An Asp–CaM complex is required for centrosome–pole cohesion and centrosome inheritance in neural stem cells

Todd Schoborg, Allison L. Zajac, Carey J. Fagerstrom, Rodrigo X. Guillen, and Nasser M. Rusan

Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

The interaction between centrosomes and mitotic spindle poles is important for efficient spindle formation, orientation, and cell polarity. However, our understanding of the dynamics of this relationship and implications for tissue homeostasis remains poorly understood. Here we report that Drosophila melanogaster calmodulin (CaM) regulates the ability of the microcephaly-associated protein, abnormal spindle (Asp), to cross-link spindle microtubules. Both proteins colocalize on spindles and move toward spindle poles, suggesting that they form a complex. Our binding and structure–function analysis support this hypothesis. Disruption of the Asp–CaM interaction alone leads to unfocused spindle poles and centrosome detachment. This behavior leads to randomly inherited centrosomes after neuroblast division. We further show that spindle polarity is maintained in neuroblasts despite centrosome detachment, with the poles remaining stably associated with the cell cortex. Finally, we provide evidence that CaM is required for Asp’s spindle function; however, it is completely dispensable for Asp’s role in microcephaly suppression.

Introduction

Faithful chromosome segregation relies on the collective effort of the mitotic spindle and hundreds of macromolecules that regulate its structure, behavior, and function (Walczak and Heald, 2008). In a simplified view, the spindle is a two-component system consisting of microtubules (MTs) and MT-associated proteins. The interplay between these components dictates spindle architecture and harnesses its dynamics to ensure proper ploidy.

A large number of genes play a role in various aspects of spindle biology (Goshima and Vale, 2003; Goshima et al., 2007). Despite this, spindle phenotypes that arise from mutations in these genes fall into a limited number of categories, suggesting that spindle form and function is dictated by only a handful of basic principles. These phenotypes manifest as defects in spindle length and shape, centrosome number and positioning, kinetochore function, and chromosome congression and segregation (Goshima et al., 2007). Some spindle assembly genes have been well studied, providing insight into the mechanism by which they influence the spindle; however, mechanistic insight into most spindle genes remains lacking.

Spindle pole focusing is one example in which many essential genes have been identified with little understanding of how it is achieved. Loss of MT focusing at spindle poles has been documented after perturbation of several MT-associated proteins and motors, including the kinesins Ncd/Kar3 and Eg5 (Sawin et al., 1992; Endow et al., 1994; Gaglio et al., 1996; Matthes et al., 1996) in addition to dynein, dynactin, and nuclear mitotic apparatus protein (NuMA; Merdes et al., 1996). This latter complex is the best understood, primarily because of biochemical data that support a model where NuMA oligomerizes and binds directly to MTs and dynein/dynactin complexes, leading to MT cross-linking and pole focusing (Harborth et al., 1999; Merdes et al., 2000).

In Drosophila melanogaster, several nonmotor proteins, such as the kinase regulator Mob4 and the microcephaly-associated protein abnormal spindle (Asp), are required for pole focusing (Ripoll et al., 1985; Wakefield et al., 2001; Trammell et al., 2008), but the mechanism of pole focusing remains less clear. In Drosophila, mushroom body defect (Mud) has been suggested to be an ortholog of vertebrate NuMA; however, there is little sequence similarity, and data suggest the primary role of mushroom body defect is to maintain spindle orientation through interaction with the Pins-Gui cortical polarity proteins (Bowman et al., 2006). Instead, Asp is a more likely candidate as a “functional” ortholog of NuMA given its spindle pole localization, its ability to bind MTs, and phenotypes associated with asp mutations, such as centrosome detachment and loss of pole focusing (Gonzalez et al., 1990; Saunders et al., 1997; do Carmo Avides and Glover, 1999; Wakefield et al., 2001; Morales-Mulia and Scholey, 2005). Interestingly, vertebrates possess a true ortholog of asp, known as abnormal spindle-like...
microcephaly associated \((\text{ASPM})\), which is the most commonly mutated gene in patients afflicted with autosomal-recessive primary microcephaly, characterized by reduced head and brain size and mental retardation (Bond et al., 2002, 2003). \(\text{Asp}\) and ASPM play key roles in neural development in both flies and mice (Fish et al., 2006; Rujano et al., 2013). However, the mechanism of \(\text{Asp}\) and ASPM function remains largely unexplored.

Underlying our deficit in understanding \(\text{Asp}\) function is the lack of a null allele to afford robust genetic analysis. Here, we use CRISPR, live cell imaging of \(\text{Drosophila}\) neural stem cells (neuroblasts [NBs]), and mutant analysis to investigate the underlying mechanism of \(\text{Asp}\) regulation. We show that Calmodulin (CaM) forms a complex with \(\text{Asp}\) that dynamically associates with MTs and regulates its role in centrosome–pole cohesion, pole focusing, and proper centrosome inheritance, but not its role in suppressing microcephaly.

