Current Biology 17, 1735–1745, October 23, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.09.031

Article

Asterless Is a Centriolar Protein Required for Centrosome Function and Embryo Development in *Drosophila*

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Summary

Background: Centrosomes, the major organizers of the microtubule network in most animal cells, are composed of centrioles embedded in a web of pericentriolar material (PCM). Recruitment and stabilization of PCM on the centrosome is a centriole-dependent function. Compared to the considerable number of PCM proteins known, the molecular characterization of centrioles is still very limited. Only a few centriolar proteins have been identified so far in *Drosophila*, most related to centriole duplication.

Results: We have cloned *asterless* (*asl*) and found that it encodes a 120 kD highly coiled-coil protein that is a constitutive pancentriolar and basal body component. Loss of *asl* function impedes the stabilization/maintenance of PCM at the centrosome. In embryos deficient for Asl, development is arrested right after fertilization. Asl shares significant homology with Cep152, a protein described as a component of the human centrosome for which no functional data is yet available.

Conclusions: The cloning of *asl* offers new insight into the molecular composition of *Drosophila* centrioles and a possible model for the role of its human homolog. In addition, the phenotype of *asl*-deficient flies reveals that a functional centrosome is required for *Drosophila* embryo development.

Introduction

As the major organizer of the microtubule cytoskeleton in most animal cells, the centrosome provides essential functions required for cell proliferation, differentiation, and development [1–3]. Centrosomes consist of a pair of centrioles surrounded by a matrix of pericentriolar material (PCM) that contains the proteins involved in microtubule nucleation and other cellular processes regulated by the centrosome. A significant number of PCM proteins have been identified in different experimental species by genetic analysis, via antibodies raised against purified centrosomes, or by homology with centrosomal proteins identified in other species [4]. In *Drosophila*, examples include the Polo and Aurora-A kinases [5, 6], founding members of the Polo/Plk and Aurora families; CP190 and CP60 [7, 8]; Cnn [9, 10]; DTACC and Msps [11–13]; and components of the γTURC [14–18].

Data on the molecular composition of centrioles and the pathways that control their assembly are very limited [19]. Most of our current knowledge on centrioles comes from studies in *C. elegans* where genome-wide RNAi and genetic screens have identified a number of proteins essential for centriole duplication. These include SAS-4, SAS-5, SAS-6, ZYG-1, and SPD-2 [20, 21]. Some of these proteins seem to be conserved in evolution. *Drosophila* Sak and its human orthologs Plk4 are related to *C. elegans* ZYG-1 [22, 23], and DSas-4 and CenpJ/CPAP are the suspected orthologs of *C. elegans* SAS-4 in *Drosophila* and humans [24]. Orthologs of SAS-6 have also been identified in humans [25] and *Drosophila* [26]. Other proteins necessary for centriole duplication in *Drosophila* are Ana1 and Ana2 that were identified in an RNAi screen in S2 cells [27]. In addition to these, the only other known centriolar proteins in *Drosophila* are Unc and D-Plp, reported to be required mostly for ciliogeneis [28, 29].

The gene *asterless* (*asl*) was identified by B. Wakimoto in a screen for mutants that affect male fertility [30]. Cytological studies showed that in larval neuroblasts and spermatocytes mutant for *asl*, γTUB accumulation and aster nucleation were highly defective [30–32]. We have cloned *asl* and found that it encodes a constitutive pancentriolar protein. The *asl* mutant phenotype reveals that Asl function is required for PCM recruitment and that a functional centrosome is mandatory for embryo development. The *asl* gene encodes a large, highly coiled-coil protein that shares significant homology

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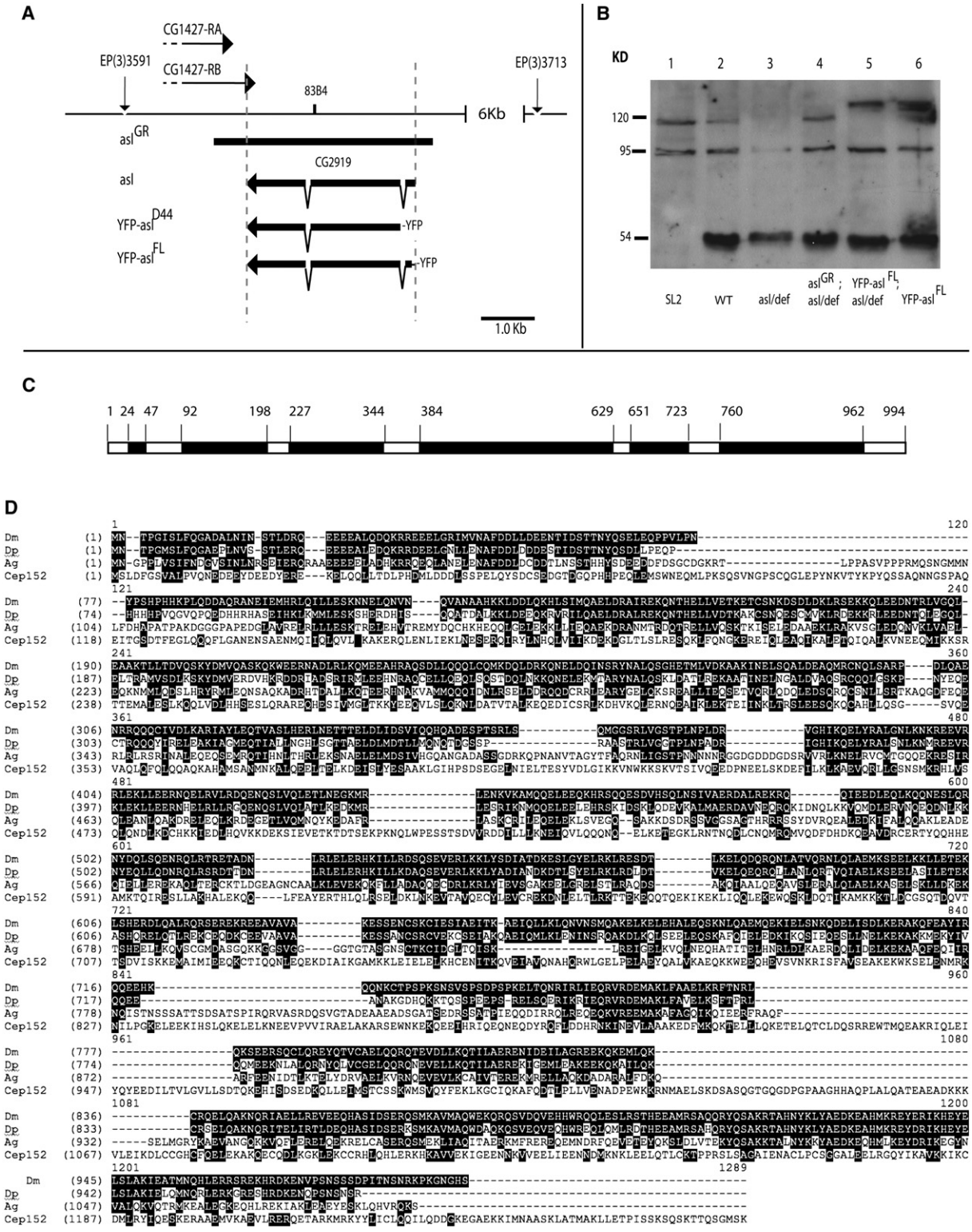

