Published in final edited form as:

Cytoskeleton (Hoboken). 2010 August ; 67(8): 504–518. doi:10.1002/cm.20462.

Influence of centriole number on mitotic spindle length and symmetry

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Abstract

The functional role of centrioles or basal bodies in mitotic spindle assembly and function is currently unclear. Although supernumerary centrioles have been associated with multipolar spindles in cancer cells, suggesting centriole number might dictate spindle polarity, bipolar spindles are able to assemble in the complete absence of centrioles, suggesting a level of centriole-independence in the spindle assembly pathway. In this report we perturb centriole number using mutations in *Chlamydomonas reinhardtii*, and measure the response of the mitotic spindle to these perturbations in centriole number. Although altered centriole number increased the frequency of monopolar and multipolar spindles, the majority of spindles remained bipolar regardless of the centriole number. But even when spindles were bipolar, abnormal centriole numbers led to asymmetries in tubulin distribution, half-spindle length and spindle pole focus. Half spindle length correlated directly with number of centrioles at a pole, such that an imbalance in centriole number between the two poles of a bipolar spindle correlated with increased asymmetry between half spindle lengths. These results are consistent with centrioles playing an active role in regulating mitotic spindle length. Mutants with centriole number alteration also show increased cytokinesis defects, but these do not correlate with centriole number in the dividing cell and may therefore reflect downstream consequences of defects in preceding cell divisions.

Keywords

basal body; centrosome; cytokinesis; vfl1; vfl3; *Chlamydomonas*

INTRODUCTION

The fact that the number of centrioles in interphase (two per cell) matches the number of spindle poles in mitosis suggests that centriole number determines the number of spindle poles. Consistent with this idea, supernumerary centrioles and multipolar spindles tend to co-occur in tumor cells [Boveri, 1929; Pihan et al., 1998; Lingle and Salisbury, 1999; Duensing et al., 2000; Kramer et al., 2002; Nigg, 2006; Sankaran and Parvin, 2006; Basto et al., 2008]. But if centriole number dictates spindle pole number, cells with supernumerary centrioles would fail in mitosis and thus could not contribute to tumor growth [Sluder and Nordberg, 2004]. In some cases extraneous centrioles are seen to coalesce together into two clusters, one at each spindle pole [Ring et al., 1982; Brinkley, 2001; Quintyne et al., 2005; Basto et al., 2008], implying that the effective number of microtubule organizing centers may not be directly proportional to the number of actual centriole pairs. In addition, cells have mechanisms to prevent re-duplication of centrioles [Wong and Stearns, 2003; Tsou and Stearns, 2006] as well as active error correction mechanisms to restore number following perturbation [Marshall 2007], all of which appear designed to enforce a centriole copy

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number of two per cell. This strong preference for two centrioles seems to underscore the notion that cells seek to match the number of centrioles to the number of spindle poles.

On the other hand, bipolar spindles can form without any centrioles [Debec et al., 1995; Heald, 1996; de Saint Phalle and Sullivan, 1998; Hyman and Karsenti, 1998; Compton, 2000; Khodjakov et al., 2000; Basto et al., 2006] and the bipolar spindle is now understood to be a self-organizing structure [Nedelec et al., 2003; Burbank et al., 2007; Clausen and Ribbeck, 2007]. Thus the presence of exactly two centrioles per cell is not strictly required for forming a bipolar spindle, and indeed it has been speculated that centrioles evolved primarily for the production of cilia rather than spindles and hence may best be viewed as epiphenomena of mitosis. But this dispensability of centrioles for spindle assembly leaves open the possibility that centrioles, when present, may provide biasing signals to guide the position and orientation at which spindles will form. What would happen if the number of centriole pairs does not match the number of spindle poles? The results of such numerical mismatch could provide an indicator of the influence that centrioles exert over spindle assembly.

The unicellular green alga *Chlamydomonas reinhardtii* is a powerful genetic model system for analysis of centriole duplication [reviewed by Marshall and Rosenbaum, 2000]. A number of mutants have been identified in this organism in which centriole number is variable from cell to cell, ranging between zero and six, resulting in cells with variable numbers of flagella per cell. This variable flagellar number (*vfl*) phenotype results from defects in centriole segregation. All known *vfl* mutants are viable, confirming the idea that spindle assembly is robust enough to continue in the face of centriole numerical variation. One such mutant is *vfl2*, caused by a point mutation in the gene encoding the EF-hand protein centrin [Taillon et al., 1992]. In this mutant, centrioles can become detached from the spindle poles [Kuchka and Jarvik 1982; Koblenz et al., 2003] such that during mitosis and cell division, the centrioles are randomly partitioned to the two daughter cells. As a result, cells inherit a random number of centrioles, between zero and six. This random cell to cell variation in centriole number provides the basis for our tests, described below, that seek to correlate mitotic defects with centriole number. We have previously used a genetic assay for chromosome loss to show that mitotic chromosome loss rate is 100-fold higher in *vfl2* mutant cells than in wild-type, but is still low enough to allow high viability [Zamora and Marshall, 2005]. Three other mutants with similar cell-to-cell variation in centriole number, *vfl1*, *vfl3*, and *asq2*, have been described [Wright et al., 1983; Adams et al., 1985; Silflow et al., 2001; Feldman and Marshall 2009], all of which encode proteins localized in or around centrioles. All of these mutants are viable even though more than half the cells in each mutant have an incorrect number of centrioles. Clearly bipolar spindles can still form in the face of abnormal centriole number, but are these spindles structurally normal?