## Results

### Asp and CaM are required for pole focusing and centrosome attachment.

In agreement with previous studies (Morales-Mulia and Scholey, 2005), two prominent spindle phenotypes were observed after RNAi depletion of \(\text{Asp}\) from cultured S2 cells: unfocused spindle poles and centrosome detachment from spindles (Fig. 1, A, E, and F; and Fig. S1 A). We found centrosomes randomly positioned throughout the cell, and in cells with more than two centrosomes (common in S2 cells), they fail to cluster in mitosis (Fig. 1 A). To probe the underlying molecular basis of these phenotypes, we localized GFP-tagged full length (FL) and truncations of \(\text{Asp}\) in S2 cells depleted of endogenous \(\text{Asp}\) (Fig. 1, B and C). FL \(\text{Asp}\) (\(\text{Asp}\) FL) rescued pole focusing, centrosome detachment, and unclustering; however, \(\text{Asp}\)\(^N\) and \(\text{Asp}\)\(^C\) did not (Fig. 1, C, E, and F). Interestingly, in addition to \(\text{Asp}\) localization to spindle poles, we identified a previously unreported population decorating the entire spindle (Fig. 1 C). This MT localization can be divided into two populations as revealed by \(\text{Asp}\) truncations: \(\text{Asp}\)\(^N\) formed discrete spindle puncta (similar to \(\text{Asp}\) FL), whereas \(\text{Asp}\)\(^C\) localized weakly throughout the spindle, consistent with very weak affinity found in vitro between MTs and \(\text{Asp}\)\(^{621–1196}\) (Saunders et al., 1997), which partially overlaps with our \(\text{Asp}\) C (976–1954). We believe this \(\text{Asp}\) C localization is normally masked by the stronger spindle pole and punctate localization of wild type (WT). Therefore, our truncation analysis uncovered two modes of MT attachment: (a) punctate attachment, likely mediated by the known MT-binding domain at the N terminus, and (b) a diffuse, weak attachment mediated by an unknown region within \(\text{Asp}\) C. Importantly, MT localization of either \(\text{Asp}\)\(^N\) or \(\text{Asp}\)\(^C\) alone is insufficient for \(\text{Asp}\) function, suggesting proper pole focusing and centrosome attachment require the coregulation of the N and C termini.

Based on our data, we hypothesized that two key domains of \(\text{Asp}\) are required for its localization and function: the high-affinity MT-biding domain within \(\text{Asp}\)\(^N\) and an IQ motif–rich region in \(\text{Asp}\) C that was computationally identified, yet remains unexplored (Fig. S2 A; Saunders et al., 1997; Franke et al., 2006). Given CaM is known to bind IQ motifs and was shown to be required for pole focusing (Goshima et al., 2007), we hypothesized that CaM directly binds and regulates Asp. In support of this, RNAi depletion of \(\text{cam}\) in S2 cells phenocopied \(\text{asp}\) depletion in our measurements (Fig. 1, D–F; and Fig. S1 A). Similar phenotypes were also observed on acute drug treatment of S2 cells using W-7, a cell-permeable CaM inhibitor (Fig. S1 B; Osawa et al., 1998). Furthermore, depletion of both \(\text{asp}\) and \(\text{cam}\) simultaneously did not lead to a more severe phenotype.
Asp–Cam complex facilitates centrosome–pole attachment • Schoborg et al. 989

Asp–Cam complex facilitates centrosome–pole attachment • Schoborg et al. 989

To test if loss of pole focusing is a consequence of centrosome detachment, we repeated our knockdown in cultured cells lacking centrosomes (sas4−/−; Lecland et al., 2013). Loss of asp, cam, or both led to a significant increase in lateral pole distance (Fig. S1, C–E). This is in agreement with previous work that showed poles are focused in centrosome-less asl2 mutant NBs and unfocused in the asl2,asp1 double mutant (Wakefield et al., 2001). These results suggest that CaM and Asp cooperate to perform two independent roles at spindle poles: MT focusing and centrosome–pole attachment. However, these two roles are likely mediated by the same lateral MT–MT interaction mechanism.

Asp and CaM interact and display identical dynamics

To test if Asp and CaM form a complex in vivo, we used a mitochondria targeting assay, which uses colocalization to assess the interaction between two proteins expressed off the same plasmid after the artificial tethering of one to the mitochondria (Galletta et al., 2014). In addition to AspFL, AspN, and AspC, we generated a FL construct in which five highly predicted IQ motifs in the C terminus were deleted (AspFLΔIQ; Fig. S2 A; Franke et al., 2006). In this assay, CaM interacted strongly with Asp FL and AspC, but not with Asp N (CaM remained exclusively nuclear). Interestingly, the AspFLΔIQ construct was still able to bind CaM, but this interaction was reduced as evident by its inability to pull most of CaM out of the nucleus (Fig. 2 A). This suggests that other predicted IQ motifs in the C terminus of Asp (Rujano et al., 2013) contribute to CaM binding. Nevertheless, our phenotypic analysis (see Fig. 5) indicates the perturbation of CaM interaction in AspFLΔIQ is significant. We verified that the AspC–CaM interaction is direct using yeast two-hybrid analysis (Galletta and Rusan, 2015), in agreement with previous Asp-CaM Y2H analysis in Caenorhabditis elegans (van der Voet et al., 2009). However, unlike the mitochondrial targeting assay, an interaction with CaM was only revealed on separating the N and C termini (Fig. 2 B). This suggests that the Asp FL–CaM interaction requires a specific Asp tertiary structure only afforded in Drosophila cells.