Figure 1. Cloning the *asl* Gene

(A) The *asl* locus maps in the region between P elements *EP(3)3591* and *EP(3)3713* in band 83B4 of the third chromosome, which includes loci *CG2919* and *CG1427*. *CG1427* has two isoforms (RA and RB). The genomic construct (*asl*^{GR}), as well as the full-length coding region from *CG2919* (2.99 kb) fused to *YFP* (*YFP-asl*^{FL}), rescue the *asl* mutant phenotypes, while a truncated version lacking the first 44 C-terminal amino acids (*YFP-asl*^{D44}) does not, suggesting that the *asl* locus corresponds to gene *CG2919*.

(B) In western blot, Asl antibody Rb5110 recognizes a 120 kDa protein, corresponding to the expected Mw of Asl, in SL2 cells and wild-type 1–2 hr embryo extracts (lanes 1 and 2). The 120 kDa protein is absent in extracts of embryos from *asl*^{1}*/Df(3R)ED1557* females (lanes 3 and 5) but is present in embryos from *asl*^{1}*/Df(3R)ED1557* females carrying the *asl*^{GR} construct (lane 4). In embryos expressing *YFP-asl*^{FL}, a band with a rMW

with Cep152, one of the proteins identified in a proteomic analysis of human centrosomes [33]. No functional data have yet been published on CEP152. The cloning of *asl* now offers a new tool to further characterize centriole function in *Drosophila* and a possible model for the role of its human homolog.

Results

Drosophila Asl Is a Highly Coiled-Coil Protein and Shares Homology with the Human Centrosomal Protein Cep152

The gene *asl* was originally mapped by meiotic recombination 0.03 cM proximal to *Ki* (47.3), within region 83A1-83D3 of the polytene chromosomes [30]. Three alleles of *asl* have been described: asl^1 (or *asl*), asl^2, and asl^3, all three EMS induced [30]. As a first step toward cloning the *asl* gene, we decided to map it more precisely by P element-induced recombination of the asl^1 allele in the male germline [34]. By taking advantage of a series of available P elements inserted in this region, we were able to narrow down the position of *asl* to a short stretch of 11.735 base pairs, between the insertion points of *EP(3)3591* and *EP(3)3713* (Figure 1A). Only two genes have been predicted to be encoded by this region: *CG1427* and *CG2919* (Figure 1A). No mutant alleles of these genes have been described. A fragment of a third gene, *Rga*, is also within this region, but it is truncated by *EP(3)3713* and it is not allelic to *asl*. We therefore focused our attention on *CG1427* and *CG2919*. We found that $P[asl^{GR}]$, a transgene carrying a copy of the genomic region that spans from 300 nucleotides upstream to 500 nucleotides downstream of the *CG2919* coding sequence (Figure 1, asl^{GR}), restored viability of the lethal *asl* transheteroallelic combinations, as well as male and female fertility in all sterile combinations (Table S1 in the Supplemental Data available online). Because only a truncated form of *CG1427*, which lacks all the 5′UTR and the first 45 nucleotides of the ORF, is present in $P[asl^{GR}]$, the rescue of *asl* mutant phenotypes by the $P[asl^{GR}]$ transgene strongly suggested that *CG2919* was the *asl* gene. To confirm this possibility, we tested another transgene, $P[YFP\text{-}Asl^{FL}]$, which expresses a fusion between YFP and the coding sequence of *CG2919* under the control of the *poly-Ubiquitin* promoter. We found that $P[YFP\text{-}Asl^{FL}]$ was also able to rescue viability and fertility in all transheteroallelic combinations, including those over *Df(3R)ED1557* that uncovers *CG2919* (Table S1). Another transgene, $P[YFP\text{-}Asl^{D44}]$, similar to $P[YFP\text{-}Asl^{FL}]$ but lacking the sequences encoding the first 44 amino acids and the first intron of *CG2919*, did not rescue any of the *asl* phenotypes. These results confirmed that Asl is encoded by *CG2919*.

In extracts made from S2 cells or from embryos derived from wild-type females, western blotting with Rb5110, an antibody raised against the C-terminal 16 amino acid peptide of *CG2919*, recognizes one band of about 120 kD that corresponds to the MW predicted from the *asl* coding sequence and a second band of about 95 kD. A third band with a rMW of 54 kD is recognized in extracts from embryos, but not in S2 cells (Figure 1B, lanes 1 and 2). The 120 kD band is absent in embryos laid by $asl^1/Df(3R)ED1557$ females (Figure 1B, lanes 3 and 5), as well as in embryos laid by females carrying any of the viable transheteroallelic combinations asl^1/asl^1, asl^1/asl^2, or asl^1/asl^3 (not shown). In embryos derived from transgenic $asl^1/Df(3R)ED1557$ females expressing the genomic rescue construct $P[asl^{GR}]$, the 120 kD band is restored to endogenous levels (Figure 1B, lane 4). In embryos derived from $asl^1/Df(3R)ED1557$ females expressing the $YFP\text{-}asl^{FL}$ fusion, the 120 kD band is replaced by a slightly overexpressed, higher molecular weight band of the size expected for this fusion (Figure 1B, lane 5), which can also be observed, together with the 120 kD band in embryos derived from wild-type females expressing the YFP-asl^{FL} fusion (Figure 1B, lane 6). These results strongly suggest that the 120 kD protein recognized by the Rb5110 antibody is Asl. Upon sequencing of the genomic region of the asl^1 and asl^2 alleles covered by the $P[asl^{GR}]$ transgene, no significant polymorphism were identified. This is a surprising result given the fact that asl^{GR} contains all sequences required to provide *asl* function. However, a 71 base pair deletion (from bp 2381 to 2452) was observed in the coding sequence of asl^3. This deletion shifts the reading frame and introduces a premature stop codon at position 795.

