Cells were then transformed with either glass beads (Kindle, 1990) or by electroporation (Shimogawara et al., 1998). For glass bead transformation, cells and DNA were mixed with 100 µL of 20% PEG 8000 and glass beads and vortexed for 1 minute. For electroporation, cells and DNA were added to a GenePulser cuvette (Biorad) with a .4 cm gap distance. Cells were electroporated using a Biorad GenePulser apparatus at 1kV and 25 µF. Transformed cells were added to 10 mL of TAP and incubated overnight at 21°C.
in a roller drum under continuous light. Cells were then concentrated and plated onto TAP plates with appropriate selection media and grown under continuous light for 7 to 10 days.

**Antibody Production**

To generate antibodies to TBCCd1, a 600 bp fragment was amplified from wild-type cells. Using Ligase-Independent Cloning (Doyle, 2005), this fragment was introduced into a vector containing an in-frame maltose binding protein and 6xHis tag. This construct was transformed into chemically competent Rosetta (DES) pLysS cells (Novagen). The fusion protein was purified under soluble conditions using Talon metal affinity resin (Clontech). Two rabbits were injected intradermally with 50-100µg of the fusion protein at day 0, 14 and 28 and boosted every 28 days thereafter with 25-50µg. All treatment and care of rabbits was preformed by Pocono Rabbit Farm and Laboratory, Inc.

**Measurements**

Cells were fixed and stained as described above. For $\theta_{\text{centriole}}$ measurements (Feldman et al., 2007), cells were labeled with DAPI and antibodies against centrin (diluted 1:100, generous gift from J. Salisbury), acetylated $\alpha$-tubulin (diluted 1:100, Sigma), and Bld10p (diluted 1:100, generous gift from M. Hirono). For $\theta_{\text{spindle}}$ measurements, cells were labeled with DAPI and antibodies against $\alpha$-tubulin, phosphorylated histone H3, and acetylated $\alpha$-tubulin or centrin. A three-dimensional stack through each cell was generated and used in the analysis.
Using Softworx software, the center of mass of the pyrenoid and cell was defined. The center of mass was determined by obtaining the centroid, approximated by the midpoint of the three orthogonal edges of a bounding box containing the structure of interest and whose edges were parallel to the x-, y-, and z-axes of the 3D image. The appropriate structure for each specific $\theta$ measurement (e.g. the centrioles for $\theta_{\text{centriole}}$) was also marked. These coordinates were entered into a PERL script to calculate $\theta$. Because we cannot differentiate the two poles during our analysis of $\theta_{\text{spindle}}$, we cannot judge these angles as absolute. Therefore, for all statistical analysis, we compare the deviation from 90º of the angular measurement.

Statistical Analysis

Comparison of means was performed using a one-tailed Student’s t-test in Excel. Comparison of variance using the F-test was performed in Excel. Unless indicated, error is shown as the standard deviation.
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Figure Legends

Figure 1.

*asq2 cells have defects in centriole cohesion*

(A-D) are stained with DAPI (blue) and antibodies against centrin and acetylated α-tubulin (green) and Bld10p (red). A) Wild-type cells have two centrioles that each makes a flagellum at the apical end of the cell. Each centriole recruits centrin and acetylated α-tubulin (green) and Bld10p (red). (B-D) *asq2* mutants make variable numbers of flagella due to their variable numbers of centrioles. B) *asq2* cell with one centriole nucleating one flagellum. C) Biflagellate *asq2* cell with two unpaired centrioles at a distance from one another. D) Triflagellate *asq2* cell with a pair of centrioles at the apical surface of the cell and one lone centriole off to the side. E-H) VFL1-HA expressing cells are labeled with antibodies against HA (red), acetylated α-tubulin (green), and DAPI (blue). (E’-H’) show only HA localization. E) Wild-type cells expressing HA-tagged Vfl1p have an existing centriolar pair that localizes acetylated α-tubulin (green) and two new pro-centrioles forming nearby that localize Vfl1p (red). F) *asq2* cells expressing HA-tagged Vfl1p have newly forming pro-centrioles forming near (red, arrow) and at a distance (G, arrow) from older centrioles (green and red staining). H) Newly forming centrioles can also appear in the absence of existing centrioles (arrow).
Figure 2.

*asq2 cells have defects in spindle morphology, but make bipolar spindles*

(A-G) are stained with DAPI (blue) and antibodies against acetylated $\alpha$-tubulin (green), $\alpha$-tubulin (red), and phosphorylated histone H3 (CY5, not shown). (A'-G') show $\alpha$-tubulin localization alone.

A) Wild-type cells form a bipolar spindle with two centrioles (green) at each pole of the spindle (red). B) The majority of *asq2* cells form bipolar spindles (62.5%, H, blue). Some of these spindles have slight irregularities in morphology, including stray microtubules (C) and asymmetric focusing/brightness of the poles (D). A minority of *asq2* cells form monopolar spindles (E and H, 27.4%), tripolar spindles (F and H, 4.8%), and end-on-end spindles (G and H, 3.2%).
Figure 3.

*Centriole cohesion is necessary for proper spindle orientation*

A) Cartoon of a metaphase *Chlamydomonas* spindle. During mitosis in wild-type cells, a pair of centrioles (green) migrates to each pole to form a bipolar spindle (red). Rootlet microtubules (one two-membered pair shown in grey and one shown in black) are connected to each pair of centrioles through an interaction with one of the centrioles at each pole. B) Cartoon depiction of $\theta_{\text{spindle}}$ measurement. To measure the position of the spindle in *Chlamydomonas* cells, we use the pyrenoid (blue) center of mass (yellow) and the cell center of mass (purple) to define the central axis of the cell. We then pick a point at each end of the spindle (black) to define the spindle axis. $\theta_{\text{spindle}}$ is defined as the angle at which the spindle axis intersects the central cell axis. (C,F,I,L) DIC images and (D,G,J,M) immunofluorescence images labeled with antibodies against $\alpha$-tubulin (red), acetylated $\alpha$-tubulin (green), and DAPI (blue) of dividing *Chlamydomonas* cells were used to generate $\theta_{\text{spindle}}$. E) Wild-type cells have a mean $\theta_{\text{spindle}}$ of 94.5 ± 17.2 (n=12). H) *asq2* cells have a mean $\theta_{\text{spindle}}$ of 78.9 ± 36.4 (n=36). This distribution differs significantly from wild-type (F-test on the variance, $p < 6.6 \times 10^{-4}$). K) *vfl2* cells have a mean $\theta_{\text{spindle}}$ of 80.5 ± 41.3 (n=54), which differs significantly from wild-type (F-test on the variance, $p < 2.3 \times 10^{-4}$). N) *vfl1* cells have a mean $\theta_{\text{spindle}}$ of 90.3 ± 38.2 (n=33), which differs significantly from wild-type (F-test on the variance, $p < 4.3 \times 10^{-6}$).
Figure 4.