To further validate Asp–CaM interactions in a physiologic context, we simultaneously imaged RFP-CaM and GFP-Asp on mitotic spindles in S2 cells. Both proteins localized in a near identical pattern with strong enrichment at spindle poles and weaker foci throughout the spindle (Fig. 2, C and D). Remarkably, live imaging revealed that Asp and CaM foci moved concertedly poleward along the metaphase spindle at similar velocities (Asp: 1.3 ± 0.5 µm/min; CaM: 1.5 ± 0.6 µm/min), gradually accumulating at the poles (Fig. 2, E and F; and Video 1). The concomitant movement of Asp and CaM toward the poles argues these proteins are organized as a complex. Furthermore, the similarity of their velocities to MT flux in S2 cells (1.1–1.2 µm/min; Matos et al., 2009; Rath et al., 2009) suggests these complexes bind MTs directly and not through a minus end motor traveling along the MT.

CaM is required to stabilize AspC, which can form higher-order structures with AspFL

Evidence in vertebrates suggests NuMA can oligomerize, generating an insoluble pole matrix that facilitates focusing

Figure 2. Asp and CaM interact to form a complex that streams along spindles. (A) Mitochondria targeting assay for each of the indicated Asp fragments. White dotted line corresponds to cell outline, and yellow dotted lines show nuclei. Bar, 2 µm. (B) Yeast two-hybrid analysis of Asp constructs and CaM. Left column indicates growth, and right column indicates interaction. (C) Single frame from Video 1 of live S2 cell expressing RFP-CaM and GFP-Asp. Boxed region (yellow) denotes inset, bottom panel. Colored lines represent position of kymograph in E. Arrowheads denote colocalized foci. Bar, 5 µm; inset, 1 µm. (D) 2D histograms from colocalization analysis. (E) Kymograph along positions denoted in C. Time bar, 1 min; distance bar, 2.5 µm. (F) Histogram of movement rates for CaM and Asp (µm/min). n > 200 tracks from more than nine cells.
We hypothesized that Asp might mediate pole focusing in *Drosophila* using an analogous mechanism, with CaM acting as the lynchpin. We used our mitochondria targeting assay and cotransfected FLAG-tagged versions of AspFL, AspFLΔIQ, AspN, and AspΔ and found that the AspC fragment could interact with both AspFL and AspFLΔIQ. We did not observe an interaction with AspC or AspN (Fig. S2 B). Although the AspC-AspFLΔIQ suggest otherwise, it is still possible that CaM is required for this interaction by binding the five major IQ motifs in AspC, or binding the other nonmajor IQ motifs that remain in AspFLΔIQ. We attempted to investigate this further by depleting CaM using RNAi in the aforementioned assay; unfortunately, AspC was not detected in cells (Fig. S2 C), suggesting CaM is required for AspC stability. Therefore, our data show that CaM can oligomerize in vivo via its C terminus, but the interaction appears to require a structural feature present within the FL protein, and it remains to be determined if CaM is required for this interaction.

The failure to detect the AspC fragment after CaM depletion suggested that CaM might regulate Asp behavior through stabilization of Asp protein. We tested this hypothesis by expressing CaM-GFP and AspFL-FLAG constructs in S2 cells treated with control or CaM RNAi. We were unable to quantify AspFL-FLAG stability via Western blotting because of our inability to obtain a reproducible migrating band on SDS-PAGE gels from these extracts. We therefore quantified the percentage of cells expressing AspFL-FLAG using immunostaining and found a significant decrease in the number of interphase cells expressing AspFL-FLAG after CaM depletion (Fig. S2 D). These data suggest that a potential mechanism of Asp regulation by CaM involves protein stability; however, more biochemical analysis will be required to verify and extend these findings, particularly within the spindle lattice itself.