CG2919 encodes a 994 amino acid protein with six predicted coiled-coil motifs that span 86.7% of the protein (Figure 1C). Stretches of low sequence complexity link these domains. Comparison with public protein databases by BLAST identified Asl homologs in different *Drosophila* species like *D. pseudoobscura* (64% sequence identity) as well as in mosquito (*A. gambiae*; 32% identity; Figure 1D). PSI-BLAST search based on the cluster of highly conserved Asl homologs in insects identified the human centrosomal protein Cep152 [33] as an Asl homolog in humans (E = 6e-20; see Experimental Procedures). In addition, *Drosophila* Asl and human Cep152 were identified as putative orthologs by reciprocal best hit analysis with BLAST, as well as by the orthology prediction software of the Ensembl Genome Browser and the eukaryotic ortholog database Inparanoid (see Experimental Procedures). Identity throughout the entire protein sequence between Asl and Cep152 is 13% (Figure 1D).

Asl Is a Constitutive Centriolar Protein

To determine the subcellular localization of Asl, we first immunostained wild-type testes with the Asl antibody Rb5110. PCM and chromatin were counterstained with γTUB antibodies and DAPI. Figure 2A summarizes the main stages of PCM reorganization that take place

corresponding to that of the fusion protein is observed (lines 5 and 6). Two additional bands of 95 kDa and 54 kDa (the latter absent in S2 cells) are also recognized by this antibody.

(C) Black boxes correspond to the six predicted coiled-coil domains of Asl, which cover 86.7% of the protein.

(D) Clustal IW alignment of the Asl protein sequence from *Drosophila melanogaster* (Dm) against the most closely related proteins in *Drosophila pseudoobscura* (Dp), *Anopheles gambiae* (Ag), and *Homo sapiens* (Cep152). Amino acid similarities (gray boxes) between *Drosophila melanogaster* Asl and its homologs in *Drosophila pseudoobscura*, *Anopheles gambiae*, and *Homo sapiens* is 64%, 34%, and 13%, respectively.

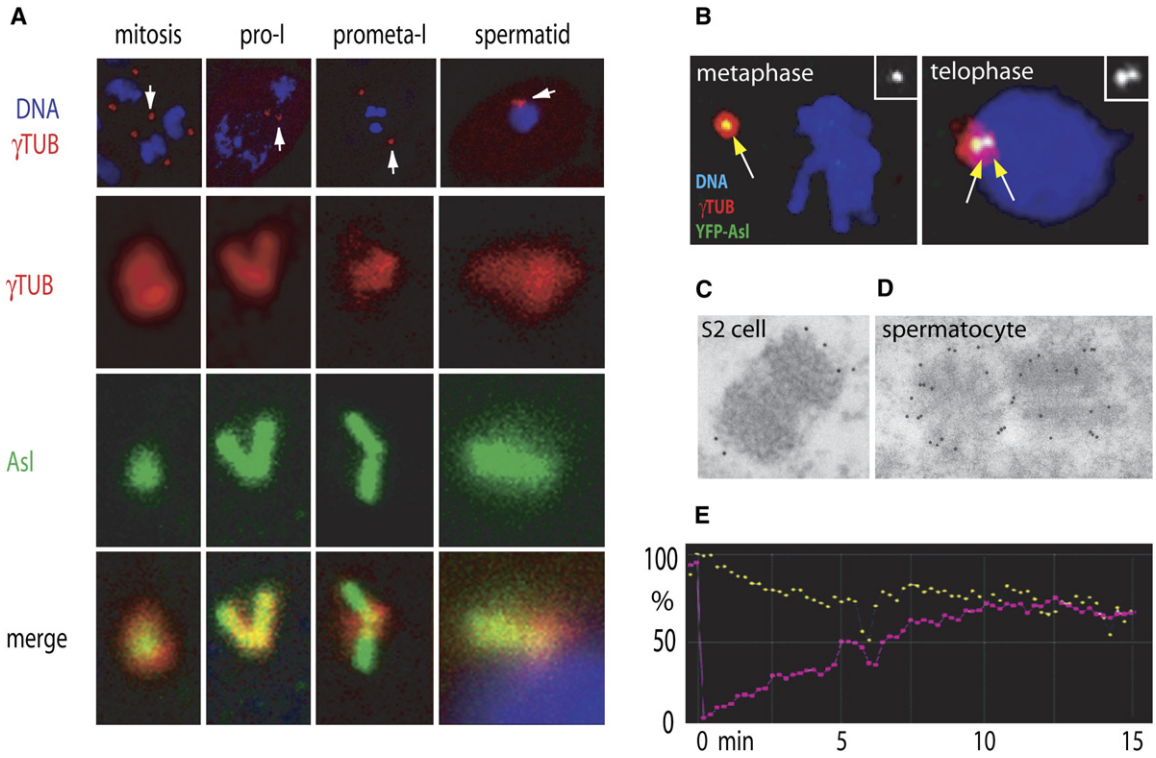

Figure 2. Asl Is a Centriolar Protein

(A) Projection of confocal sections from cells labeled with antibodies against γ-tubulin (red) and Asl (green) and counterstained for DNA (DAPI, blue). The first row includes a low-magnification view of cells at different stages of spermatogenesis, including spermatogonial mitosis, prophase I, prometaphase I, and early spermatid. The rows below are insets showing high-magnification views of the centrosomes marked with an arrow in those cells. The PCM, as revealed by the localization of γ-tubulin (red), is notoriously reshaped during spermatogenesis: globular in the mitotic cycles that precede meiosis, V-shaped like the pair of centrioles described by EM in prophase, then globular again, corresponding with the proximal ends of the two centrioles in prometaphase I, and finally, after completion of meiosis, localized over the basal body. Asl localization (green) is significantly different as it remains constrained to the centrioles throughout this process.

(B) Projection of confocal sections from syncytial embryos showing YFP-Asl^{FL} (green), γ-tubulin (red), and DNA (blue). YFP-Asl^{FL} is tightly concentrated at the core of the metaphase centrosome inside the γ-tubulin signal (arrow). In late telophase, as the centrioles begin to split in preparation of the next cell cycle, YFP-Asl^{FL} reveals two dots within each centrosome (inset, double arrows).

(C) Immunogold labeling of *Drosophila* SL2 cells with Asl antibody. Asl was detected along the periphery of the centriole.

(D) Immunodetection of Asl on a section of plastic-embedded spermatocytes expressing YFP-Asl and immunostained with GFP antibodies. On a cross section through a centriole pair, YFP-Asl^{FL} was observed at the periphery of centrioles along the entire centriole length. No YFP-Asl^{FL} was detected in the PCM.