In this report we test the effect of centriole number abnormality on the structure of mitotic spindles using *Chlamydomonas* mutants. These mutants allow the centriole number effects to be examined in a genetically well-defined system; as opposed to tumor derived cell lines which can also have abnormal centriole numbers, but in which interpretation of centriole-specific effects is complicated by the myriad of other defects such as aneuploidy or cell-cycle misregulation. In contrast, we employ only mutants in genes encoding centriole-localized proteins to minimize side effects. These mutants affect either centriole number or centriole structure, which mimic the two predominant types of centriole abnormalities in tumor cells [Nigg, 2006]. The ability to analyze large numbers of cells in a single experiment makes this approach more suitable for detecting rare events than micromanipulation or ablation based approaches that must be done one cell at a time. A disadvantage of a genetic approach is the possibility of pleiotropic effects of mutants, however we were able to correlate defects with centriole number on a cell by cell basis to

distinguish centriole-related defects from centriole-independent side effects of the mutations. Within each mutant, a subset of cells have the normal wild-type number of centrioles (2 in interphase, 4 at metaphase) and these serve as an internal control against centriole number-independent effects of the mutations. Our results indicate that spindle bipolarity is highly robust and only minimally perturbed by variations in centriole number, but that alterations in centriole number can lead to bipolar spindles whose organization is unbalanced and asymmetric, which may have substantial consequences for genomic instability.

MATERIALS & METHODS

Cell Culture

This study employed the following *Chlamydomonas reinhardtii* strains: wild-type strain cc-124, flagella-less strain *bld1* (cc-2506), basal body-deficient strains *bld2* (cc-478), *uni3* (cc-2508), and *bld10* (cc-4076), variable centriole number strains *vfl1* (cc-1388), *vfl2* (cc-2530) and *vfl3* (cc-1686), uniflagellar mutant strain *uni1* (cc-1926), ts flagellar assembly mutant *fla10* (cc-1919), and ts *vfl2* strain *vfl2*-R15. All strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). For normal growth, cells were grown and maintained in Tris-Acetate-Phosphate media [Harris, 1989]. Growth was at 25°C with continuous aeration and constant light. For cell synchronization, cells were grown in M1 (Sager and Granick Medium I) medium in a 14:10h light/dark cycle and were analyzed after two days near the end of the light cycle.

Immunofluorescence and imaging of mitotic spindles

Cells were allowed to adhere to polylysine-coated coverslips prior to fixation in methanol at −20°C for five minutes. Coverslips were then transferred to a solution of 50% methanol/50% TAP for an additional five minutes. After fixation, cells were blocked in 5% BSA, 1% fish gelatin and 10% normal goat serum in PBS. Cells were then incubated in primary antibodies overnight: anti-centrin (a generous gift from J. Salisbury) 1:100, anti-acetylated-tubulin (T6793, Sigma) 1:500, anti-alpha-tubulin FITC conjugated (F2168, Sigma) 1:100, anti-phospho-histone H3 (06-570, Upstate) 1:500, anti-Bld10p (a generous gift from M. Hirono) 1:100, and anti-POC1 1:200 [Keller et al., 2009]. Coverslips were then washed six times in PBS before staining with secondary antibodies from Jackson ImmunoResearch at a dilution of 1:1000. Cells were then incubated with DAPI (1 µg/ml in water) and mounted in Vectashield mounting media. The anti-alpha tubulin FITC conjugated antibody was added only after the other antibodies were stained with both primary and secondary antibodies to prevent cross-reactivity.

Antibodies recognizing centrin [Salisbury et al., 1988], acetylated-tubulin [LeDizet and Piperno, 1986], Bld10p [Hiraki et al., 2007] and POC1 [Keller et al., 2009] were all used for the identification and counting of centrioles in images. Cells were imaged using a Deltavision deconvolution system with a 100× N.A. 1.4 oil immersion lens and a 1.5× optivar, with Z-sections taken every 0.2 µm.

Quantitative image analysis

A 3D stack through each cell was generated and used for all spindle analyses. Carefully stepping through each Z-section, we counted the number of centrioles per spindle pole (based on centrin, acetylated-tubulin, Bld10p, and/or POC1 staining) and determined whether spindles were monopolar, bipolar, multipolar or unorganized (based on alpha-tubulin staining in conjunction with centriole staining). Using Softworx software, spindle lengths were measured using the centers of alpha-tubulin staining intensity at both poles. Distances from centrioles to nearest spindle pole were measured using the center of centriole

staining intensity and the center of polar alpha-tubulin staining intensity. Half spindle length was measured from the center of tubulin staining at the pole to the center of mass of the chromatin. Shape factor and chromatin area were measured using a custom MATLAB program that thresholds the DNA staining and automatically detects the edge of the chromatin region and uses the shape of the outline to calculate the area and perimeter. Quantification of tubulin content was performed by calculating projection images, masking out each half spindle using the Edit Polygon function of Softworx, and then measuring the average intensity and total area of each polygon using the statistics table feature of Edit Polygon.

Cytokinesis in individual cell divisions

To determine frequency of cytokinesis defects in live *vfl* cells, cells were embedded in 0.4% agarose in R/2 media [Harris, 1989] and mounted under coverslips sealed with Vaseline, as previously described [Marshall et al., 2001], and imaged with a Zeiss Axioskop with DIC optics using 20× air objective lens. Cells that were well separated from neighboring cells were selected and their position noted on the stage micrometer. The number of flagella was then scored and recorded. Slides were then transferred to a humid chamber and re-checked periodically for 36 hours. Cytokinesis defects were scored based on visual analysis of cell morphology after division.

Cell Death Analysis

A 0.25% solution of Erythrosine B (E9259, Sigma) in TAP was made and mixed 1:1 with *Chlamydomonas* suspension. Dead cells stain bright pink by light microscopy. Approximately 500 cells were counted during each cell death experiment. Averages were pooled from at least three separate experiments. We have obtained equivalent cell death results by staining *vfl* mutants with phenosafranin, an alternative stain that is also excluded from live cells (data not shown). For measuring cell death rates in living cells, agarose embedded cells were observed and the appearance of vacuolated cell morphology combined with failure to undergo subsequent divisions was used as an indicator of death.