### CaM and Asp dynamics in NBs

Asp and its vertebrate orthologue ASPM are key determinants of neural development (Bond et al., 2002, 2003; Fish et al., 2006; Rujano et al., 2013), providing a relevant system to probe the role of the Asp–CaM interaction in the context of a developing tissue. To begin, we analyzed endogenous CaM localization by immunostaining *Drosophila* larval central NBs (Morin et al., 2001), which undergo repeated rounds of rapid asymmetric cell divisions. Our fixed analysis of prophase NBs shows CaM distinctly localized to the centrosomes (Fig. 3, A and A′). During metaphase, CaM redistributed proximally from the centrosomes to the spindle pole region, with smaller punctae throughout the spindle (Fig. 3, B and B′). Similar CaM localization on spindles was observed in mitotic S2 cells overexpressing GFP-CaM (Fig. S3). To gain further insight into the dynamic behavior of these two spindle populations of CaM in NBs, we used live-cell imaging. Metaphase NBs show endogenous CaM streaming within the spindle, leading to incorporation into a pool of immobilized CaM at the poles (Fig. 3, C and D′). This streaming behavior ceases during late anaphase as the chromosomes separate (Videos 2 and 3). Critically, we observed identical dynamics of AspFL in NBs (Video 4), consistent with a pole focusing mechanism that relies on Asp–CaM complexes. Finally, by telophase, CaM localizes near the cleavage furrow (Fig. 3, C and C′; and Video 2), suggesting that CaM also plays an important role in Asp’s function during cytokinesis (Wakefield et al., 2001; Riparbelli et al., 2002), possibly aiding the cross-linking and stabilization of midbody MT minus ends.

### Asp null mutations cause spindle defects in NBs

Previous work has shown various *asp* allelic combinations lead to small fly brains, similar to human microcephaly (Rujano et al., 2013). We also found *aspC* and *aspCΔIQ* adults with significant brain size reduction; conversely, *aspFLΔIQ* (reduced CaM interactions) and *aspCΔIQ* (no CaM interaction) rescued brain size comparable with *aspFL* and WT animals (Fig. 6). Therefore, CaM is required for Asp’s spindle assembly role, but not its role in microcephaly suppression.

### Centrosome inheritance in NBs is randomized in *asp* mutants

Maintaining centrosome–pole attachment is an intriguing, yet underappreciated function for Asp and CaM. Previous studies have noted this phenotype for other *asp* alleles in the embryo and brain (Gonzalez et al., 1990; Wakefield et al., 2001); however, the mechanism of detachment and its consequences have not been explored. To this end, we used live imaging of GFP-Tubulin to monitor centrosome and spindle dynamics in *aspΔIQ* mutant NBs. Early stages of mitosis, including events up to and including NPB proceeded normally, similar to WT NBs (Fig. 7, A and B). Shortly after NPB, centrosomes detach from the poles and move randomly around the cell (Fig. 7, B and C; and Videos 5–8). Polarity establishment in these NBs
Asp–CaM complex facilitates centrosome–pole attachment

was not impaired, consistent with early mitotic events being normal in asp t25 mutants. Even more interesting is that cell polarity was maintained through mitosis and was always associated with spindle poles, not the wandering centrosomes (Fig. 7 D and Fig. S4 A).

We followed NBs as they exited mitosis to determine the fate of the nomadic centrosomes and found that their location at anaphase onset determined inheritance. We observed instances in which mother and daughter centrosomes were correctly inherited (Fig. S4, B and B'; and Video 5) and others in which they swapped positions before segregation (Fig. 7, C and C'; and Video 7). Previous work has highlighted asymmetry in composition and function between the mother and daughter centrosomes, with NBs retaining the daughter and ganglion mother cells (GMCs) inheriting the mother (Rebollo et al., 2007; Rusan and Peifer, 2007; Conduit and Raff, 2010; Januschke et al., 2011, 2013; Lerit and Rusan, 2013). Although the purpose of this asymmetry remains unclear (Lerit et al., 2013), our asp t25 mutant provides an excellent model for testing such questions. Additionally, we observed cases where both centrosomes were inherited by the NB (Fig. 7, B and B'; and Video 6) or the GMC (Fig. S4, C and C'; and Video 8).

Given these defects, we predicted that the duration of mitosis would increase. Indeed, we find many asp t25 NBs with extended metaphase duration (Fig. 7, B and C; and Fig. S4 C), whereas others proceed with near WT timing (Fig. S4 B). Importantly, we did not observe any cases of complete mitotic arrest in NBs as determined by our living imaging and mitotic index analysis (Fig. 7 D and Fig. S4 D), in contrast with previous studies for other asp alleles (Ripoll et al., 1985; Carmena et al., 1991; Wakefield et al., 2001). It is not clear why a longer metaphase does not lead to an increase in mitotic index, but it is not because of a change in NBs numbers (Fig. S4 E), suggesting that the entire NB cell cycle is extended, not just metaphase. Nevertheless, the downstream consequence of receiving too many or too few centrosomes are well documented, including chromosome instability, tumor formation, and cell death (Basto et al., 2006; Rusan and Peifer, 2007; Castellanos et al., 2008; Lerit and Rusan, 2013; Sir et al., 2013; Poulton et al., 2014).

Discussion

The results presented here provide insight into how Asp, a key protein involved in mitotic spindle function, is regulated by the ubiquitous calcium-sensing protein CaM. CaM was localized near the spindle poles over 35 yr ago (Welsh et al., 1978); our data now assign a role for this CaM localization in directly regulating Asp to cross-link spindle MTs. The Asp–CaM interaction is conserved because it has also been biochemically identified in other eukaryotes, such as nematodes and mice, suggesting that this complex performs an essential spindle function (van der Voet et al., 2009; Xu et al., 2012). The work presented here extends our functional understanding of the Asp–CaM complex.
in spindle pole focusing and centrosome–pole cohesion, in addition to the cell biology of microcephaly.