(E) Upon photobleaching, the centriolar YFP-Asl^{FL} signal (red dots) shows a half-turnover rate of ~5 min. Yellow dots show signal from unbleached neighbor centrosomes.

through spermatogenesis. During the four rounds of mitosis in spermatogonia, two dots of γTUB signal, one at each side of the metaphase plate, reveal the PCM of each centrosome in these cells (Figure 2A, mitosis). At this stage, Asl signal is located at the core of each centrosome, surrounded by the PCM. During prophase of meiosis I, the PCM is dramatically reshaped and enlarged (Figure 2A, pro-I), appearing as two rods, about 2 μm in length each, joined to form a V-shaped structure. At this stage, Asl largely colocalizes with the PCM. A second reorganization of the PCM is observed during prometaphase, as the PCM coalesces back into a compact mass (Figure 2A, prometa-I). At this stage, Asl remains as a V-shaped figure, the vertex of which overlaps with the PCM. After completion of meiosis, early spermatids contain a spot of γTUB that is close to the nucleus (Figure 2A, spermatid) and is made of two domains of different γTUB concentrations, the proximal being more heavily labeled by the γTUB antibodies than the distal. In these cells, the Asl antibody stains one rod that spans the entire length of the low-γTUB density domain and partially colocalizes with the high-γTUB density domain. Asl was also detected in individualized, fully mature sperm (not shown) where, as previously reported, γTUB cannot be observed [35]. EM studies have shown that in spermatocytes, before meiosis, the centriole pair acquires a distinctive V shape that is large enough to be observed by light microscopy and remains such until the end of meiosis I, when each of the two rods are segregated apart in preparation for meisosis II [36]. Thus, throughout spermatogenesis, Asl colocalizes with centrioles and remains basal body bound in fully matured sperm. No signal could be detected by immunofluorescence with the Asl antibody Rb 5110 in spermatocytes from *asl*^{1} males (Figure S1).

We then followed YFP-Asl^{FL} localization in embryos during early cleavage mitosis. At metaphase, YFP-Asl^{FL} (Figure 2B, yellow) reveals a core structure surrounded by PCM (Figure 2B, red). The centrosome cycle during these nuclear divisions, which proceed very rapidly

without intervening gap phases, is somewhat different from the canonical cycle. In these syncytial cycles, centrosomes split apart and start to segregate by late anaphase [37]. At this stage, YFP-Asl^{FL} labels two dots at the core of the replicating centrosomes (Figure 2B, double arrows). Asl also localizes to the centrioles throughout the cell cycle in larval discs and brains (not shown; [38]).

To determine the precise localization of Asl at the ultrastructural level, we used immuno-electron-microscopy. Three different experimental conditions were tested. First, we immunostained Schneider's S2 cells with the Asl antibody Rb5110 (Figure 2C). Second, we immunostained sections of testes expressing YFP-Asl^{FL} with an GFP antibody (Figure 2D). Finally, we immunostained whole-mounted embryo centrosomes purified by centrifugation through sucrose gradients with the Asl antibody (not shown). All three tests revealed that Asl and the YFP-Asl^{FL} fusion are closely bound to centrioles, largely located on the periphery of the centriole barrel along its entire length. All centrosomes studied, whether by immunofluorescence or immuno-EM, contained Asl. Altogether, these data strongly suggest that Asl is a constitutive component of *Drosophila* centrioles and basal bodies and that the centriolar localization of Asl is not cell cycle dependent.

FRAP analysis in living embryos expressing YFP-Asl^{FL} showed that photobleached centrioles recover endogenous levels of centriole-bound YFP-Asl^{FL} signal with a half-turnover rate of ~5 min (Figure 2E). A similar rate of YFP-Asl^{FL} turnover was observed in larval neuroblasts (not shown). This dynamic behavior of Asl is markedly different from that of the other two centriolar proteins subjected to FRAP analysis so far in *Drosophila*. In the case of PACT-GFP, a fusion between the Pericentrin/AKAP450 centrosomal targeting (PACT) domain of *Drosophila*, FRAP of the PCM-bound signal occurs rapidly, with a half-turnover rate of 1–2 min, while the centriole-bound signal recovers only in the following round of centriole replication [29]. The same applies to Unc-GFP that shows no sign of recovery 1 hr after photobleaching [28].

Asl Function Is Required for PCM Recruitment and Centriole Orientation

To further characterize the function of Asl, we analyzed *asl^{1}* mutant spermatocytes expressing the centriolar marker PACT-GFP [29] and immunostained with antibodies against γTUB to reveal the PCM. In wild-type spermatocytes from prophase to prometaphase, PACT-GFP reveals the centrioles as two pairs of rods that partially overlap with the PCM (Figure 3A). In *asl^{1}* spermatocytes, where no Asl protein could be detected (Figure S1), the centrosomes were severely perturbed in two regards (Figure 3A). First, two PACT-GFP-decorated, V-shaped centriole pairs were rarely observed. Rather, the PACT-GFP signal in most *asl^{1}* spermatocytes appeared as one cluster of irregularly shaped structures (Figure 3A). In some unfixed, living *asl^{1}* spermatocytes where the PACT-GFP signal is sharper, these clusters seems to be made of rods like those seen in wild-type cells, but randomly arranged (Figure S2). Consistent with these observations, nonserial EM sectioning of these cells showed that most *asl^{1}* spermatocytes contained a single cluster of up to four centrioles (Figure 3B). Centriole ultrastucture was normal in most cells (Figure S3), with only a very small fraction of the sections showing minor alterations at this level (Figure S3). Therefore, the irregularly shaped centriolar material revealed by PACT-GFP in *asl^{1}* spermatocytes, rather than reflecting gross structural centriole abnormalities, seem to correspond to groups of up to four clustered centrioles that fail to segregate and may have lost the geometric arrangement stereotypical of wild-type centriole pairs. Loss of D-plp has also been reported to compromise the orthogonal orientation of mother and daughter centrioles [29].

The centrosomes in *asl* spermatocytes were also abnormal in that the amount of PCM material associated with the centrioles was highly reduced: in 70% of the cells, the γTUB signal was below 25% of the average signal observed in control cells, the remaining 30% of the cells showing no significant accumulation of γTUB around the centrioles (Figures 3A and 3C). Such loss of PCM recruitment and the resulting failure to nucleate microtubules have direct consequences in spindle assembly (Figure 3C). In cells where the centrosomes do not recruit detectable amounts of PCM, microtubule organization is anastral and chaotic (Figure 3C, asterisk). In cells where some PCM is recruited, loss of centrosome segregation results in either one single aster (Figure 3C, arrow) or two (Figure 3C, double arrows) that remain very close to each other. In a few instances, the asters separate and bipolarity is established (Figure 3C, arrowheads). Thus, the expressivity of the *asl^{1}* phenotype varies from virtually acentrosomal cells to cells that contain two segregated asters before NEB. However, even in those cases where PCM was recruited and microtubule asters were organized, the asters were always much smaller and less dense than in control cells. Video recording in living *asl^{1}* spermatocytes expressing GFP-α-tubulin revealed that in the few cells that initiated spindle assembly with well-separated centrosomes, biastral bipolar spindles were organized (Figure S4). In the case of cells with nonseparated centrosomes, the microtubule arrays got organized into a monastral spindle. Monastral spindle figures were the main spindle type observed in *asl^{1}* spermatocytes (Figure S5). Thus, the abnormal centrosome function caused by the *asl* mutation results in reduced microtubule nucleation and severe defects in meiotic spindle assembly, which in turn results in a high incidence of aneuploidy (Figure S5) [30].