RESULTS

Mitotic spindle aberrations in *vfl* mutants

If centrioles play a role in organizing the mitotic spindle, then mutants with abnormal centriole number should show defects in spindle organization. Centriole number can in principle be altered by physical manipulation such as microdissection, cell fusion, and laser ablation, but such approaches run the risk of unintended side-effects and are hard to apply to large numbers of cells. We therefore turned to a genetic approach using the unicellular green alga *Chlamydomonas reinhardtii*. A class of *Chlamydomonas* mutants has been described in which centriole ultrastructure is abnormal. These include *bld2*, in which centrioles are flat discs of singlet microtubules [Goodenough and St. Clair, 1975; Dutcher et al., 2001]; *uni3* in which centrioles have doublet rather than triplet microtubules [Dutcher et al., 1998]; and *bld10* in which centrioles are largely absent [Matsuura et al., 2004]. Analysis of spindle organization in such mutants allows us to test whether centriole structure affects mitosis. A second class of *Chlamydomonas* centriole-related mutants consists of the VFL mutants *vfl1*, *vfl2*, and *vfl3*, in which centriole number is variable from cell to cell [Adams et al., 1985; Wright et al., 1989; Kuchka and Jarvik, 1982]. In these mutants, centriole ultrastructure is apparently normal, but some cells lack centrioles, or have just one, others have supernumerary centrioles, while others have the normal wild-type number and serve as an internal control for centriole-independent effects of the mutations. These mutants allow us to explore the effect of centriole number on spindle morphology.

Using these two classes of mutants, we examined the three-dimensional organization of mitotic spindles by fluorescence microscopy. Mitotic cells were identified during initial scanning using the anti-phospho-histone antibody staining. Among the mitotic cells, metaphase cells were identified according to the description by Doonan and Grief [1987] who defined metaphase as the stage in which spindle poles are well separated, chromosomes are condensed, and microtubules of the spindle run all the way through the nucleus. A similar convention is followed by Ehler et al. [1995] who diagram prophase as a stage with condensed chromosomes but microtubules not fully extending through the nucleus. All of our analyses were conducted using spindles at this stage. We found that centriole mutants in *Chlamydomonas* displayed a range of abnormal spindle phenotypes, as illustrated in Figure 1. Defects in spindle morphology included monopolar spindles, multipolar spindles, split or multiradial spindles, and asymmetric bipolar spindles with unequal pole focus. These defects occur rarely, if ever, in wild-type cells, but were seen in all mutants examined. Similar defects also occur in *asq2* mutant cells, which have defects in mother-daughter centriole cohesion [Feldman and Marshall, 2009]. We frequently observed centrioles in these mutant cells which were not associated with any spindle pole. These detached centrioles, defined as those further than 1 μm from a pole, still appeared able to induce microtubule organizing centers (MTOCs) as judged by the appearance of small asters around each detached centriole during metaphase. We have previously observed that centrioles unassociated with spindles are able to form MTOCs (Feldman and Marshall, 2009). We never observed clustering of extraneous centrioles such as has been reported in some mammalian cell lines [Quintyne et al., 2005].

Comparing different types of mutants, we find that a similar spectrum of defects was seen in mutants with abnormal centriole ultrastructure and in mutants with abnormal centriole number distributions (Figure 2). The frequency of monopolar spindles was significantly increased for all mutants when compared with wild type ($P < 0.05$ by Fisher's exact test corrected for multiple comparison). In contrast, the frequency of multipolar spindles was not statistically different from wild-type in any mutant except for *vfl1*, in which the difference was significant ($P < 0.02$ by Fisher's exact test). This result suggests that formation of multipolar spindles is a relatively rare event in these mutants, whereas formation of monopolar spindles is far more common. Overall, however, the majority of cells were still able to form bipolar spindles in all of the mutants examined (Figure 2A), suggesting that the robustness of bipolar spindle self-assembly can generally overcome the influence of centriole number variation.

Even when bipolar spindles formed, in many cases these showed clear abnormalities in appearance (Figure 1B). As shown in Figure 2B, the frequency of spindles with unequal distributions of tubulin in the two half spindles (as judged by eye - see below for quantification of asymmetry) was significantly increased in the set of mutants ($P < 0.05$ for *vfl1*, *vfl3*, and *bld2* individually, $P < 0.007$ for the group of mutants collectively), as was the frequency of spindles with unequal pole focus ($P < 0.05$ for all mutants individually, $P < 0.0006$ for the whole set of mutants collectively), and the frequency of detachment of centrioles from the poles ($P < 0.05$ for all mutants individually, $P < 0.0001$ for the set of mutants collectively). The high frequency of detachment suggests the effect of supernumerary centrioles may be to some extent buffered by the inability of all centrioles to become associated with spindle poles.

Defects in spindle morphology may lead to defects in chromosome segregation, but because we rely on imaging of fixed cells to score spindle morphology, we cannot follow the subsequent chromosome segregation mediated by such spindles. However, as another function of the mitotic spindle is to mediate congression of chromosomes to the metaphase plate [Khodjakov et al., 1999], we asked whether the defective spindles that form in

centriole mutants might also be defective in congression. We scored the compactness of the metaphase plate by measuring the area of the phospho-histone specific staining in two-dimensional image planes in which both poles are in focus, with results graphed in Figure 3A. By this measure, several centriole mutants show a small but statistically significant increase in chromatin area ($P < 0.0001$ among the group by ANOVA, $P < 0.05$ for *vfl1*, *vfl3*, *bld2*, and *bld10* considered individually by Holm-Sidak test). These three mutants that showed alteration in chromatin area also showed inequality in tubulin between the half spindles (Figure 2). We note that the area analyzed here is not the area of individual chromosomes, but the area subtended by the entire metaphase plate. In addition to area, a "shape factor" was calculated by taking the ratio of the circumference of the entire chromatin mass to the square root of the area (Figure 3B). This is a unit-less quantity that takes its minimum value if the metaphase plate region has a circular cross-section, and has a larger value if the chromosome-containing region becomes more elongated. The alteration in chromatin mass shape factor was significantly different from wild type in *vfl1*, *vfl2*, *bld2*, and *bld10* mutants ($P < 0.05$ in all cases individually by Holm-Sidak test and $P < 0.0001$ among the entire group of mutants considered versus wild type by ANOVA), which we interpret as an average elongation of the metaphase chromosome cluster along the axis of the spindle, as though the chromosomes had not fully congressed to the center. These defects in metaphase plate size and shape may suggest that the alterations in spindle morphology seen in these mutants result in subtle defects in chromosome congression, although our data cannot rule out an effect on chromatin compaction.