Previous work in Drosophila, C. elegans, and mice has suggested a link between Asp and CaM. Goshima et al. (2007) were first to highlight the similar spindle phenotypes observed after RNAi depletion of either protein in Drosophila S2 cells. In C. elegans, analysis of meiotic spindles in the early embryo showed spindle defects after asp depletion and Asp’s dependence on CaM (CMD-1) for pole localization. Furthermore, yeast two-hybrid analysis identified an Asp fragment containing a single IQ motif that could interact with CMD-1 (van der Voet et al., 2009). This interaction between CaM and Asp on meiotic spindles was later identified in mouse oocytes using immunoprecipitation (Xu et al., 2012). However, in all cases, details of the underlying mechanism of the Asp–CaM association and a direct test of its contribution to spindle architecture remained unexplored.

Our results demonstrate that CaM functions as the critical factor that dictates Asp’s ability to cross-link MTs. This is supported by the fact that Asp transgenes that localize to the spindle in a manner identical to that of the FL protein, yet are defective in CaM binding (AspN and AspFLΔ), fail to maintain pole focusing and centrosome–pole cohesion. Further, our transgene analysis also highlighted a second mode of MT binding by Asp, mediated through its C terminus, and is independent of its known N-terminal MT binding domain. This interaction, though clearly weaker and distinct from the punctate signals observed for N-terminal containing transgenes, is supported by previous studies in vitro (Saunders et al., 1997). We believe the stronger spindle pole and punctate localization of WT Asp normally masks this AspN localization and possibly contributes to Asp’s ability to cross-link MTs (see model in Fig. 8).

Furthermore, we also uncovered a novel mode of Asp–CaM complex behavior on spindles, highlighted by dynamic streaming of foci through the spindle lattice toward the pole. Previous work suggested that Asp associates with MT minus ends based on its accumulation at spindle poles where their density is highest (do Carmo Avides and Glover, 1999; Wakefield et al., 2001). Our localization of the Asp–CaM complex in live cells supports this hypothesis. However, we further suggest that Asp–CaM complexes, seen as discrete puncta that move poleward, reside at MT minus ends distributed throughout the spindle that are collectively transported and organized at poles. These observations are consistent with work showing γ-tubulin–marked minus ends present throughout the spindle that stream toward the poles (Lecland and Lüders, 2014). Additionally, vertebrate NuMA displays similar streaming behavior (Kisurina-Evgenieva et al., 2004), indicating a shared mechanism in which pole focusing is achieved through the concerted movement of protein complexes along the spindle toward the pole. Biochemical analysis will be critical for establishing the relationship between the distribution of minus ends within the spindle, the ability of the Asp–CaM complex to bind MT minus ends, and how the dynamic nature of their movement contribute to pole focusing and centrosome–pole cohesion.

The complete detachment of centrosomes from the spindle and random movement within the NB could have substantial long-term effects that are not fully appreciated by our limited...
aspt
treatment in NBs. [A] asp^FL/DF NBs expressing indicated transgenes were fixed and stained for β-Tubulin, pH3, and centrosomin (Cnn). Outline denotes NB cortex, and arrowheads mark centrosome position. (B) Snapshots from live movie of indicated genotype. Localization of Asp is similar to samples in A, except here the weak Asp^C localization to spindle MTs is clear (yellow arrowheads). Bars, 2 µm.

Figure 6. Analysis of microcephaly phenotypes in asp^FL mutants. (A) Fixed adult brains from the indicated genotype. (B) Quantitative analysis of head size from A (n > 8; error bars are SD). Bar, 1 mm.

analysis of third-instar larval brains. Although the swapping of mother–daughter centrosome position and improper inheritance is interesting, its significance is unknown (Lerit et al., 2013). It could be that centrosome position after detachment, rather than detachment, per se, negatively influences mitotic events. One would predict, for example, that centrosomes positioned anywhere in the cell other than the poles could influence the MT architecture within the spindle. In fact, we do see a significant number of aberrantly bent spindles, and our live imaging showed that wandering centrosomes transiently interact laterally along the entire length of the spindle. One might also predict that this lateral centrosome position would influence the dynamics and tension across the kinetochores, triggering the spindle assembly checkpoint and an extended metaphase, which we also document in asp^FL mutants. Therefore, the wandering centrosomes and their improper inheritance could have many negative downstream effects. If these results of inheriting too many or too few centrosomes are extrapolated to mammalian cells, one would predict detrimental effects on cilia formation in addition to mitotic defects, as previously documented in other mutant backgrounds (Mahjoub and Stearns, 2012).