Centrioles may be required at the poles for proper spindle positioning

A) Distance from each centriole to the nearest pole was measured in fixed cells. Wild-type cells have a mean centriole-to-pole distance of .80 ± .19 µm (black, n=40). asq2 cells have a mean distance of 1.51 ± .96 µm (blue, n= 96), which differs significantly from wild-type (one-tailed t-test: p < 1.9e-10). vfl2 cells have a mean distance of 1.68 ± 1.07 µm (red, n= 193), which differs significantly from wild-type (one-tailed t-test: p < 4.7e-22). (B-E) Immunofluorescence images of fixed cells labeled with DAPI (blue) and antibodies against acetylated α-tubulin (green), α-tubulin (red), and phosphorylated histone-H3 (CY5, not shown). (B'-E') DIC images of cells shown in (B-E). The majority of asq2 cells (B) or vfl2 cells (D) do not have centrioles (green) at both poles (distance is greater than one standard deviation from wild-type mean). These cells have severe defects in spindle positioning (F, asq2 mean = 77.1 ± 33.4°, n=31, H, vfl2 mean = 78.5 ± 45.3°, n=44). A small subset of asq2 and vfl2 cells have centrioles at both poles (Figure 4C and E, respectively, distance is < 1 standard deviation from the wild-type mean distance). In these cases, the spindles are correctly positioned (asq2: Figure 4G, mean = 90.4 ± 11.2°, n=5, and vfl2: 4I, mean = 89.5 ± 9.7°, n=10).
Figure 5.

ASQ2 encodes the conserved gene TBCCd1

A) *asq2* maps to a <.3 cM region on linkage group IX (grey box defines the genetic interval that is constrained by recombination events). The hygromycin cassette (HYG) inserted on scaffold 22, while the *asq2* mutation maps to scaffold 23. Both scaffolds are part of the same chromosome (Linkage Group IX). Within the genetic interval containing *asq2* is the previously unannotated gene TBCCd1, a conserved protein named for its TBCC domain. B) PCR across the exon-intron boundary of exon 8 (A, arrow) demonstrates that *asq2* cells have an insertion in genomic TBCCd1 and in the cDNA (C) between exon 8 and 9. D) TBCCd1 is contained within a family of proteins that is divided into three clades as previously described (Stephan et al., 2007), canonical TBCC (blue box), retinitis pigmentosa 2 (RP2, green box), and TBCCd1 (yellow box). TBCC domains from mutual best-hit proteins were aligned and organized into a phylogenetic tree using *Clustalw*. (CR, *Chlamydomonas reinhardtii*, VC, *Volvox carteri*, HS, *Homo sapiens*, TB, *Trypanosoma brucei*, and AT, *Arabidopsis thaliana*).
Figure 6.

Expression of wild-type TBCCd1 rescues the phenotype in asq2 cells

A) Wild-type TBCCd1 was expressed in asq2 cells using a rescue construct containing the cDNA of TBCCd1, the first intron to enhance expression (Lumbreras et al., 1998), 507 bp of upstream genomic sequence and 191 bp of downstream sequence. This construct also contained the PAR construct (Sizova et al., 2001) containing the aphVIII gene that confers resistance to paromomycin (PAR). Pertinent restriction enzymes site for plasmid construction are noted (EV = EcoRV, E = EcoRI, and S = SpeI). B) Rescued cells have wild-type appearance (acetylated \( \alpha \)-tubulin (green), Bld10p (red) and DAPI (blue)). C) Cartoon depicting \( \theta_{\text{centriole}} \) measurement to define centriole position. F) Mean \( \theta_{\text{centriole}} \) for rescued cells is 21.0 ± 9.6° (n=60), which is similar to wild-type (D, mean = 20.5 ± 9.0°, n = 59), but differs significantly (one-tailed t-test, p < 2.0e-16) from asq2 (E, mean = 61.7 ± 32.3°, n = 71). (G) DIC image and (H) immunofluorescence image labeled with antibodies against \( \alpha \)-tubulin (red), acetylated \( \alpha \)-tubulin (green), and DAPI (blue) of a spindle in a rescued cell. I) Rescued cells have a mean \( \theta_{\text{spindle}} \) of 94.2 ± 14.2 (n=23). J) Rescued cells have a wild-type flagellar number distribution (asq2: blue, wt: black, rescue: green).
Figure 7.

TBCCd1 localizes to centrioles

A) Cartoon of TBCCd1 protein. TBCCD1 is a 681 amino acid protein with a predicted glycine-rich domain (blue box) and a TBCC domain (purple box). An antibody was raised against an N-terminal 200 amino acid fragment of the protein. (B-E) Cells are labeled with DAPI (blue) and antibodies against TBCCd1 (red) and acetylated α-tubulin (green). (B'-E') Isolated channel showing TBCCd1 localization in cells in (B-E). B and C) In wild-type cells, TBCCd1 localizes to centrioles and pericentriolarly. D and E) In asq2 cells, TBCCd1 localization is significantly reduced or absent.
Figure 8.

*TBCCd1 may regulate de novo centriole assembly*

Mutation of TBCCd1 in *asq2* cells leads to newly forming centrioles appearing at a distance from existing centrioles, suggesting that they have assembled *de novo*. The function of TBCCd1 might normally be to suppress *de novo* centriole assembly either directly (A) or indirectly (B). One mechanism for this suppression would be to bias the kinetics of template-driven centriole assemble, thereby out-competing the slower *de novo* assembly, which normally occurs at about half the rate of templated assembly (Marshall et al., 2001). Alternatively, TBCCd1 might be impacting centriole cohesion directly, which in turn may allow for the licensing of new centrioles near (template-driven) or at a distance (*de novo* assembled) from existing centrioles.
Template-Driven Assembly

TBCCd1

A

B

de novo Synthesis
Supplemental Material

Supplemental Figure 1.