Spindle polarity versus centriole number

The results of the previous section indicate that *vfl* mutants in *Chlamydomonas*, which have variable numbers of centrioles per cell, can form both monopolar and multipolar spindles. These defects might reflect a direct role for centrioles in spindle assembly, but two alternative explanations exist - the defects might reflect aberrations in cellular organization caused in earlier divisions, or the defects might reflect additional, centriole-independent functions of the *VFL* genes. To distinguish these possibilities, we scored the frequency of monopolar, bipolar, and multipolar spindles as a function of centriole number (Figure 4). For this plot we grouped all three *vfl* mutants, *vfl1*, *vfl2*, and *vfl3*, together on the grounds that they have indistinguishable distributions of centriole number. This result shows that cells with fewer than four centrioles at the time of mitosis are more likely to form monopolar spindles, while cells with more than four centrioles are more likely to form multipolar spindles. However, in some cases cells with less than four centrioles still formed multipolar spindles, and cells with more than four centrioles still formed monopolar spindles.

The frequency of bipolar spindles was maximum for cells containing exactly four centrioles. The fact that polarity correlated with number of centrioles is not consistent with a centriole-independent side effect of the *vfl* mutations, because the cells analyzed shared the same pool of genetic defects and differed only in centriole number.

Spindle geometry versus local centriole number at the pole

If centrioles exert a direct influence on spindle assembly, then we might expect to see a correlation between the centriole number at a given pole and the morphology of the half-spindle containing that pole. Because the primary proposed function for centrioles at the poles is to recruit a functional MTOC and thus contribute microtubules for spindle assembly, we specifically tested whether increased centriole number would correlate with either the width of the pole or the length of the half-spindle. If centrioles help recruit microtubule nucleating activity at the poles, one would expect that more centrioles would lead to more microtubules and thus a wider or thicker half-spindle. On the other hand, if

centrosomes play a key role in pulling spindle microtubules into a tight focus [Silk et al., 2009], then more centrioles at a pole might lead to a narrower pole. In order to explore these possibilities, we describe half-spindle morphology by two parameters, the width of the pole and the length of the half-spindle, as illustrated in Figure 5A, and ask if either parameter varies as a function of centriole number associated with each pole. We define the pole-associated centriole number as the number of centrioles that are within one micron of the nearest pole.

A clear trend was seen relating centriole number at a pole to the width of the pole (Figure 5B), such that when the number of centrioles at a pole exceeded 2, the width of the poles became significantly increased (average width $0.53 \mu\text{m} \pm 0.03 \mu\text{m}$ SEM versus $0.75 \mu\text{m} \pm 0.09 \mu\text{m}$ SEM, $P < 0.02$). For numbers between 0 and 2, there was no difference in the average width, indicating that the presence of too few centrioles at a pole, or even none at all, has little or no effect on pole focusing. Overall the correlation coefficient between the number of centrioles at a pole and the width of the pole was 0.92. The ability of poles to focus in the absence of centrioles, even in a cell type normally containing centrioles at the poles, is consistent with previous studies (Manning and Compton, 2007).

A clear trend was also seen relating the centriole number at a pole to the length of the corresponding half-spindle (Figure 5C). When examining this relation, we found no variation in the average spindle half length for centriole numbers between 1 and 3, but found that poles lacking centrioles had a somewhat increased length for the corresponding half spindle (average half length $2.16 \mu\text{m} \pm 0.09 \mu\text{m}$ SEM for zero centrioles versus $1.99 \mu\text{m} \pm 0.04 \mu\text{m}$ SEM for one to three, $P < 0.05$), while poles with 4 or more centrioles had a decreased average half-spindle length (average half length $1.71 \mu\text{m} \pm 0.15 \mu\text{m}$ SEM for four or more centrioles versus $1.99 \mu\text{m} \pm 0.04 \mu\text{m}$ SEM for one to three, $P < 0.07$). The difference in half length corresponding to poles with too few centrioles and poles with too many centrioles was highly significant ($P < 0.02$ for zero centrioles versus four or more centrioles). Overall the correlation coefficient was -0.92 for spindle half length versus the number of centrioles at a pole. In contrast to these clear trends relating centriole number to half-spindle length and pole width, we found no evidence for a correlation between total tubulin content of each half spindle, as judged by background-corrected integrated tubulin immunofluorescence intensity, and the number of centrioles at the pole (Figure 5CD), for which we calculated a correlation coefficient of 0.18. This negative result does not lead to a strong conclusion because quantification of protein content based on immunofluorescence is problematic, due to the inherent nonlinearity of antibody-epitope interactions as well as high variability in fixation and antibody penetration between samples. It is therefore quite possible that this high variability might mask an actual trend, if present.

The results of Figure 5B and 5C suggest a link between centriole number at a pole and the local geometric properties of the spindle at that pole, but they ignore interactions between the two half spindles. We therefore asked whether inequality in the number of centrioles at the two poles of a spindle might lead to increased asymmetry between the two halves of the spindle. To do so, we plotted the average ratio between the lengths of the longer and shorter half spindles (a measure of asymmetry in spindle length) versus the absolute difference in centriole number between the two poles of each spindle (Figure 6A). The graph indicates that length asymmetry as defined here increases monotonically with increasing centriole imbalance, with an overall correlation coefficient of 0.96 between length asymmetry and centriole imbalance. This result indicates that increased imbalance in centriole number does in fact correspond with increased asymmetry in the spindle half lengths. In contrast, we see no such relation between imbalance in centriole number and asymmetry of spindle pole widths (Figure 6B), for which we calculate a correlation coefficient of only 0.31.