Our analysis of apical determinants in NBs highlighted a possible role for spindle poles (not centrosomes) in the maintenance of cell polarity. Despite centrosome detachment in the...
asp^{25}/Df NBs and long curving spindles, we did not observe misaligned spindles. This was true in fixed tissue using the api-
cal polarity marker aPKC, in which, despite pole splaying and cur-
vature, minus ends of MTs appeared to remain stably associ-
ated with the crescent at the cell cortex. Furthermore, we never
observed significant spindle rotation after centrosome detach-
ment during the course of live imaging, and NBs divided asym-
metrically. These observations support the prevalent model that
centrosomes initiate NB polarity (Siegrist and Doe, 2006; ja-
nuschke and Gonzalez, 2010) but further add that centrosomes
are neither necessary nor able to alter polarity once established.
This is corroborated by the fact that we did not observe a sig-
nificant difference in NB number in the asp^{25}/Df mutant, sug-
gest that cell fate determinants were correctly partitioned
during asymmetric division.

Our results also shed light on the role of Asp in mi-
crocephaly (Bond et al., 2002). Interestingly, this phenotype is
not dependent on the Asp–CaM complex. Both AspN and
Asp^{LΔIQ} rescued the brain size defects of the asp^{25}/Df despite
showing no or reduced binding to CaM. These results are
in agreement with previous work from the Basto laboratory
that demonstrated normal head size in animals expressing an
N-terminal Asp fragment in the hypomorphic asp allele back-
ground (Rujano et al., 2013). Importantly, our data using the
null allele show that microcephaly is a result of the loss of
Asp function and not a dominant-negative effect of the hy-
pomorphic asp alleles. Furthermore, we show that the micro-
cephaly phenotype is not a consequence of unfocused spindle
poles or detached centrosomes, because the AspN and Asp^{LΔ
IQ} rescue fragments displayed both of these defects. Taken
collectively, our analysis of the null asp allele uncovered a
separation of function that requires both termini of Asp to
maintain MT cross-linking and an unknown region of the N
terminus to specify proper brain size.

In closing, we propose two possible models by which the
Asp–CaM complex could function (Fig. 8). In both models,
CaM exerts its influence on the spindle through directly bind-
ing the C terminus of Asp and is required for its stability. The
first model proposes that CaM aids Asp oligomerization within
the spindle. Putative higher-order Asp assemblies would be
analogous to NuMA oligomerization shown to facilitate MT
focusing in vertebrate cells (Dionne et al., 1999; Harborth et
al., 1999; Merdes et al., 2000). A second model proposes that
CaM might regulate the weak association of Asp's C terminus
to MTs. In this model, Asp would bind MT minus ends via its
N terminus and the MT lattice via its C terminus, effectively
bridging and zippering MTs. In both models, CaM might pro-
mote a structural conformation that allows for oligomerization or
for a single Asp molecule to bind two separate MTs. Both
models are not mutually exclusive, because elements of each
can cooperate to ensure proper cross-linking between spin-
dle MTs and centrosome MTs for robust pole focusing and
centrosome attachment. Future biochemical and structural
studies will be required to more fully understand the influ-
ence of CaM binding to Asp and the role of this complex in
spindle MT cross-linking.

Figure 7. Centrosome inheritance is ran-
domized in asp NBs. Live imaging of WT (A)
and asp^{25}/Df mutant (B and C) NBs (dotted
outline) expressing GFP-tubulin. (B) Colored
arrowheads differentiate and mark positions of
the two centrosomes; both are inherited by the
NB after asymmetric division. Tracks of cen-
trosome trajectory are shown in B ′. (C) An ex-
ample of mother–daughter centrosome swap.
Tracks of centrosome trajectories are shown in
C ′. (D) Central brain NBs stained with the
polarity marker aPKC (PKCζ; magenta) and
β-tubulin (green). n = 15 NBs for each phe-
totype were scored for spindle alignment; a
pole touching/oriented toward the aPKC cres-
tend was considered to be properly oriented.
EP, end point; SP, start point at prophase.
Bars: (A–C) 5 µm; (D) 3 µm.
Asp-Cam complex facilitates centrosome-pole attachment • Schoborg et al. 995

Materials and methods

Fly stocks and husbandry
All stocks and crosses were maintained on standard cornmeal-agar media at room temperature (20–22°C). The following lines were obtained from the Bloomington Stock Center: w1118, Df(3R)BSC519/ TM6C, Stb CW (asp deficiency mutant, stock 25023); w+; PPTF-un-CamP00695/CyO (CaM-GFP Trap Line, stock 50843). Microinjection of Asp+, Asp*, Aspβ, and AspβQ transgenes into w1118 embryos was performed by BestGene Inc.

Asp CRISPR
Two guide RNAs flanking the asp promoter (gRNA1-3R:24753685..24753707) and part of the second exon (gRNA2-3R:24754455..24754477) were cloned into separate U6 plasmids (pU6-BbsI). Equimolar amounts (250 ng/μl final) were injected into Cas9 embryos (NIG-FLY CAS-0004) by BestGene Inc. Individual lines were double balanced and progeny screened for small adult head size as homozygotes.