Mitosis in wild-type Chlamydomonas cells. Cells are labeled with antibodies against acetylated α-tubulin (green), α-tubulin (red), phosphorylated histone H3 (CY5, not shown), and DAPI (blue). A) At the beginning of mitosis, cells reorganize their cytoplasmic microtubules into rope-like bundles near the rootlet microtubules. B) Microtubules are then drawn up near the separating centrioles and then form the spindle (C). Mitosis in Chlamydomonas is semi-closed and microtubules penetrate the nucleus at large fenestrae on either side (Johnson and Porter, 1968). D) The DNA is divided and the two new nuclei are connected by internuclear microtubules. Microtubules also retrace the form of the rootlets microtubules as they reach down the middle of the dividing cell to form the cleavage furrow.
Supplemental Figure 2.

*bld2* cells have defects in spindle positioning (A-C) are stained with DAPI (blue) and antibodies against acetylated $\alpha$-tubulin (green), $\alpha$-tubulin (red), and phosphorylated histone H3 (CY5, not shown). (A’-C’) DIC image of stained cells in (A-C). A) *bld2* cell, $\theta_{\text{spindle}} = 34.2^\circ$. B) *bld2* cell, $\theta_{\text{spindle}} = 100.5^\circ$. C) *bld2* cell, $\theta_{\text{spindle}} = 163.9^\circ$. 
Supplemental Figure 3.

asq2 cells have an insertion on scaffold 22 of linkage group IX.

A) The cassette carrying the aphVII gene, which confers resistance to hygromycin (HYG) inserted at position 1487009 on scaffold 22, between predicted genes 79905 and 191060. Using Restriction Enzyme Site Directed Amplification (Gonzalez-Ballester et al., 2005), we identified the location of the HYG cassette insertion in asq2 cells. B) Using primers homologous to the insertion cassette and flanking genomic sequence (F: CCGCATGTTTGCCAATTACTA, R: CGATCGTAGGGAGTGCAACTG), we can amplify a product in asq2 cells, but not in the wild-type parent strain.
A

scaffold 22, version 3.0

79905

1487009

191060

F

R

B

wt asq2
Supplemental Figure 4.

The TBCC domains from TBCC (blue), RP2 (green), and TBCCd1 (yellow) proteins represented in Figure 4D were aligned using Clustalw. The corresponding residue numbers are listed in the headings of each sequence. The previously identified conserved arginine residue (Bartolini et al., 2002; Scheffzek et al., 1998) in the TBCC and RP2 classes of proteins is highlighted in red. The putative arginine residue that may fulfill the same purpose in TBCCd1 proteins is highlighted in purple. CR, Chlamydomonas reinhardtii, VC, Volvox carteri, HS, Homo sapiens, TB, Trypanosoma brucei, and AT, Arabidopsis thaliana
Supplemental Figure 5.

TBCCd1 may regulate de novo centriole assembly

To further explore the possibility of de novo synthesis in asq2 as well as in the other vfl mutants, we turned to a computational model for centriole duplication. Using a model that predicts centriole number distribution changes caused by changes in centriole duplication or inheritance (Marshall, 2007), we were able to determine if the actual centriole distribution seen in asq2 and the other vfl mutants is consistent with de novo assembly.

We find that if only de novo synthesis is employed by the cell (“no temp”), but not templated duplication, the resulting distribution of centriole numbers would be similar to those seen for asq2 mutants as well as for vfl1, vfl2, and vfl3. The combination of this theoretical prediction with the identification of pro-centrioles located far away from pre-existing centrioles suggests that template-driven assembly may be lacking or extremely impaired in these mutants.

A) Flagellar distribution in vfl1 (red), vfl2 (green), vfl3 (yellow), and asq2 (blue) is consistent with the distribution of centriole numbers predicted from a theoretical inheritance matrix in which de novo assembly, number limiting, and maximally symmetric inheritance are preserved, but templated duplication is lacking (no temp, black). These data suggest that the vfl/asq mutants may lack or have severely impaired templated duplication.
Supplemental Experimental Procedures

*In silico Modeling*

We employed a Markov model for centriole inheritance that we have previously described (Marshall, 2007). This model represents the centriole number distribution as a vector of probabilities of a cell having a particular number of centrioles. Centriole duplication and inheritance are represented as a transition probability matrix whose elements represent the probability of a cell with n centrioles producing a daughter cell with m centrioles. We started with the transition matrix previously derived for wild-type *Chlamydomonas* cells (Marshall, 2007) which includes four general features: (a) duplication, (b) segregation by the spindle, (c) de novo assembly when centrioles are lacking, (d) number-limiting, a process that shuts off centriole assembly when more than 2 centrioles are initially present. To predict centriole number distribution for a hypothetical mutation in which duplication is blocked but de novo assembly, number limiting, and centriole segregation are normal, we modified the wild-type transition matrix as follows. First, we assumed that cells with 3 or 4 centrioles will show perfect number-limiting so the fourth and fifth columns of the matrix are the same as in the ideal wild-type matrix including number limiting described above. We assumed that cells with no centrioles will perform de novo assembly at least as efficiently as *vfl2* mutants and we therefore set the first column of the matrix to be identical to the first column of the experimentally derived matrix for *vfl2* (Marshall, 2007). In the case of cells with one or two centrioles, we assumed that they may still form centrioles via *de novo* assembly (since the *de novo* pathway is no
longer out-competed by the duplication pathway), and that the number of centrioles formed de novo follows the same distribution as that seen in vfl2 cells with zero centrioles. The resulting matrix has only a single eigenvalue equal to 1, corresponding to a steady state solution. All other eigenvalues have magnitude less than 1, indicating transient states that will not persist. The steady-state solution is found from the elements of the corresponding eigenvector as previously described (Marshall, 2007), and this solution is plotted in Supplemental Figure 5.
Chapter 5

*blb* mutants reveal insight into centriole and flagellar assembly
Abstract

Centrioles are complex structures composed of nine triplet microtubule blades arranged around a central cartwheel. One main function of centrioles is to nucleate the assembly of flagella or cilia, microtubule-based structures that project from the cell and are involved in sensation and motility. Although proteomic and genomic strategies have been employed to understand centriole composition, little is known about the mechanism by which these structures assemble and eventually nucleate the assembly of flagella. Using a motility-based assay in the unicellular green alga *Chlamydomonas*, we have identified 57 mutants that lack flagella (the so-called *bld* phenotype.) To further characterize these mutants, we have employed a strategy involving cytoplasmic rescue of flagellar assembly. We have also characterized a number of these *bld* mutants by immunofluorescence and electron microscopy and have discovered different perturbations in the centrole/flagellar structure. Finally, we have identified the insertion site in eight *bld* mutants.
Introduction

Centrioles are complex structures composed of nine microtubule blades arranged around a central cartwheel. Centrioles have two main functions in the cell. First, together with pericentriolar material, they comprise the centrosome. Second, they serve as basal bodies to nucleate the assembly of cilia and flagella. Although proteomic and genomic strategies have been employed to understand centriole composition, little is known about the mechanism by which these structures assemble and eventually nucleate the assembly of flagella.