Likewise, we failed to detect any significant correlation between centriole number imbalance and asymmetry in tubulin content as judged by immunofluorescence intensities (Figure 6C). We found a correlation coefficient of only 0.15 for tubulin asymmetry versus centriole number difference. As discussed above, quantification of tubulin by immunofluorescence is inherently problematic, so no strong conclusion can be drawn from this negative result. We note also that the variation of half-spindle geometry with centriole number does not necessarily require a variation in tubulin quantity, since as centriole number increases, half-spindle length decreases but pole width increases. If we imagine that each half spindle is shaped like a truncated cone, we see that increasing the width will tend to increase the volume of the cone, while decreasing the length will tend to decrease the volume. The combined effect of a width increase and a length decrease will therefore only lead to a slight overall variation in the volume of the half spindle, which, combined with the difficulties of quantifying protein content from immunofluorescence intensity, would be unlikely to be detected in our experiments.

Cytokinesis defects in *vfl* mutants

In addition to a putative role in mitosis, centrioles have also been implicated in regulating cytokinesis, either by signaling to the cytokinesis machinery itself [Gromley et al., 2003; Piel et al., 2001; Murata-Hori and Wang, 2002] or by coordinating the orientation and position of the spindle with the cleavage furrow [Costello 1961; Ehler et al., 1995; Hinchcliffe et al., 2001; Von Dassow et al., 2009]. As with mitosis, it is well established that cleavage can progress without centrioles and even without spindles at all [Hiramoto 1965], so if centrioles contribute to the process at all, they would only be expected to act in a regulatory manner. Defects in cytokinesis have been observed in some instances after centriole ablation [Khodjakov and Rieder, 2001], but only in a fraction of cells. Therefore, cells with structural or numerical centriole abnormalities might only show a small increase in cleavage defect frequency over normal cells, requiring a statistical test on a large number of cells, an approach facilitated by genetic the genetic mutations available in *Chlamydomonas*.

To test for an effect of abnormal centriole number on cytokinesis we apply the same strategy as above, using mutants with variable centriole number to ask whether cytokinesis defects occur at a significantly increased rate when centriole number is abnormal. Observations of populations of *vfl* mutant cells show frequent failures of cytokinesis, especially incomplete cleavage furrows (Figure 7A). To quantify cytokinesis failure in *vfl* mutants, we embedded single *vfl* mutant cells and observed them before and after cell division. As tabulated in Figure 7B, we see a dramatic increase in the frequency of cleavage failure in *vfl* mutants as compared with wild-type cells. The increase in frequency is statistically significant ($P < 0.05$ for both *vfl2* and *vfl3* compared to wild-type using Fisher's exact test corrected for multiple comparison). For *vfl1* mutants, there was no statistically significant increase in cytokinesis defects compared to wild type. Given that in all other respects, such as centriole number distribution, *vfl1* and *vfl3* seem to have identical phenotypes, we were surprised to see this difference and we currently do not know why this difference exists.

These results indicate that variable centriole number mutants in *Chlamydomonas* show a significant increase in cytokinesis defects. These defects might reflect a direct role for centrioles in cytokinesis, but as with the mitotic analysis described above, two alternative explanations exist - the defect might reflect aberrations in cellular organization caused in earlier divisions, or the defects might reflect additional, centriole independent functions of the *VFL* genes.

One approach for distinguishing direct effects of centriole number from centriole independent side-effects of the mutations is to use a conditional *vfl2* allele [Taillon, 1993].

We grew *vfl2ts* mutants for 1 week at the nonpermissive temperature, shifted them back to the permissive temperature for 2 days, and then embedded and tracked individual cells in agarose pads. We have previously demonstrated [Marshall, 2007] that under this growth regime the *vfl2ts* mutant strain shows a complete restoration of centrin fiber assembly and centriole segregation within 1 day of growth after returning the strains to the permissive temperature, suggesting that by the time we started observing cells, the molecular function of centrin was restored. However, we have also found the number of centrioles per cell takes additional generations to become fully corrected [Marshall, 2007]. If such cells showed normal cleavage despite having variable numbers of centrioles, it would indicate that the cleavage failures observed in constitutive *vfl2* mutants must have been caused by a centriole-independent effect of the *vfl2* mutation. However, as shown in Figure 8A, we found that in *vfl2ts* mutants shifted back to the permissive temperature and grown for over 1 day, the defects in cytokinesis continue to occur at a frequency that was not significantly different from the constitutive *vfl2* mutant. This result suggests the defects are caused by the abnormal centriole number and not the loss of centrin function, since by this time the latter had been restored following the downshift to permissive temperature, as judged by centrin fiber assembly.

A second approach to testing whether the cytokinesis defects are centriole-dependent is to ask whether cytokinesis defects correlate with centriole number. We tested this correlation by embedding single *vfl2* cells in agarose pads and observing them before and after division, making note of the number of flagella in the parent cell prior to division. Since in *vfl2* all centrioles are active as basal bodies, the number of flagella can be taken as a reliable indicator of the number of centrioles, as previously demonstrated using multiple protein markers as well as electron microscopy [Marshall et al., 2001]. We then asked if cytokinesis defect rates correlate with flagellar number, and hence centriole number. If the cytokinesis failure was caused by a centriole-independent effect of the *vfl2* mutation, the failure should occur in all cells at a rate independent of the number of centrioles that happen to be present. As seen in Figure 8B, the rate of cleavage failure appears to vary only weakly with the number of centrioles, and does not reflect a statistically significant difference ($\chi^2=6.66$)

Taken together these results suggest that the cytokinesis defects may have been caused by centriole number abnormalities in previous cell divisions, because the defect is still seen when the conditional *vfl2* mutant is shifted back to permissive temperature, but does not correlate strongly with the number of centrioles present in the cell at the time of division.

Cell death in centriole mutants

Consistent with the increased frequency of abnormalities in mitosis and cytokinesis described above, we have observed a significantly increased proportion of dead cells in cultures of all centriole mutants relative to wild-type cells (Figure 9A, $P<0.0001$ for difference between groups, $P<0.05$ for each mutant individually compared with wild type), although from these data alone it is not possible to determine the proximal cause of death. Presence of increased cell debris has previously been noted for *vfl3* mutants [Wright et al., 1983]. An accumulation of dead cells could be due to a decreased rate of cell degradation or clearance from the culture, rather than an increase in death rate. To distinguish these possibilities we observed individual mutant cells embedded in agarose and measured the frequency with which a normal-looking cytokinesis gives rise to dead progeny cells. This analysis (Figure 9B) showed increased cell death rate in mutants with abnormal centriole numbers.