Vectors
For S2 cell expression, modified Gateway cassette vectors from the Drosophila Gateway Vector Collection were used to generate GFP (pAGW)-, RFP (pATRW)-, or FLAG-HA (pAFHW)-tagged N-terminal linker (GFP-40aaLinker-CaM), which was cloned from an unpublished vector that was a gift from T. Megraw (Florida State University, Tallahassee, FL). To generate the vector for the mi-

Cell culture, transfection, and double stranded RNA treatment
Drosophila S2 cells were obtained from Life Technologies and maintained in SF900 insect media supplemented with 1x penicillin/streptomycin at 25°C. The acentriolar dSas4; Jupiter::GFP cells (Line 131; Lecland et al., 2013) were obtained from the Drosophila Genomic Resource Center and maintained in SF900 containing 1x P/S and 5% FBS. Transfection of S2 cells was achieved using Amaxa Nucleofector technology (Lonza). 2 μg vector was diluted in 100 μl nucleofection solution (50 mM d-mannitol, 15 mM MgCl2, 5 mM KCl, and 120 mM NaPO4, pH 7.2) and used to resuspend a pellet of ~4 × 106 cells. This

Asp constructs and prediction of putative IQ motifs
Five asp constructs were generated pertaining to the FL version of the protein (Aspβ, aa 1–1,954), the N-terminal half (Aspβ*, aa 1–975), the C-terminal half (Aspβ*, aa 976–1,954), and a FL version lacking five of the most highly predicted IQ motifs (Aspββ); IQ 1 (aa 1,011–1,041), IQ 2 (aa 1,087–1,103), IQ 3 (aa 1,329–1,342), IQ 4 (aa 1,528–1,550), and IQ 5 (aa 1,719–1,731). IQ motif predictions were performed with the FL Drosophila Asp protein using the Calmodulin Target Database using default settings.

Yeast two-hybrid
Asp constructs and CaM were introduced into pDEST-pGADT7 and pDEST-pGBK7 (Rossignol et al., 2007) using the Gateway cloning system (Life Technologies). Before use in cloning, the kanamycin resistance cassette in pDEST-pGBK7 was replaced with an ampicillin resistance cassette using yeast-mediated recombination. Fragments in pGADT7 or pGBK7 were transformed into yeast strains Y187 and Y2HGold, respectively (Clontech) using standard techniques. Cultures of yeast carrying these plasmids were grown to OD600 ~0.5 at 30°C in SD Leu or SD –Trp media as appropriate to maintain plasmid selection. For mating, 20 μl of a Y187 strain and a Y2HGold strain were added to 100 μl of 2x yeast extract/peptone/dextrose medium in the well of a 96-well plate. Mating cultures were grown for 20–24 h at 30°C with shaking. Approximately 3 μl of cells were then pipetted onto SD –Leu –Trp (DDO) plates using a Multi-Blot Replicator (VP 407AH; V&P Scientific), and plates were grown for 5 d at 30°C. These plates were replica plated onto four plates: (a) DDO, (b) QDO (SD –ade –leu –trp –ura), (c) DDO XA (SD –leu –trp plates containing Aureobasidin A; Clontech) and X-α-Gal (Gold Biotechnology), and (d) QDOXA (SD –ade –leu –trp –ura with Aureobasidin A and X-α-Gal). Plates were grown for 5 d at 30°C. Interactions were scored based on growth and development of blue color as appropriate.

Figure 8. Model of CaM’s possible role in Asp function. (1) CaM might mediate oligomerization of Asp to cross-link MTs, or (2) CaM might regulate Asp C-terminal interaction with MTs, a mechanism that would provide the necessary MT cross-links for both pole focusing and centrosome attachment.
solution was added to a cuvette and electrophoresed using the S2 cell (G-030) setting. Transfected cells were maintained in six-well plates with 2 ml SF900 at 25°C for 48 h before imaging. For double stranded RNA treatment, transfected cells were treated with 10 μg of double stranded RNA added directly to the well immediately after electrophoresis and then again on day 3. Cells were then fixed on day 5. The following primers were used to generate DNA templates for T7 RNA synthesis reactions (Promega): CaM, 5′-AACGGCAATAAGTCTTTC-3′; 5′-ACCGTGGG CATCGATATC-3′; AspN, 5′-GTTGAGACTCCGCTCAGTCCG-3′; 5′- CATAAGGCTTGACGGAAGGC-3′; AspC, 5′-GGAAACAGCCAGA CTTAGC-3′; 5′-GCTTGTGCAGGCAGATAACA-3′.

Immunostaining

S2 or dsSas4-: Jupiter::GFP cells were allowed to adhere to coverslips coated with 0.5 mg/ml Concanavalin A for 60 min in a covered 35-mm dish. Transfected S2 cells were fixed with 4% PFA diluted in PBS for 10 min. dsSas4-: Jupiter::GFP cells were fixed with 0.25% glutaraldehyde diluted in PBS for 1 min, extracted for 1 min in Karsenti’s buffer (80 mM Pipes, 1 mM MgSO4, 5 mM EDTA, and 0.5% Triton X-100, pH 6.9), fixed again with 0.25% glutaraldehyde for 10 min, and then postfixed in NaBH4 (1 mg/ml in H2O) for 10 min. Cells were counterstained with DAPI for 1 min and then mounted in Vectashield (Vector Laboratories).