Flagella are built from the distal ends of centrioles, extending the nine doublet microtubules from the A- and B-tubules of the basal bodies. Flagella are built using a process called intraflagellar transport (IFT), which employs flagellar kinesin to bring components to the tip of flagella, where they can be assembled. The flagella are connected to the basal bodies by a transition zone. During flagellar severing, the transition zone remains attached to the basal bodies, indicating that it is more a part of the basal body structure rather than the flagellar structure.

Using a motility-based assay in the unicellular green alga, Chlamydomonas, we have identified 57 mutants that lack flagella (the so-called bld phenotype.) To further characterize these mutants, we have employed a strategy involving cytoplasmic rescue of flagellar assembly. bld mutants may lack flagella because of a defect in flagellar components or due to a defect in the basal body. By fusing bld cells to wild-type cells, we can supply wild-type protein in the cytoplasm, thus complementing the genetic defect, and ask whether wild-
type protein is sufficient to restore flagellar assembly. Based on this assay, we have been able to place these \textit{bld} mutants in three different categories. \textit{bld} mutants with immediate rescue of flagellar assembly presumably have defects in peripheral aspects of the flagellar assembly machinery. \textit{bld} mutants with delayed rescue of flagellar assembly presumably have defects in core flagellar components. \textit{bld} mutants in which flagellar assembly is not restored may have defects in centriole structure or in flagellar nucleation. We have characterized a number of these \textit{bld} mutants by immunofluorescence and electron microscopy and have discovered different perturbations in the centrole/flagellar structure. We have also identified the insertion site in 8 of these mutants.

\textbf{Results}

\textit{Phototaxis screen uncovers mutants in centriole/flagellar assembly}

We previously underwent a screen for mutants with defect in centriole number, placement, and assembly (Feldman et al., 2007). We used a motility-based assay that took advantage of the phototactic nature of \textit{Chlamydomonas}. Following, the initial phototaxis screen, 252 ptx- lines were re-screened by DIC microscopy to identify the specific cellular defect in each mutant line. Among these mutants was a class of mutants that lack flagella, the so-called bald (\textit{bld}) phenotype.

\textit{Quadriflagellate sub-screen reveals four classes of bld mutants}
We originally obtained 57 mutants from our phototaxis screen with defects in flagellar assembly (bld). The bld phenotype can be indicative of a host of different problems in centriole or flagellar assembly. In order to further classify the defect in these mutants we employed a sub-screen strategy involving the quadriflagellate state of cells following mating.

During mating, *Chlamydomonas* cells use their flagella to intertwine and fuse to form a dikaryon. For a brief period, these cells have four flagella. After about 2 hours, the cells resorb their flagella and enter into a meiotic program. If our specific bld mutant were to lack flagella due to a defect in a component of flagellar assembly, then upon mating these cells to wild-type cells, the wild-type protein in the cytoplasm would be able to build a flagellum from the otherwise normal basal bodies supplied by the bld strain (Figure 1). The resulting dikaryon would therefore be quadriflagellate. If the bld mutant lacks flagella due to a defect in centriole assembly, then mating the mutant to a wild-type cell would not result in building flagella from the mutant basal bodies. As a result, the resulting dikaryon would be biflagellate. This strategy is a variation on diakaryon rescue experiments that have been conducted in the past (Luck et al. 1977).

We used this strategy to re-screen all of the bld mutants from our phototaxis screen. As *Chlamydomonas* cells require their flagella for mating, we used dibutyryl cAMP and IMBX to induce mating between bld and wild-type cells (Harris, 1989). Cells were mated and visualized using iodine staining at 10 minutes and 1.5 hours following mating and flagellar number distribution was recorded.
We found four classes of defects that resulted from this strategy. First, we obtained a class of mutants that rescued the flagella-less phenotype after mating for 10 minutes (Figure 2A, class I) and 1.5 hours (Figure 2B, class II). We also obtained a class that initially rescued the flagellar growth phenotype (after 10 minutes), but after 1.5 hours, no quadriflagellate cells were visible in the population (Figure 2D, class IV). Finally, we obtained a class of mutants in which mutant cells never acquired the ability to grow flagella upon mating with wild-type and therefore never formed quadriflagellated cells (Figure 2C, class III).

**Linkage in bld Mutants**

*bld* mutants were generate by transforming cell wall-less cells (cc-849) with the aphVII cassette that confers resistance to hygromycin (hyg) (Berthold et al., 2002). Previous mutants identified in this phototaxis screen have a mutation that is at a distance from the hyg insertion site (Feldman et al., 2007). Therefore, we wanted to determine the linkage of the insertion to the phenotype causing mutation in *bld* mutants. *bld* mutants were outcrossed to wild-type line cc-232 to establish linkage. Tetrad products were grown in TAP and the phenotype was tested by phototaxis, while hyg resistance was tested by streaking cells onto TAP plates supplemented with hyg. Table 2 summarizes these linkage results. “Co-segregation” indicates if the *bld* phenotype segregates with hyg resistance or if the wild-type phenotype co-segregates with hyg sensitivity.
Identifying the insertion site in bld mutants

*bld* mutants were generated by transforming a cassette carrying the aphVII gene into a cell wall-less *Chlamydomonas* strain (cc-849) (Berthold et al., 2002). Using a site directed degenerate PCR strategy (Gonzalez-Ballester et al., 2005), we were able to identify the insertion site in eight *bld* mutants (Table 3).

Mutant 1042 has an insertion that removes a large region on scaffold 2 of the *Chlamydomonas* genome. Among the genes in this region is the gene for neudesin, a secreted factor involved in neural cell proliferation and differentiation (Kimura et al., 2006; Kimura et al., 2005). The cDNA for neudesin is not expressed in mutant 6141 (Table 3). Neudesin may be a new molecule involved in flagellar assembly.