As with the spindle and cytokinesis defects discussed above, the increase in cell death could either be due to the centriole abnormalities or to a centriole-independent side effect of the mutations. We therefore correlated the frequency of cell death with the number of centrioles

present in *vfl2* cells, using the number of flagella as an indicator of centriole number, as was done above for cytokinesis defects. The results of this analysis (Figure 9C) indicate that the frequency with which a normal cleavage division produces one or more dead progeny is a function of centriole number, with the frequency of cell death much higher for cells with one or four centrioles compared to cells with two ($P < 0.02$, $P < 0.01$, respectively according to Fisher's exact test). Our results do not allow us to distinguish cell death that results from mitotic failures from death due to other causes such as increased stress sensitivity that has been reported in cells lacking centrosomes [Uetake et al., 2007].

DISCUSSION

Robustness of spindle bipolarity

Our first main result is that, although a range of spindle defects were observed in mutant cells with different numbers of centrioles, in the majority of cases the spindles were still bipolar. This was true for cells with too many centrioles as well as for cells with too few. Bipolar spindles also predominated in the *bld2*, *uni3*, and *bld10* mutants that contained structurally abnormal centrioles. It has been known for some time that bipolar spindles can self-organize in the absence of centrosomes. Our results are, to our knowledge, the first to show that this bipolar organization appears to be equally robust to quantitative perturbations in centriole number, as well as to alterations in centriole ultrastructure. Presumably, much of this robustness arises from the self-organizing capacity of spindles [Nedelec et al., 2003; Burbank et al., 2007; Clausen and Ribbeck, 2007]. However this is not to say that other cellular components might contribute to the robustness of spindle assembly in our system. We particularly note that unlike the case in mammalian cells, the nuclear envelope does not break down in *Chlamydomonas*, but instead only acquires fenestrations at the poles, the rest of the envelope remaining intact throughout mitosis [Johnson and Porter, 1968]. Experimental and theoretical analyses in *Xenopus* and *Drosophila* indicate that the nuclear lamina can serve as a mechanical stabilizer for spindle structure [Tsai et al., 2006; Civelekoglu Scholey et al., 2010]. It is therefore possible that such a stabilizing function might help increase the robustness of spindle morphology during metaphase in organisms such as *Chlamydomonas* that have persistent nuclear lamins throughout mitosis.

Our second main result is that monopolar spindles are more frequent when centriole number is low, and multipolar spindles are more frequent when centriole number is high. This result was not unexpected but it does represent a clear experimental demonstration that variation of centriole number over a wide range correlates with a switch from monopolar to multipolar spindles. Experiments in sea urchin eggs showed that cells inheriting two centrioles after division were most likely to form bipolar spindles while cells inheriting a single centriole were most likely to form monopolar spindles [Sluder and Rieder, 1985], which is consistent with the trend we see here in *Chlamydomonas* cells. In our case, however, cells having a single centriole (Figure 4) still could form bipolar and, interestingly, multipolar spindles. This result implies that multipolarity does not require direct nucleation of extraneous poles by supernumerary centrioles. Such a conclusion is consistent with a recent report of multipolar spindles in the absence of supernumerary centrioles, induced by defects in nuclear export [Rousselet, 2009]. It has previously been reported that when centrosome integrity is disrupted, multipolar spindles can result, and this was interpreted as reflecting dispersion of microtubule nucleating material to multiple foci [Oshimori et al., 2006]. Our results suggest that the loss of functional centrosomes itself, rather than creation of dispersed foci of pericentriolar material, may have accounted for the multipolar spindles in those experiments. We propose that the normal centriole copy number may have co-evolved with the bipolar spindle organization so as to reinforce each other, such that imbalances in centriole number may somehow destabilize bipolar self-organization, such that both

monopolar and multipolar spindles can form when there are not enough centrioles present to reinforce bipolarity.

Our third key result is that when bipolar spindles form in the presence of abnormal centriole numbers, the spindles themselves lose their structural symmetry between the two half-spindles, and begin to show differences in spindle pole focus and half-spindle length. We speculate that centrioles may contribute a regulatory biasing input to the self-organization process that drives spindle assembly, such that an imbalance between the bipolar symmetric spindle and an unequal number of centrioles may lead to subtle structural alterations in the resulting spindle. The structural defects we observed would thus represent a biological example of the physical phenomenon of frustrated symmetry [Toulouse, 1981; Shin et al., 2009]. Our data do not address the mechanistic function of centrioles/centrosomes at the poles, and there are potentially many ways that centrioles could influence spindle dynamics. Centrosomes help gather microtubules to form a focused pole [Goshima et al., 2005; Manning and Compton, 2007; Silk et al., 2009], hence variation in number of centrioles may result in variations in microtubule-gathering efficacy. Indeed, an increase in spindle length has been reported following dissociation of centrosomes from spindle poles by knockdown of dynein/dynactin complex components [Morales-Mulia and Scholey, 2005], which is consistent with our observation that maximum half-spindle length was seen for poles lacking centrioles. Centrioles may also play more mechanical roles; for example, it has been proposed that spindle poles are sites of mechanical integration that help regulate spindle length [Dumont and Mitchison, 2009a], as well as acting as mechanical anchors to help spindle microtubules resist forces exerted by motors during spindle dynamics [Gordon et al. 2001; Abal et al., 2005]. Attachment of centrioles, which are large and mechanically stable structures, to the poles might thus affect spindle morphology by altering spindle pole mechanical properties. Finally, centrioles and centrosomes may act as “gathering points” [O’Connell and Khodjakov, 2007] for factors that influence spindle assembly, which could include both signaling molecules [Quarmby and Mahjoub, 2005; Cuschieri et al., 2007; Fielding et al., 2008; Greenan et al., 2010] as well as microtubule depolymerizing or severing factors [Buster et al., 2002; Zhang et al., 2007]. In the latter case we can envision a mechanism whereby increased number of centrioles at a pole might lead to elevated microtubule severing, thus shortening the corresponding half-spindle. This would be consistent with the fact that katanin inhibition affects spindle length by reducing the rate of spindle shortening [McNally et al., 2006]. In any case, our observed correlations between centriole number and spindle half length and pole width indicate that centrioles/centrosomes cannot be neglected in models of spindle length control, and indeed we suggest our data could serve as a test dataset for theoretical models of spindle assembly and function that incorporate centrosome function in the process [Mogilner et al., 2006; Dumont and Mitchison, 2009b]. A similar conclusion has been reached concerning the role of the nuclear lamina in spindle length control [Civelekoglu-Scholey et al., 2010], and it will be interesting to see, in the long run, how much of spindle assembly is strictly a function of the spindle microtubules themselves, and how much is imposed by additional structures such as the lamina or centrosomes.