A Functional Centrosome Is Required to Initiate Development

Fertilization contributes the first centriole of the developing *Drosophila* embryo via the sperm basal body [37]. We found Asl to be a constitutive element of the sperm basal body and hence to be paternally contributed (not shown). However, Asl is also maternally provided in quantities that significantly exceed the amount of paternal centriole-bound Asl. This fact, together with the high turnover rate of centriolar Asl that we have documented before, results in the fast replacement of the basal body-bound Asl by the maternal pool. Thus, in unlabelled eggs fertilized by males expressing the YFP-Asl^{FL} fusion, the basal body loses its YFP signal

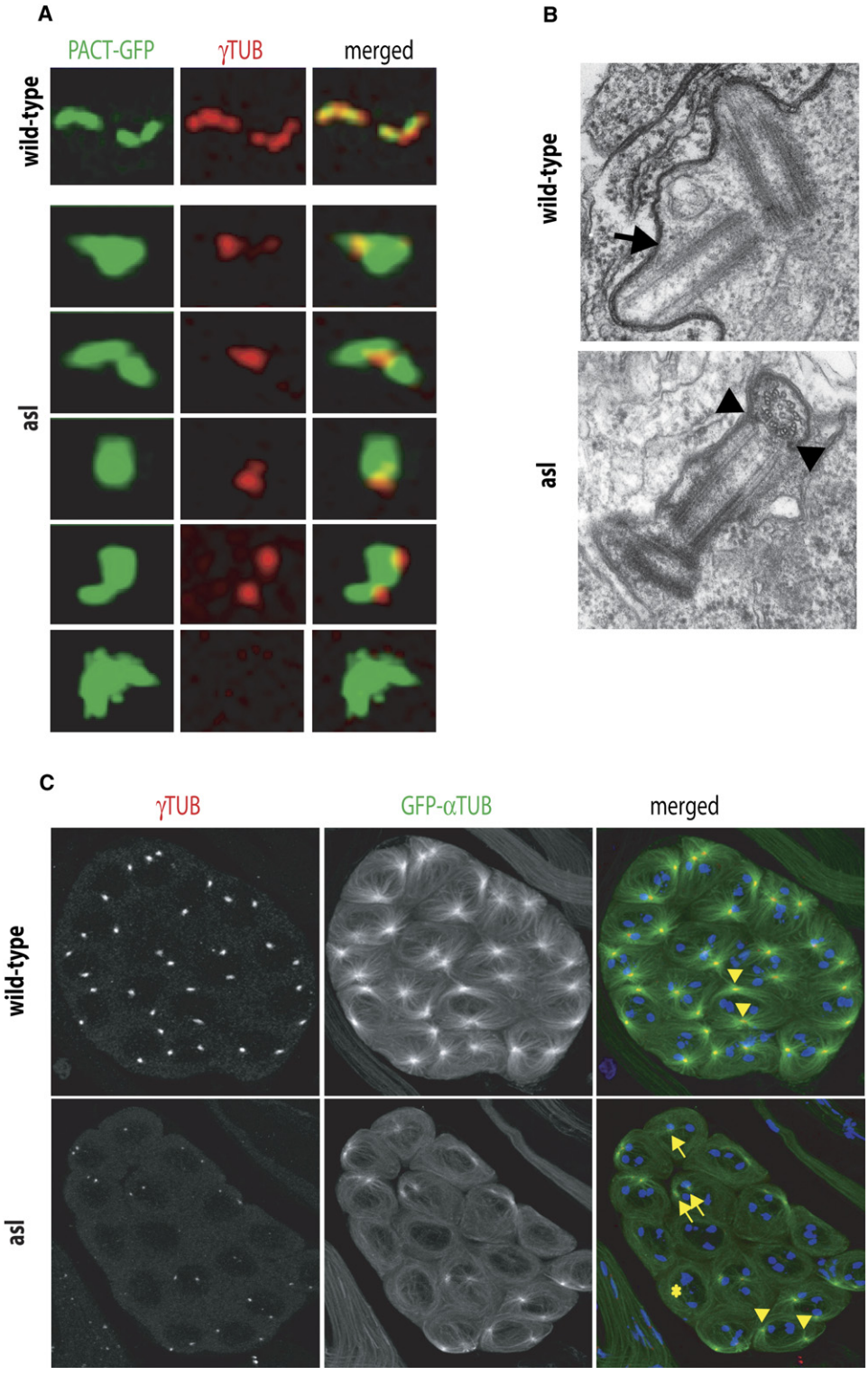

Figure 3. PCM Stabilization, Centrosome Segregation, and Microtubule Nucleation Require *asl* Function

(A) Projection of confocal sections from spermatocytes expressing PACT-GFP (green) and immunolabelled with γ-tubulin antibodies (red). Wild-type meiotic prophase I spermatocytes contained four centrioles organized into two pairs of orthogonally oriented centrioles. In *asl* mutants, PACT-GFP reveals a cluster of centriolar signal. γTUB localization in these clusters is highly irregular, ranging from undetectable to one or a few small dots over the much larger PACT-GFP signal.

(B) Ultrastructural analysis of centrioles in wild-type and *asl* primary spermatocytes. In wild-type premeiotic spermatocytes, the V-shaped centriole pairs are positioned with the distal part of the orthogonally oriented centrioles protuding slightly under the plasma membrane (arrow). In *asl* spermatocytes, the two centriole pairs are clustered so that the distal end of a centriole may be found in close contact with the centriole of the other pair (arrowheads).