Mutant 800 has an insertion in the beginning of the gene encoding radial spoke protein 6 (RSP6), a component of the radial spokes of the flagella. The cDNA for this gene is no longer present in the mutant (Table 3), suggesting that gene function has been lost. Perturbation of RSP6 function was previously thought to lead to paralyzed flagella, so this mutant may reveal new insight into the role of RSP6 in flagellar assembly.

Mutant 1382 has an insertion in the 5’ genomic region of a cyclin protein, cycD3. The cDNA is still present for this gene (Table 3). Mutant 1869 has an insertion in the mating type locus next to the gene EZY2. The cDNA is not present in this mutant (Table 3). Mutant 8471 has an insertion in a guanylate cyclase (C_189432). The cDNA is missing in this mutant (Table 3), but insertion in this gene does not co-segregate with the *bld* phenotype (Table 2). This mutant
has two insertions. Outcrossed products of 8471 cells that only carry the insertion into the guanylate cyclase are not *bld*. Mutant 6141 has an insertion adjacent to FAP84, a component of the flagellar proteome (Pazour et al., 2005). The cDNA for FAP84 is still present in this mutant (Table 3). Mutant 4249 has an insertion near the gene DYF-13, a ciliary protein whose knock-down in *C. elegans* results in ciliary defects. Mutant 278 has an insertion near FAP233, another component of the flagellar proteome (Pazour et al., 2005).

*Immunofluorescence analysis of bld mutants*

To further characterize the defects in *bld* mutants, we investigated the recruitment of centriole and flagellar proteins using immunofluorescence. Looking at the localization of acetylated tubulin, all mutants tested exhibited normal rootlets microtubules and centrioles. However, one mutant 8699, demonstrated significantly reduced acetylated tubulin localization. Furthermore, this mutant has the round morphology and acetylated tubulin staining that is a hallmark of centriole mutants such as *bld2* and *bld10* (Feldman et al., 2007). Based on this morphology and the quadriflagellate assay results for 8699, it is likely to be a bone fide centriole assembly mutant and warrants further analysis. We also investigated the recruitment of flagellar kinesin, fla10, as well as IFT particles. One mutant 8471 had a defect in IFT139 recruitment, but relatively normal fla10 localization (data not shown).

*Analysis of bld mutants using electron microscopy*
To further characterize the specific defects in *bld* mutants, we used electron microscopy (in collaboration with Stefan Geimer) to inspect the centriole structure. We were only able to characterize a limited number of these mutants, so a more exhaustive characterization will be necessary in the future. Of the mutants, we characterized mutant 4249 and 8471. Both mutants appear to have defects in their transition zones. The transition zone has a characteristic H structure (Figure 4A) and is postulated to be the docking region for IFT particles being loaded into the flagellum. Mutant 4249 appears to have a break in the proximal part of the H structure. Mutant 8471 has an elongated H region, with an apparent duplication of the distal portion of the H. 8471 also has defects in the connecting fibers that join the two basal bodies, resulting in elongated and buckled looking fibers.

**Discussion**

Using a phototaxis assay, we have identified 57 putative *bld* mutants. We have been able to extensively characterize these mutants using a quadriflagellate based subscreen, immunofluorescence, and electron microscopy. We have identified the insertion site in a number of mutants and elucidated several new and previously identified potential candidates for controlling centriole and flagellar assembly.

As several of the mutants have a causative mutation at a distance from the insertion site, more work will be needed to refine the genomic lesion in these
mutants. However, likely candidates based on genetic distance relative to the insertion site can be used to infer the identity of the genes that are perturbed in these mutants (Table 3). The molecular identity will have to be confirmed by testing for rescue of the phenotype. The data presented here will serve as a starting point for the further dissection of the assembly of cilia and flagella.

These data reveal four classes of centriole mutants. In class I mutants, flagellar assembly is rapidly rescued. Thus, mutants in this class may represent mutants with defects in peripheral assembly, such as an IFT particle or an axonemal structural component. Cells appear ready to assemble flagella, and when supplied with the necessary component, are able to rapidly execute this task. Class II mutants can rescue flagellar assembly after prolonged incubation with wild-type proteins. These data indicate that class II mutants might have a defect in more integral components of flagellar assembly such as a motor component. In class III mutants, flagellar assembly is never rescued upon mating with wild-type cells. This class may represent bone fide centriole mutants, however, it may also contain mutants with defects in mating or the mutation may be a dominant negative mutation. Class IV mutants in which flagellar assembly is rescued early, but not late, may also have dominant negative mutations. If the mutant protein poisons flagellar assembly, wild-type protein might initially be able to rescue flagellar growth, but over time might fail to continue this process. This strategy of bld mutant classification will hopefully serve as an important test for characterizing future mutants.
Table 1. Summary of quadriflagellate subscreen results

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Table 1 (continued). Summary of quadriflagellate subscreen results

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ND: Not determined
Table 2. Linkage Analysis in *bld* Mutants

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<th># of insertions</th>
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### Table 3. Summary of insertion sites in bld mutants

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Table 4. Summary of immunofluorescence data for potential centriole (class III) mutants

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<td>9055</td>
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Figure 1. Strategy for quadriflagellate subscreen

A) Cartoon depicting wild-type mating. Mated cells fuse and form cells with four flagella (quadriflagellates). B) bld cells mated to wild-type cells have two possible fates. Cell fusion either rescues flagellar assembly and forms a cell with four flagella (quadriflagellate, left) or fails to rescue flagellar assembly and forms a cell with two flagella (biflagellate, right). C) Flagellar assembly is rescued in a mutant defective in intraflagellar transport, bld1, upon mating with wild-type. Mated cells do not form quadriflagellates after 10 minutes (blue, 407), but do form quadriflagellates after 1.5 hours (red, n=417). D) Flagellar assembly is not rescued in a centriole mutant, bld2, upon mating to wild-type cells. Mated cells do not form quadriflagellates after 10 minutes (blue, n=335) or 1.5 hours (red, n=224).
A

Flagellar Assembly

B

Flagellar Assembly

Mutant

Potential Structural

Mutant

C

D

Number of Flagella

Fraction of Cells

0 flagella

2 flagella

3 flagella

4 flagella

Fraction of Cells

0 flagella

2 flagella

3 flagella

4 flagella

0

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1

0

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1

0 flagella

2 flagella

3 flagella

4 flagella
Figure 2. Quadriflagellate sub-screen reveals four classes of *bld* mutants