A recent study has analyzed the relation between centrosome size and spindle length, and concluded that larger centrosomes correlated with longer half spindles [Greenan et al., 2010], an effect that is apparently mediated by a long-range diffusional gradient of TPXL-1. Based on this result, we had expected that spindles with additional centrioles at a pole would have longer half-spindle lengths, since multiple centrioles might in some sense mimic larger centrosomes. However our results are just the opposite. As shown in Figure 5C, more centrioles seem to correlate with a shorter, rather than longer, half spindle. At the present we do not know how to reconcile these results.

We note that mismatches between centriole number at the two poles of a bipolar spindle have previously been reported in mutants of the mitotic kinesin-5 KLP61F in *Drosophila* [Wilson et al., 1997]. In these mutants, bipolar spindles were formed in which one spindle pole had a centrosome and one did not. These spindles, dubbed “bipolar anastral spindles”, showed many of the same abnormalities in spindle organization as those reported here, including a more diffuse organization of chromatin on the metaphase plate and variable pole focus at the acentrosomal pole.

Centrioles and cell division in *Chlamydomonas*

The role of centrioles in mitosis in *Chlamydomonas* was formerly the subject of some controversy, due to initial electron microscopy studies claiming that centrioles were not located at the mitotic spindle poles [Johnson and Porter, 1968]. Subsequent electron microscopy studies, however, did find centrioles at the poles [Coss, 1974; O'Toole et al., 2007], and our immunofluorescence analysis leaves no doubt that in wild-type cells, each spindle pole has a pair of centrioles in metaphase. The presence of centriole pairs at mitotic spindle poles in *Chlamydomonas* has also been observed at the light microscopy level by many other groups [Vashishtha et al., 1996; Dutcher et al., 2002; Lechtreck et al., 2002; Harper et al., 2004]. It is thus beyond dispute that centrioles are located at spindle poles in *Chlamydomonas*. Do they have a mitotic function? A previous genetic analysis [Zamora and Marshall, 2005] investigated the role of basal bodies in mitotic spindle function by assaying chromosome segregation in *vfl2* mutants, and while that study showed an increase in chromosome loss, the genetic method employed was population based and could not distinguish a direct effect of centriole number on segregation from a centriole-independent effect of the mutation. A careful electron microscopic analysis of mitosis in a related green alga has suggested that centrioles may play a role in initiating spindle pole formation [Lechtreck and Grunow, 1999]. Our present observation that monopolar versus multipolar spindles correlates strongly with the number of centrioles strongly supports the idea that centrioles can help bias the formation of mitotic spindle poles, such that cell with more centrioles are more likely to form additional poles. The strong correlation between centriole number at a pole and the width and half-length corresponding to that pole also indicate that centrioles are having a functional influence on spindle formation. Thus, not only do centrioles spatially colocalize with spindle poles in *Chlamydomonas*, they also are functionally important for spindle assembly. Moreover, when centrioles are detached from the pole in *vfl* mutants, we clearly see them acting as microtubule organizing centers that nucleate independent cytasters (see examples in Figure 1). These results taken together with those of others indicates that *Chlamydomonas* can be used as a genetic model system for investigating the role of centrioles in mitotic spindle formation, by taking advantage of the growing number of centriole structural mutants in this organism.

In *Chlamydomonas*, cleavage furrow position is predicted by a pair of rootlet microtubule bundles which emerge from the vicinity of the centrioles. The rootlets apparently direct formation of a metaphase band of microtubules followed by a contractile actin ring [Doonan and Grief, 1987; Gaffal, 1988; Ehler et al., 1995]. Mutants in which centrioles are displaced in the cell, including the *vfl* mutants, form rootlets in corresponding displaced positions [Feldman et al., 2007]. The cytokinesis defects observed in this report may be due to misplaced or disorganized rootlets as previously suggested for the *bld2* mutant [Ehler et al., 1995]. A cell with an abnormal number of centrioles presumably cannot form the usual pair of cleavage-predicting rootlets, and would end up with abnormal numbers or positions of furrow initiating events. It is also possible that the observed defects arise from the role of centrioles as temporary recruitment sites for factors that ultimately direct cleavage furrow formation or action. For example the *Chlamydomonas* ortholog of a kinesin-like calmodulin binding protein involved in cytokinesis in plants accumulates at the centrioles in early

mitosis but then relocates to the site of cleavage during cytokinesis [Dymek et al., 2006]. The fact that numerical perturbations of centrioles in *Chlamydomonas* leads to cytokinesis defects may provide an entry point for dissecting the link between centrioles and cleavage furrow assembly.

Possibility of indirect effects of mutations

A genetic approach to testing centriole function has the danger that centriole mutations may have other, centriole-independent effects on the cell. The mutants analyzed in this report correspond to genes encoding proteins that localize to centrioles [Salisbury 1995; Schiebel and Bornens 1995; Silflow et al., 2001; Matsuura 2004; Dutcher et al., 2002; Geimer and Melkonian 2005], and are thus less likely to produce side effects than over-expression of upstream cell-cycle regulatory signals of unclear specificity, such as Plk4/SAK [Basto et al., 2008] or viral oncogenes [Duensing et al. 2000], all of which induce supernumerary centrioles but target multiple downstream molecules with unpredictable side effects. Despite our selective use of genes encoding centriole-localized proteins, the possibility remains that some of these proteins may have other cellular functions that could affect spindle dynamics in a centriole-independent manner. For instance, the *vfl2* mutation causes abnormal centriole numbers, but the affected protein, centrin, has also been reported to be involved in nuclear RNA transport [Resendes et al., 2008; Fischer et al., 2004] as well as cell wall integrity [Ivanovska et al., 2000] and nucleotide excision repair [Molinier et al., 2004] in various organisms.