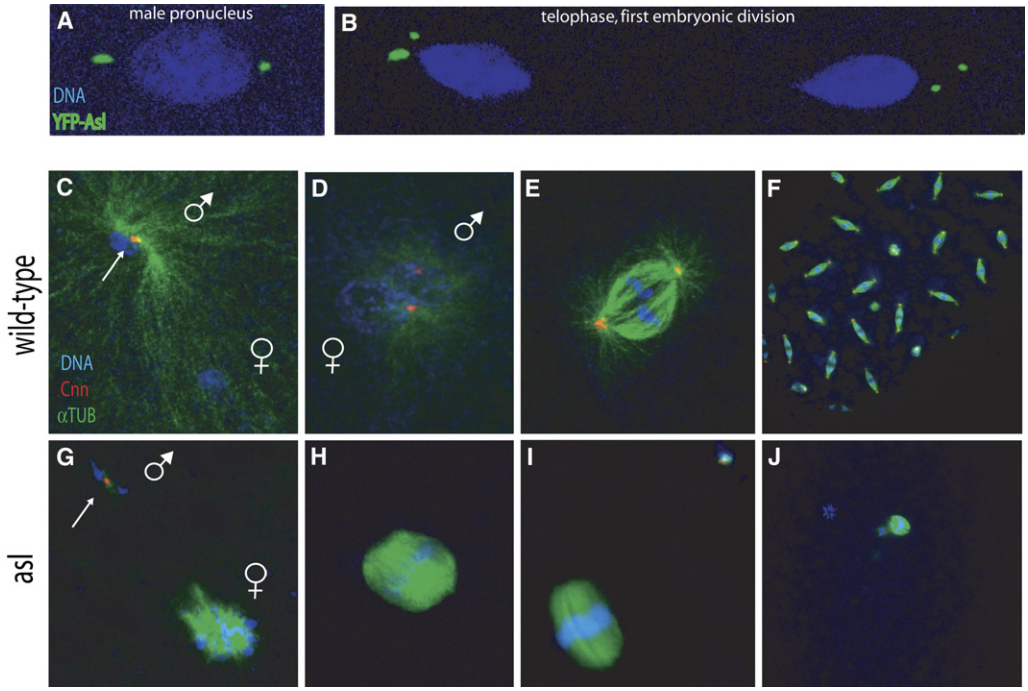

Figure 4. Maternally Provided Asl Is Required for Embryo Development

(A) Male pronucleus derived from wild-type unlabelled sperm in an egg derived from a fly expressing YFP-Asl. Because of the relatively high turnover of the Asl protein, the sperm basal body incorporates the YFP-Asl label immediately after fertilization and can be seen serving as a centriole in one of the centrosomes (long green rod).

(B) After centrosome duplication, the paternally derived centriole is still visible during the first telophase as the longest of the four YFP-Asl-labeled structures.

(C) In the wild-type, the basal body-derived centrosome (cnn, red, white arrow) associated with the male pronucleus (DAPI, blue) nucleates an aster (α-Tub, green) while the female pronucleus is still at a distance.

(D and E) After pronuclear fusion and centrosome duplication (D), the first mitotic spindle is assembled (E). This first zygotic division is gonomeric: the maternal and paternal chromosome sets are still clearly separated.

(F) In less than an hour, exponential nuclear proliferation fills the wild-type embryo with hundreds of nuclei.

(G–I) Assembly of the sperm aster, centrosome duplication, and pronuclear fusion are absent in embryos derived from *asl*^{1}/*Df(3R)ED1557* females fertilized by wild-type males (G). An anastral, barrel-shaped microtubule array is formed around the female-derived chromosomes (H and I), but chromosome segregation was never observed.

(J) As a consequence thereof, development was completely arrested at this early stage in *asl*^{1}/*Df(3R)ED1557* embryos.

soon after fertilization and becomes untraceable by fluorescence microscopy (not shown). Likewise, when YFP-Asl^{FL}-expressing females are fertilized by wild-type males, the basal body incorporates YFP-Asl^{FL} immediately after sperm entry. This is illustrated in Figure 4A in which the basal body-derived centriole can be seen as a rod-like YFP-Asl^{FL}-labeled structure, significantly larger than the dot-shaped newly synthesized centriole. In the following telophase, after a further round of replication, the rod-like, basal body-derived centriole can still be seen together with the three dot-like new centrioles (Figure 4B). Indeed, the long centriole that originates during spermatogenesis perdures and serves as a centriole beyond the first zygotic mitosis (not shown). These observations show that Asl is associated with centrioles from the first stages of development and that paternal basal body-bound Asl is quickly exchanged with the maternally provided Asl pool.

We then decided to determine whether Asl plays a function during these first stages of zygotic development. After sperm entry and activation, female meiosis is resumed and four haploid nuclei are produced in wild-type eggs [37]. The most internal of these nuclei, which is the closest to the sperm nucleus, becomes the female pronucleus, while the others, usually clustered into a polar body, remain inactive and eventually disappear. Recruitment of PCM around the paternally contributed centriole results in the assembly of the first zygotic centrosome (Figure 4C) and in the organization of a prominent aster, which is thought to mediate male and female pronuclear fusion. Soon afterwards, the duplicated centrosomes migrate apart over the male pronucleus, and fusion with the female pronucleus takes place (Figure 4D). The first mitotic spindle is then assembled (Figure 4E), and repeated rounds of nuclear division cycles result in the exponential proliferation of syncytial

(C) Confocal projections of intact cysts of wild-type and *asl* spermatocytes that expressed GFP-α-tubulin (green) and were immunolabeled with antibodies against γ-tubulin (red). In the wild-type, during prometaphase, centrosomes have segregated and organize dense microtubule asters. In *asl* cells, γ-tubulin accumulation and aster nucleation was highly reduced and the extent of centrosome separation varied greatly among the cells within a cyst. In some cells, one centrosome cluster producing a single aster was seen (single arrow). In others, the two centrosomes were slightly separated (double arrows). A few cells had well-separated centrosomes positioned at opposite sides of the nucleus prior to nuclear envelope breakdown (arrowheads). In some cells, no γ-tubulin accumulation or aster nucleation could be detected (asterisk).

nuclei (Figure 4F). In embryos derived from *asl*^{1}/*Df(3R)ED1557* mothers, female meiosis proceeds normally: distinct polar bodies and female pronuclei can be identified, sperm entry takes place, and upon fertilization, all five nuclei are present and arranged in a seemingly wild-type configuration. Cnn accumulates at a point near the male pronucleus, presumably around the paternally provided basal body that is wild-type for Asl, but a functional MTOC is not organized, development is brought to a halt, and the first zygotic mitosis never occurs (Figure 4G). Instead, nonfunctional, anastral spindle-shaped structures are organized around the chromatin (Figure 4H). These spindles, which do not segregate chromosomes, persist in embryos aged for 1–2 hr (Figures 4I and 4J), a stage at which wild-type embryos contain hundreds of nuclei (Figure 4F). Thus, maternal Asl is needed to facilitate the centrosome function required for initiation of cleavage cycles in the fly. This terminal phenotype is indistinguishable from the phenotype of embryos derived from γ*TUBTW1* homozygous females, which lack the maternal γ*TUB37C* gene [39].