*bld* mutants fall into four classes. A) Class I, mutant 5751: After 10 minutes (blue, n=402) and 1.5 hours (red, n=431) mated cells form quadriflagellates. B) Class II, mutant 414: Mated cells do not form quadriflagellates after 10 minutes (blue, n=319), but do form quadriflagellates 1.5 hours (red, n=377) after mating. C) Class III, mutant 8699: Mated cells do not form quadriflagellates after 10 minutes (blue, n=421) or after 1.5 hours (red, n=436). D) Class IV, mutant 4388: Mated cells form quadriflagellates after 10 minutes (blue, n=335), but not after 1.5 hours (red, n=457).
Figure 3. Immunofluorescence analysis of Class III *bld* mutants

Cells labeled with DAPI (blue) and antibodies against acetylated α-tubulin (green) and Bld10p (red). A) Wild-type cell has two flagella (green) extending from the centrioles (red). Rootlet microtubules are also present (green). (B) *bld1* cells, which have defective intraflagellar transport have normal centrioles, but lack flagella. (C) *bld2* cells have defective centriole assembly and, as a result, have a round cell morphology and disorganized rootlet microtubules. (D-L) *bld* mutants lack flagella but have centrioles. All panels, except J are similar to *bld1* cells. (J) Mutant 8699 has the stereotypic cell morphology of a centriole mutant (compare to C), along with disorganized rootlet microtubules (green).
Figure 4. Electron microscopy of bld mutants reveals transition zone and connecting fiber defects

A) Centriole and cilium from a wild-type cell. The transition zone is visible extending from the distal end of the centriole. This structure forms a bridge between the centriole and flagellum that extends from the cell. (A’) 2.7x magnification of the transition zone in A. The stereotypical H-structure is present. B) Centriole from mutant 4249. The transition zone is broken in its proximal region (B’, arrow). The structure terminates shortly after the H-structure, resulting in the bld phenotype. C) Centriole from mutant 8471. A transition zone duplication appears off of the distal end (C’, arrows) and flagellar assembly is clearly absent. The centriole connecting fibers are also perturbed, appearing buckled (C, arrow).
CHAPTER 6

Summary and Perspectives
The mother centriole controls cell geometry

In summary, I hope that I have revealed some insight into cell architecture in the green alga *Chlamydomonas reinhardtii*. Specifically, the mother centriole acts as a spatial organizer, transmitting positional information to several subcellular structures, including the daughter centriole and the nucleus. The mother may also play a role in spindle orientation. Centrioles at the poles correlate to proper orientation of the spindle. These data combined with the fact that there is differential attachment of centrioles to the spindle poles in *asq2/vfl* mutants suggest that the mother centriole may be required for proper positioning of the spindle. As centrioles lacking distal ends are improperly positioned, it is clear that the mother centriole exerts her authority over cell geometry through structures on the distal end.

The role of VFL/ASQ genes

By revealing the identity of the gene encoded by ASQ2, I have begun to identify the molecules involved in centriole positioning in *Chlamydomonas*. Furthermore, as I have shown that all of the *vfl* mutants have defects in centriole positioning, the gene products encoded by the VFL genes can also be added to this list. However, although *asq2* and the *vfl* mutants have defects in centriole positioning, these gene products may not be directly involved in positioning the centrioles. The disconnection of centrioles in these mutants revealed that differently aged centrioles have a differential potential to interpret or establish positioning cues. The mispositioning in most of these mutants many therefore
only be a result of disconnection rather than positioning per se. However, we must consider why the centrioles are disconnected in these mutants. If lone daughter centrioles arise from the abolition of centriole connecting fibers or through *de novo* assembly as was discussed in Chapter 2 and 4, then the daughters are only mispositioned because they are immature. The positioning defects in these mutants should instead be considered centriole cohesion defects.

Another alternative is that the lone daughters arise from cohesion defects due to problems in rotational orientation of the centrioles. Vfl1p has been shown to localize asymmetrically on the centrioles (Silflow et al., 2001). Thus, cohesion defects in the *vfl1* mutant have been thought to arise via problems with rotational orientation of the mother during duplication. While I do not believe these defects to be the cause of lone centrioles in *vfl2* or *asq2*, I do have some data to suggest that the *vfl1* phenotype might be different with regards to centriole positioning. While I showed that the *uni1* mutation rescues the centriole positioning defect in *asq2* and *vfl2* mutants (Chapter 2), this does not seem to be the case for *vfl1*. Interflagellar distance in *vfl1uni1* cells appears similar to that of *vfl1* (data not shown), indicating that mother centrioles in this mutant can be found all over the cell. This experiment needs to be repeated and *asq* measurements need to be taken to confirm these results. These data can be explained by the fact that Vfl1p might actually be one of the molecules directing positioning of the mother centrioles. Thus, cells have a *vfl* phenotype because of problems in positioning during duplication, not because of the type of cohesion defects seen in *vfl2* or
asq2. These data are exciting as they indicate that Vfl1p might be the first direct centriole positioning molecule revealed in *Chlamydomonas*. As Vfl1p is a conserved molecule, it may also control centriole positioning in higher organisms. The role of Vfl1p in centriole positioning and its subsequent importance in development should be tested in the future.

*Centriole positioning in vertebrates*

While it is interesting to understand the mechanism of centriole positioning in *Chlamydomonas* cells, it is perhaps even more intriguing to understand the general mechanism of centriole positioning and its impact and importance in patterning an organism. Cilia have been found on a large number of tissues in vertebrate embryos, including the node, kidney, and heart. In these locations, cilia seem to be specifically poised to carry out their function. This is especially true in the node, where the orientation of cilia has already been shown to be crucial for establishing left-right (L-R) asymmetry. The mouse node has a functional equivalent in many different taxa (Essner et al., 2002). In zebrafish, this analogous structure is called Kupffer’s Vesicle (KV). Because of the ease of reverse genetics in zebrafish, I originally proposed to use this system to test whether, 1) the molecules responsible for centriole positioning in *Chlamydomonas* play similar roles in zebrafish, 2) whether specific centriole positioning is important in KV in generating left-right asymmetry, and 3) whether centriole positioning in other organs, such as heart and kidney, is critical for their proper patterning and function.
To investigate the role of candidate genes in centriole positioning in zebrafish, morpholine modified antisense oligonucleotides, morpholinos (MOs), were designed against potential molecules involved in centriole positioning. These genes include the VFL genes and planar cell polarity genes. Although time did not allow the successful completion of these experiments, they should still be carried out in the future. Specifically, zebrafish embryos will be injected at the one-cell stage with MOs designed against candidate genes. Embryos will be fixed at 12, 24, and 48 hours post-fertilization. Proper L-R development will be determined using immunofluorescence and in situ hybridization to analyze asymmetric landmarks in the embryos. These include a properly looped heart, the correct side expression of nodal genes, and the correct placement of the pineal gland in the brain. In addition, form and function of other ciliated structures, including heart, kidney, and limb will be assessed.