For brain staining, third-instar larvae were quickly dissected in SF900 media, and intact brains were transferred to 0.5-ml tubes containing SF900/0.05% BSA. Brains were fixed in 9% PFA/0.5% Triton X-100/SF900 with head-tail rotation for 30 min at room temperature. Brains were rinsed 3× in PBST (PBS and 0.1% Triton X-100) and then permeabilized in 1% BSA (wt/vol)/0.5% Triton X-100/SF900. Tubes were then placed in a shaking incubator set at 37°C and then again on day 3. Cells were then fixed on day 5. The following primers were used to generate DNA templates for T7 RNA synthesis reactions (Promega): CaM, 5′-AACGGCAATAAGTCTTTC-3′; 5′-ACCGTGGG CATCGATATC-3′; AspN, 5′-GTTGAGACTCCGCTCAGTCCG-3′; 5′- CATAAGGCTTGACGGAAGGC-3′; AspC, 5′-GGAAACAGCCAGA CTTAGC-3′; 5′-GCTTGTGCAGGCAGATAACA-3′.

Adult brain size analysis

Age-matched females from each genotype were decapitated using a dissecting needle. Forceps were used to remove the mouthparts, and heads were placed in a 1.5-ml microfuge tube filled with 8% PFA/SF900. Tubles were then placed in a shaking incubator set at 37°C and 250 rpm for at least 1 h. Samples were rinsed three times in PBS and further dissected by removing eyes and the remaining cuticle using forceps. Intact brains were placed on a stage micrometer slide under a Leica stereomicroscope outfitted with an IC80 HD camera (Leica) and captured. Measurements of brain width were performed in Fiji by drawing a straight line across the outermost tips of the optic lobes.

RNA extraction, cDNA synthesis, and quantitative PCR

For fly tissue, 10 pairs of ovaries from yw and asp25Df/adult females were dissected in triplicate in SF900 media and RNA extracted using 500 μl Trizol (Life Technologies). For tissue culture, ~106 cells were pelleted at 2,500 g and homogenized in 500 μl Trizol. Samples were treated with Turbo-free DNase (Life Technologies), and 1 μg RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR runs were performed on a Light Cycler 96 (Roche) using a two-step amplification protocol at 60°C with iQ SYBR Green Supermix (Bio-Rad) and 1 μl cDNA. Relative expression was calculated after the ∆∆Ct method using Rp49 primers as the normalizer. A paired t test was used to assess statistical significance based on three biological replicates per treatment. Asp and CaM primer sequences are available on request.
Online supplemental material
Fig. S1 shows cam and asp transcript levels via quantitative PCR after RNAi treatment for the experiment outlined in Fig. 1 (A and C), the effect of W-7 or control DMSO treatment on S2 cells expressing GFP-CaM and RFP-tubulin, and the pole focusing consequences after CaM and Asp loss in acentrosomal cells. Fig. S2 highlights the IQ motifs deleted to generate AspFL, the ability of AspFL and AspΔIQ to dimerize with Asp and the lack of stability for both Asp and AspFL after CaM depletion. Fig. S3 outlines the spindle pole localization for GFP-CaM in S2 cells. Fig. S4 shows the full panel from Fig. 7 D regarding spindle polarity establishment and maintenance, examples of both correct centrosome inheritance and GMC inheritance in asp+/− NBs, and analysis of NB number and mitotic cells in WT and mutant brains. Video 1 shows S2 cells expressing GFP-CaM and RFP-Asp. Video 2 shows mitotic NBs expressing GFP-CaM. Video 3 shows the same NB described in Video 2, but with the metaphase duration only to highlight streaming. Video 4 shows NB expressing asp−/−, asp+ or aspΔIQ. Video 5 shows NB from an asp+/−/Δf mutant expressing tubulin-GFP. Video 6 shows NB from an asp+/−/Δf mutant expressing tubulin-GFP. Video 7 shows NB from an asp+/−/Δf mutant expressing Tubulin-GFP. Video 8 shows NB from an asp−/−/Δf mutant expressing tubulin-GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201509054/DC1.

Acknowledgments
We thank members of the Rusan and A. Kelly Laboratories (National Cancer Institute, National Institutes of Health) for critical discussion and comments throughout this project. We thank K. Plevock for help with RNAi experiments, D. Lerit for critically reading of the manuscript, and C. Gonzalez for discussion and sharing reagents.

N.M. Rusan is supported by the Division of Intramural Research at the National Institutes of Health/National Heart, Lung, and Blood Institute (1ZIAHL006126).

The authors declare no competing financial interests.

Submitted: 11 September 2015
Accepted: 27 October 2015

References

Asp-Cam complex facilitates centrosome-pole attachment • Schoborg et al. 997