Discussion

Asl Is a Conserved Centriolar Protein that Mediates PCM Assembly

We have found that the gene previously identified by genetic analysis as *asl* corresponds to *CG2919*. By using several fluorescencence microscopy and EM assays, we have found Asl to be a centriolar protein. The number of centriolar proteins identified so far in *Drosophila* is very limited. Aside from Unc (whose expression is restricted to certain developmental stages and is specifically required for basal body function [28]) and the PACT domain-containing protein D-plp (which is found in the PCM as well and whose main function seems to be ciliogenesis [29]), all centriolar proteins identified so far in *Drosophila* are mainly involved in centriole duplication. Such is the case of a number of proteins recently identified, including D-SAS4, D-SAS6, Ana1, Ana2, and SAK/PLK4 [22–24, 26, 27]. Asl is a ubiquitous constitutive component of centrioles and basal bodies. Even in cell types such as the developing oocyte, where centrioles are eventually disposed of, Asl remains centriole bound until the last traces of these organelles disappear. No significant accumulation of Asl is detected in the PCM or at the pole of mitotic spindles. Moreover, no Asl is found in the poles of female meiosis I or II spindles, which are known to be acentriolar, and none of these meiotic spindles is affected by loss of Asl function. Finally, loss of Asl severely diminishes PCM stabilization around centrioles. Aside from their role as basal bodies in cells with cilia or flagella, clustering of the PCM at the centrosome is the main function assigned so far to centrioles [40]. Asl is one of the first identified molecular components that mediate this key process.

Asl contains 994 amino acid residues, of which more than 80% are within coiled-coil motifs. High rMW and coiled-coil content are common traits of centrosomal proteins [4]. Relatively low levels of conservation are also common among these large coiled-coil proteins, suggesting that their function can tolerate many liberal substitutions in the coding sequence. For instance, in the cases of Pericentrin and SAS4, sequence identity between the *Drosophila* and human proteins is a mere 17% in both cases [29, 41]. Asl is no exception. The Asl protein of *Drosophila melanogaster* shares 64% identity with its homolog in the closely related species *D. pseudoobscura* and only 32% with its homolog in the mosquito *A. gambiae*. Iterated BLAST based on the cluster of highly conserved Asl sequences in insects identifies Cep152 as a likely human homolog of Asl (13% sequence identity; Evalue = 6e-20). In addition, orthology prediction algorithms based on reciprocal best-hit analysis suggest that Asl and Cep152 are putative orthologs. Cep152 was identified during a proteomic characterization of purified centrosomes, and a Cep152-GFP fusion has been shown to localize to the centrosome [33]. No functional data on CEP152 has yet been reported.

Centrosomes in Development

In terms of the possible roles that centrioles may play during development, our results show that the first zygotic division never occurs in a cytoplasm deficient for Asl, strongly suggesting that functional centrosomes are needed for embryogenesis in *Drosophila*. The same conclusion was suggested by the observation that in eggs derived from females lacking PCM components like γTUB37C or D-TACC, the first mitotic division does not take place [11, 39]. However, the possibility remained that this early developmental arrest in γTUB37C or D-TACC-deficient embryos could be a downstream consequence of the meiotic defects caused by mutations in these genes [13, 42]. This caveat is now largely circumvented by the phenotype of embryos derived from *asl* mutant females in which both meiotic divisions proceed normally. Thus, although we cannot rule out a possible noncentrosomal function of Asl, the phenotype of embryos derived from *asl* mutant females is consistent with the hypothesis that centrosomes are required for *Drosophila* embryo development.

Previous reports have shown that zygotic loss of key centrosomal proteins such as D-plp, Sas4, Sak/Plk4, or Cnn does not block progression of development into adult flies [10, 13, 22, 24, 29, 43]. However, the centrosome-less females that hatch are sterile, strongly suggesting that eggs defective for these centrosomal components cannot support embryogenesis. How development can proceed in zygotic loss-of-function conditions for these genes is not entirely clear. However, the initial stages of development of individuals homozygous for mutations in these centrosomal proteins are likely to be sustained by the wild-type RNA/protein contributed to the egg by the heterozygous females from which they derive. Thus, until a certain stage that is hard to specify, development in these mutant individuals actually takes place when cells still have centrosomes. The hatching of adults that have undergone the last stages of development without centrosomes certainly proves a certain level of centrosome dispensability in *Drosophila* development, even though such adult flies are uncoordinated and sterile and die only hours after eclosion.

Loss of centrosome function has been reported to impair a number of developmental stages in vertebrates. In humans, for instance, abnormal centrosomes have

been linked to impaired neuronal migration, hereditary spastic paraplegia, Bardet-Biedl syndrome, development of cystic kidneys, perturbed left-right asymmetry, microcephaly, and cancer [1, 44]. In mice, lack of function for Sak/Plk4 is a lethal condition, and haploinsufficiency for this gene results in a high incidence of tumors [45, 46]. The molecular dissection of centrioles in *Drosophila* may help to model the cellular basis of some of these processes.

Experimental Procedures

Drosophila Stocks and Fly Culture

Fly stocks were obtained from Bloomington and Szeged. The alleles asl^1, asl^2, and asl^3 were obtained from M. Gatti [30]. *Df(3R)ED5177* was purchased from Szeged.

Generation of Transgenic Flies

Genomic construct $P[asl^{GR}]$: A genomic fragment spanning from 300 bp upstream the initial ATG to 500 bp downstream of the STOP codon of CG2919 was cloned in the transformation vector pW8. $YFP\text{-}Asl^{FL}$ construct: The entire open reading frame of the *CG2919* gene was amplified from an embryonic cDNA library with primers 5′-ATTTGCGGCCGCTATGAACACGCCAGGTATAAGCCTC TTTC-3′ and 5′-ATTTGCGGCCGCTTAGCTGTGACCATTGCCTTT GGG-3′ and cloned into the EYFP-C1 vector (Clontech) with NotI. The EYFP-C1 vector was modified by introducing additional NotI and NheI sites between the BglII and EcoR1 sites. The resulting fusion was then cut with NheI and cloned into the SpeI site of the *Drosophila* polyubiquitin transformation vector. $YFP\text{-}Asl^{D44}$ construct: This was constructed as $YFP\text{-}Asl^{FL}$ except that primers were designed so that the resulting fusion protein contains a deletion of the 44 N-terminal amino acids. Transgenic flies were generated by standard P element-mediated transformation.

Sequencing

Genomic DNA was isolated from the different *asl* alleles and used as template for PCR with the High Fidelity PCR System (Roche). PCR products were subcloned into PBSK (Stratagene) prior to sequencing. At least two clones from different PCR reactions were sequenced.