The results from these experiments will be twofold. First, one will be able to determine the role of novel players in the centriole-positioning pathway in a vertebrate system. Additionally, one will be able to determine the impact of centriole positioning on development, specifically in establishing left-right asymmetry. It is possible that observed phenotypes could be non-specific to centriole misplacement and instead reflective of a global knock down of an important gene product. One benefit of the zebrafish system is that it is possible to specifically knock down genes in KV (Essner et al., 2005). This technique has already been demonstrated to be highly effective for knocking down ciliary genes.
in KV (Bisgrove et al., 2005) and will be essential in testing the specificity of observed phenotypes.

A sensitized screen for centriole mutants

Although many screens have been conducted in Chlamydomonas, only a handful of bona fide centriole mutants have been identified to date. Screens for centriole mutants in Chlamydomonas have relied on screening for the presence or absence of flagella. While this approach is effective in identifying scores of new mutants, these mutants actually have defects in flagellar rather than centriole assembly. I therefore tried to develop new screens for identifying centriole mutants.

One screen that is potentially very promising relies on the observation that true centriole assembly mutants, such as bld2 and bld10, will fail to dock onto the membrane (Feldman et al., 2007). The contractile vacuoles in Chlamydomonas appear at sites of centriole assembly. In bld2 and bld10 cells, contractile vacuoles appear near centrioles even when centrioles are not at the surface (Feldman et al., 2007). Thus, cells with defects in centriole docking will also potentially have defects in endocytosis at the vacuoles. A screen for endocytosis might therefore uncover new mutants in centriole assembly or position. I have tested this hypothesis by using the dye FM 4-64. Wild-type cells take up the dye and, after about 10 minutes, the internal membrane systems are stained. In contrast, bld2 and bld10 cells do not efficiently take up this dye and therefore have stain only at the cell surface. Therefore, using FM 4-64 and flow cytometry
to separate cells based on fluorescence intensity, one could efficiently and effectively screen for centriole mutants.

*Cell architecture: the future is now*

Hopefully, the data present here have revealed the importance of centriole positioning and of general control of cell architecture. To date, a number of interesting experiments have been done in ciliates to explore how patterning might occur at the single cell level (reviewed in (Frankel, 1991)). Many fewer studies exist to date that explore the question of how cell architecture is controlled in multicellular organisms and how cellular structure might contribute to function in the organism. I am excited to explore these questions in the nematode *C. elegans* in the future. *Alto Cinco!*


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The development of codon-optimized fluorescent proteins for *Chlamydomonas*
The emergence of green fluorescent protein (GFP) as a usable technology in *Chlamydomonas* required the synthesis of codon optimized GFP (Fuhrmann et al., 1999). *Chlamydomonas* DNA is highly GC rich (>60%) and therefore, standard GFP could not be expressed. In order to advance the *Chlamydomonas* toolbox, I made codon-optimized cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). To do this, I used the Quick Change mutagenesis kit (Stratagene) to introduce the necessary mutations to change the spectral character of GFP to those of other color variants. In each substitution, I used the most favored codon for *Chlamydomonas* codon bias that required the least mutations to be introduced.

For YFP, I introduced 4 mutations. These mutations are T65G, V68L, S72A, and I203Y. The I203Y mutation is in the aromatic side chain at position 203 and causes the red shift responsible for the yellow color (Pines, 1999). The V68L mutation introduces a unique *Pst*1 site that can be used for rapid identification of this mutation. For CFP, I also introduced four mutations, including Y66W, N146I, M153T, and V163A. The Y66W mutation is the most critical as the cyclization and oxidation of residues 56-67 forms the chromopore. The Y66W mutation is primarily responsible for the cyan emission (Pines, 1999). The M153T mutation introduces a unique *Eag*1 mutation that can be used for mutation verification. These mutations were entered into a plasmid containing codon optimized *Chlamydomonas* GFP, which was removed from a centrin-GFP construct (Ruiz-Binder et al., 2002) and cloned into pBluescript KS+. The start codon in this GFP has been mutated to include a linker. The GFP mutants can
be added to a gene of interest with a convenient \textit{BamHI} site in the linker region. Alternatively, the CFP or YFP can be added to constructs that are already tagged with GFP by swapping the mutated regions for the non-mutated GFP regions.

Using this strategy, I constructed a fusion of CFP or YFP to the BLE gene which confers resistance to the drug zeocine and encodes a nuclearly localized protein (bleCFP or bleYFP). These constructs were transformed into wild-type cells (cc-125) using resistance to zeocine to select for cells that had taken up the construct. BLE expressing cell were retained and observed live. Cells appear to express proteins with the proper spectral properties. BleCFP expressing cells had nuclear localization of a protein that emitted fluorescence that was visible with a CFP filter (470 nm). This fluorescence was not detectable using the GFP or YFP filters (Figure 1). BleYFP expressing cells also had nuclear localized fluorescence that was visible using the YFP filters (535 nm). However, the fluorescence was also visible using the GFP filter (528 nm) and GFP fluorescence was visible using the YFP filter. Specialized filters are necessary to distinguish GFP from YFP.