We therefore must be cautious in drawing strong conclusions from individual mutant phenotypes. The fact that similar defects are seen for mutations in unrelated genes whose only apparent common feature is abnormal centriole number argues that the centriole number, rather than non-specific side effects, may account for the spindle phenotypes, but still does not prove this point.

A more rigorous demonstration is provided in cases where we could observe a correlation between the types of defects with the number of centrioles present on a cell by-cell basis. Nonspecific effect due to the mutation would be seen with equal probability in all cells, since all cells would share the same mutated gene. Such nonspecific effects would therefore not correlate with the number of centrioles. In fact, we found strong dependence of spindle defects on centriole number. Specifically, we found that spindle pole width and spindle half-length correlated with the number of centrioles at a spindle pole, and that cells having different numbers of centrioles at the two poles of a bipolar spindle exhibited asymmetry in the lengths of the two half spindles. Because these cells all shared the same genetic mutations, but differed only in centriole number, these results imply that it is the centriole number differences, rather than centriole independent side effects of mutations, that influenced the process of spindle assembly in our studies.

CONCLUSIONS

Using mutants with variable centriole number, we found a correlation between centriole number and spindle polarity, but also observed that the majority of cells always made bipolar spindles regardless of centriole number, suggesting spindle assembly is a highly robust process. However the bipolar spindles that formed with incorrect centriole numbers showed asymmetries and reduced chromatin congression, and indicated a correlation between centriole number at a pole with the half-spindle length and width corresponding to that pole. Our results support the notion that centrioles can exert a regulatory influence that modulates the inherently self-organizing process of spindle assembly.

Acknowledgments

The authors thank Mark Chan, Benjamin Engel, Hiroaki Ishikawa, Elisa Kannegaard, William Ludington, Susanne Rafelski, and Nan Tang for careful reading of the manuscript. Antibodies were generously provided by Jeff Salisbury, Douglas Cole, and Masafumi Hirono. LCK was supported by an American Heart Association graduate fellowship. WFM was supported by a Searle Scholars Award and a WM Keck Foundation Distinguished Young Scholars in Medical Research Award. This work was funded by NIH grant R01 GM077004.

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A. Spindle Polarity

B. Spindle Morphology

Figure 1. Spindle phenotypes seen in mutants with abnormal centriole number or ultrastructure. (red) anti-POC1 to visualize centrioles. (green) anti-alpha-tubulin. (blue) anti-phospho-histone H3.

Figure 2.

Quantifying spindle defect frequency in individual mutants. Graph shows fraction of mitotic spindles in each genetic background (signified by color of the bars) that show each of the listed structural characteristics. (A) Spindle polarity. (B) Morphological abnormalities in bipolar spindles. Because categories are not all mutually exclusive, percentages can add up to greater than 100.

A

B

Figure 3. Quantifying chromosome congression in centriole mutants. (A) Area. (B) Shape Factor, as defined in text.

Figure 4.

Spindle polarity versus centriole number in metaphase spindles of *vfl* mutants. Histogram shows the fraction of mitotic cells with either monopolar (blue) or multipolar (red) spindles, as a function of the total number of centrioles in each cell. Note that the normal number of centrioles in a wild-type cell at metaphase is 4, two at each pole. A total of 125 spindles from *vfl1*, *vfl2*, and *vfl3* mutants were combined to form this histogram. The following number of total spindles was counted for each centriole number class (0, n=5; 1, n=8; 2, n=20; 3, n=19; 4, n=39; 5, n=13; 6, n=12; 7, n=9).

Figure 5.

Geometric features of bipolar spindles correlate with centriole number at poles. (A) diagram illustrating the two parameters measured, pole width and half-spindle length. (B) Spindle pole width versus the number of centrioles found at a pole. (C) Half-spindle length versus the number of centrioles found at a pole. A total of 184 bipolar spindles were analyzed from *vfl* mutants for each plot. Error bars are standard error of the mean.

Figure 6.

Imbalance in centriole number between two poles of a bipolar spindle correlates with asymmetry in half-spindle lengths but not pole width. (A) Asymmetry in half-spindle length, defined as the ratio of the larger half-spindle length to the smaller half-spindle length, plotted versus the absolute value of the difference in number of centrioles between the two poles. (B) Asymmetry in pole width, defined as the ratio of the width of the larger pole to the smaller pole, plotted versus the absolute value of the difference in number of centrioles between the two poles. (C) Asymmetry in half-spindle tubulin content, defined as the ratio of the background-corrected integrated tubulin intensity in the two half-spindles, plotted versus the absolute value of the difference in number of centrioles between the two poles. A

total of 184 bipolar spindles from *vfl* mutants were tabulated for these plots. Error bars are standard error of the mean.

Figure 7. Defects in cytokinesis in mutants with abnormal centriole number. (A) images of normal and defective cleavage. (B) Frequency of cytokinesis defects in mutants (wt n=103; *vfl1* n=129; *vfl2* n=401; *vfl3* n=60).

Figure 8.

Correlating cytokinesis defects with centriole number abnormality. (A) Defects following restoration of normal *VFL2* gene function following down-shift of a conditional mutant (*vfl2ts* n=12). (B) defect frequency versus centriole number in *vfl2* mutants (n=69, 60, 160, 93, and 17, respectively, for cells with 0, 1, 2, 3, and 4 centrioles).

Figure 9.

Cell death in centriole mutants. (A) Percent of dead cells in centriole mutant populations as assayed by erythrosine staining. (B) Frequency of cell divisions in which one or both daughter cells were dead, plotted for different mutants. (C) Frequency of cell divisions in which one or both daughter cells were dead analyzed in *vfl2* mutants and plotted versus the number of centrioles in the mother cell.