It is clear that the CFP mutations have changed the spectral characteristics of the original GFP. It is not obvious that the YFP mutations have significantly changed the fluorescence properties of GFP, but the introduced mutations do not abolish fluorescence. The CFP fluorescence is incredibly dim and the signal is rapidly silenced over time.
Figure 1. Expression of GFP mutants in *Chlamydomonas*

All fluorophores were fused to the BLE gene, allowing for nuclear localization. GFP mutants transforming it to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) were visualized using three different filters (470, 528, and 535 nm). CFP fluorescence was visible using the 470 nm filter, but not the 528 or 535 nm filters. YFP and GFP were both visible with the 528 and 535 nm filters.
APPENDIX B

Testing potential cilia drugs in zebrafish
A screen for drugs that disrupt ciliary structure (Wallace Marshall, unpublished data) identified 9 potential compounds. This screen was conducted in *Chlamydomonas* and tested the effect of each compound on swimming behavior. To test the relevance and efficacy of these compounds in higher organisms, I tested their effect on developing zebrafish embryos. Embryos were generated by intercrossing TL x Ab wild-type fish. Embryos were collected close to fertilization and treated with the compounds at 128-cell stage or at tail bud stage. Embryos were exposed to each compound diluted 1/100 in 1% DMSO dissolved in E3 embryo media. The effect of the compounds was surveyed every hour during the course of the first day following fertilization and again on the second day. The results are shown in figures 1 and 2.
Figure 1. Live imaging of zebrafish embryos following drug treatment at 128-cell stage

Wild-type zebrafish embryos were collected at fertilization and treated with each compound diluted 1/100 dissolved in embryo media supplemented with 1% DMSO at the 128-cell stage. Each row indicates a different treatment, while each column is a progressive time point of the same collection of embryos over time.
Figure 2. Live imaging of zebrafish embryos after drug treatment at tail-bud stage

Wild-type zebrafish embryos were collected at fertilization and treated with each compound diluted 1/100 dissolved in embryo media supplemented with 1% DMSO at the tail-bud stage. Each row indicates a different treatment, while each column is a progressive time point of the same collection of embryos over time.
APPENDIX C

Cilia biology in the ciliate \textit{Stentor}
*Stentor coeruleus* is a single-celled ciliate reaching up to several millimeters in size. *Stentor* has thousands of cilia lining the cell surface (Figure 1-3). *Stentor* also has a large oral apparatus, used for feeding (Figure 1 and 2). The oral apparatus is also lined with thousands of cilia. The oral apparatus is easily detached from the main cell body by treatment with 15% sucrose (Maloney, 1986). As many of the genes involved in centriole and ciliary assembly are still a mystery, *Stentor* could serve as an excellent system with which to identify new genes involved in these processes. Following oral apparatus severing, cells must re-synthesis all new basal bodies and then re-grow new cilia. This whole process takes about 8-10 hours (Tartar, 1961). Basal bodies begin to assemble at approximately 1-2 hours post-severing, while cilia reassemble 3-4 hours following oral shedding (Tartar, 1961). By collecting cells at different time points following oral severing and comparing them to untreated cells, one could identify all the unregulated genes involved in assembly of centrioles or cilia.

Currently, there is almost no genomic information for Stentor. As a first step to identifying new genes involved in centriole and cilia assembly, we must first try and obtain genomic or EST sequence for Stentor. To do this I have collected samples of untreated *Stentor* cells, as well as of regenerating *Stentor* cells and extracted RNA and synthesized cDNA. I have been able to amplify sequence for \( \alpha \)-tubulin (F1: AGACGACGCTTCAACACTT, R1: CTGTGCAGTTGTCTGCCAAT, F2: AGAAGACGCTGCCAACAAC, R2: TCCTTAGGGACGACATCC, or F3: ACCTTGCTCCCTACCCTCGT: R3:...
CACCGACATACCAGTGAACG), suggesting that these samples may be sufficient for further sequence analysis. Below I have listed the protocols that I have generated for Stentor to date.

**Stentor handling**

Stentor cells were obtained from Carolina Biological. Stentor cells were grown in buffer made in filtered spring water following the recipe of Noel de Terra. Before experiments, Stentor cells were removed from the jars that they were shipped in and washed away from the cotton matrix. Cells were removed to autoclaved glass Petri dishes. Following isolation, cells were washed through at least five washes of buffer.

**Immunofluorescence in Stentor**

Cells were transferred into microcentrifuge tubes. All steps of this protocol were done in tubes. Several fixation protocols were tried for Stentor cells, including 4% paraformaldehyde followed by methanol, iodine followed by methanol, and methanol alone. The paraformaldehyde fixation was incredibly harsh and resulted in cell disintegration over time. The iodine and methanol alone fixations worked equally well. Methanol treatment at -20º C can be performed for at least one hour. However, overnight exposure resulted in some deterioration of cell integrity. Following fixation, cells were rehydrated stepwise into PBS. Cells were then blocked in 2% BSA. Cells were incubated with antibodies against acetylated α-tubulin or α-tubulin diluted 1:200 in 20% block,
followed by incubation with the appropriate secondary antibodies also diluted in 20% block. Cells were mounted by outlining a coverslip-sized region of a slide with a hydrophobic pen. Cells were pipetted in liquid into this region. The liquid was removed and replaced with Vectashield mounting media. A coverslip was placed over the cells and sealed with nail polish.

*Oral Apparatus Removal*

Cells were treated with 15% sucrose for 3 minutes for removal of the oral apparatus and then washed through at least five washes of buffer. Cells were transferred to a microcentrifuge tube and the excess liquid was removed. Cells were taken immediately after treatment and 1 and 2 hours after treatment. Untreated cells were also collected. About 100 cells were collected per time point.

*RNA Extraction from Stentor*

For RNA extraction, cells were washed through several washes of buffer. Cells were homogenized with a hand-help homogenizer for one minute. One mL of Trizol was added to each tube and cells were frozen at -80°C. RNA was extracted per the manufacturers specifications. Residual genomic DNA was digested with RNase-free DNase. This RNA was used as a template for cDNA production. cDNA synthesis was performed according to the manufacturer’s specifications using the Superscript III Synthesis Kit (Invitrogen).
Figure 1. *Stentor* cells have complex cell architecture

*Stentor* cells were labeled with DAPI (blue) and antibodies against $\alpha$-tubulin (red) and acetylated $\alpha$-tubulin (green). The cell is lined with rows of cilia and one end of the cell has a large heart-shaped oral apparatus. This structure continues inward to form the gullet.
Figure 2. *Stentor* cells have an oral apparatus

*Stentor* cells were labeled with DAPI (blue) and antibodies against $\alpha$-tubulin (red) and acetylated $\alpha$-tubulin (green).  A) DIC image of a *Stentor* cell with its oral apparatus displaced from the cell body.  B) The oral apparatus has bundles of thousands of cilia extending from its surface.  C) Magnification of oral apparatus cilia structures.
Figure 3. *Stentor* cells are lined with rows of cilia

*Stentor* cells were labeled with antibodies against $\alpha$-tubulin (red) and acetylated $\alpha$-tubulin (green).  A) Basal portion of a *Stentor* cell. The normally extended tail has been pulled inside following detachment from a solid surface.  B) Magnification of cell surface of a *Stentor* cell. Cilia are found emanating from rows on the cell surface.
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