Identification of Asl Homologs and Sequence Alignments

BLAST search with the Asl sequence *CG2919* (AAF51993.2) identified homologs in other insect species with highly significant E-values ($E < 1e^{-80}$), including the *Anopheles gambiae* ENSANGP 00000013679 (EAA08928). PSI-BLAST, a standard algorithm for identification of remote homologs, was then used to search for potential human homologs. A first round of PSI-BLAST search with a threshold value of $1e^{-15}$ identified, aside from *CG2919* itself, *Anopheles gambiae* ENSANGP00000013679 ($E = 2e^{-81}$) and *Aedes aegypti* EAT35837 ($E = 2e^{-87}$). These sequences were then used in a second round of iterative search. The three resulting top hits from this search were two isoforms of the centrosomal protein Cep152 from chimpanzee ($E = 8e^{-21}$; accession# XP523070) and human Cep152 ($E = 6e^{-20}$; accession# O94986). In addition, standard BLAST searches with *Drosophila* Asl or human Cep152 confirmed EAA08928 as the best match among *Anopheles gambiae* proteins ($E = 2e^{-81}$). Orthology queries were run in the Ensembl Genome Browser (http://www.ensembl.org/index.html) and the eukaryotic ortholog database Inparanoid [47]. Homology alignments were carried out by Clustal IW [48].

Antibody Production

The Asl antibody Rb5110 was raised against a synthetic peptide corresponding to the 16 C-terminal amino acids of CG2919 (SIGMA-GENOSYS) and affinity purified against the immunogenic peptide.

Western Blotting of Embryo Lysates and S2 Cell Extracts

Embryo lysates: 0–2 hr embryos were collected, and the chorions were removed and boiled in SDS sample buffer. S2 cell extracts: Approximately 2×10^6 cells were pelleted, resuspended, and boiled in SDS sample buffer. Samples were then subjected to PAGE in 7% polyacrilamide gels and blotted onto a nitrocellulose filter. The blotted filter was incubated with affinity-purified Rb5110 diluted 1:100, washed three times in 0.1% PBS-T, incubated with HRP-labeled anti-rabbit IgG (Jacksons), washed as before, and treated with the ECL kit (Amersham) to reveal peroxidase activity.

Immunocytochemistry

Testes were dissected in PBS and fixed in methanol/acetone as described [30]. This fixation preserved the GFP fluorescence in cells expressing GFP-α-tubulin or PACT-GFP [29]. Fixed tissue was incubated 1 hr with primary antibodies diluted in 0.1% PBS-T containing RNAase (Boehringer), followed by three washes in 0.1% PBS-T for 5 min, a 45 min incubation with secondary antibody diluted in 0.1% PBS-T, and a final wash in 0.1% PBS-T for 5 min. DNA was labeled with 5 nM TOTO3 (Molecular Probes) in 0.1% PBS-T for 15 min. Embryos and ovaries were fixed as previously described [49]. α-TUB was detected with DM1a antibody (SIGMA). Cnn antibodies were kindly provided by T. Kauffman and E. Schejter. Other antibodies used were monoclonal mouse anti-γ-tubulin (clone GTU88, Sigma) diluted 1:50; rabbit anti-Asl Rb 5110 diluted 1:200; Alexa 594 labeled goat anti-rabbit IgG (Molecular Probes) diluted 1:200; and Alexa 488 labeled goat anti-mouse IgG (Molecular Probes) diluted 1:200. Fluorescence images were acquired in a Leica TSC SP2 confocal microscope equipped with a 63× oil objective (NA = 1.4) and further processed in Adobe Photoshop.

FRAP Analysis

Syncytial *Drosophila* embryos expressing YFP-Asl^{FL} were recorded in a Leica TSC SP2 confocal microscope equipped with a 63× oil immersion objective (NA = 1.4). Photobleaching was performed by a 15 s pulse of the 488 nm laser line at full power. Florescence recovery was imaged at a rate of 1 frame/10 s for 30 min. Florescence intensities were analyzed with NIH Image.

Electron Microscopy and Immunogold Labeling

Testes were dissected in PBS and fixed in 2.5% glutaraldehyde in PBS at 4°C overnight. The tissue was then post-fixed in 1% osmium and 1% uranyl acetate (UA) on ice, followed by room temperature dehydration in ethanol prior to infiltration and embedding in Epon. For immunogold labeling of spermatocytes, testes of Asl-YFP^{FL}-expressing males were dissected in PBS, fixed in 4% formaldehyde in PBS for 2 hr at 4°C, and cryoprotected in 33% DMF (N,N-dimethylformamid) in 4% formaldehyde/PBS for 15 min [50]. They were then jetfrozen by plunge-freezing in liquid propane, freeze-substituted in 0.5% UA in ethanol, and embedded in K11M Lowicryl. Ultra-thin methacrylate sections on 100 mesh net grids were blocked with PBG (0.2% gelatin, 0.5% BSA in PBS or TRIS) for 10 min. After removal of PBG by blotting, grids were incubated with rabbit anti-GFP antibody diluted 1:200 (Torrey Pines Biolabs, Inc.) for 30 min. Grids were then washed with PBG and incubated with Protein A-10 nm gold probe solution (York-Dieter Stierhof, University of Tübingen) diluted 1:100 in PBG. After washing with blocking buffer and PBS, the sections were fixed in 1% buffered glutaraldehyde for 5 min, washed with distilled water, and contrasted in 1% aqueous uranyl acetate for 2–5 min. In situ immunogold labeling with the Asl antibody was performed on *Drosophila* SL2 cells grown on concanavalin A (Sigma)-coated glass slides. Cells were then fixed and lysed in a 0.1 M cacodylate buffer (pH 7.2) containing 4% paraformaldehyde and 0.3% Triton X-100 (Sigma) for 10 min at room temperature. Free binding sites were blocked with 20 mM glycine and subsequent inclusion of 0.1% bovine serume albumine in all washing and antibody buffers. Incubations with the first (rabbit anti-Asl) and secondary (5 nm gold-labeled anti-rabbit, British Biocell) antibodies were performed overnight at 4°C. Fixation, contrasting, and subsequent embedding was performed in 24-well plates. Glass slides were separated from the polymerized resin by alternative temperature shifts in liquid nitrogen and boiling water. Sections were cut in parallel to the flat resin surface with a diamond knife (Diatome) and then further contrasted. Images were visualized and recorded on a Philips 400 electron microscope.

Supplemental Data

Five figures and one table are available at http://www.current-biology.com/cgi/content/full/17/20/1735/DC1/.

Acknowledgments

The authors are grateful to M. Gatti, J. Raff, T. Kaufman, and E. Schejter for providing reagents and fly strains. The technical advice from G. de Carcer and J. Pei, the assistance provided by B. Sailer and A.M. Voie, and the help from the sequencing services at IRB and EMBL are also very much appreciated. Work in C.G.'s laboratory is funded by EU and Spanish MEC grants COMBIO, MADCD, ONCASYM, SAF, and BFU. Work in B.L.'s current laboratory is funded by the Berliner Senat für Kultur, Wissenschaft und Forschung (EFRE), and NGFN2 (SMP Protein).

Received: July 12, 2007
Revised: August 31, 2007
Accepted: September 12, 2007
Published online: October 11, 2007

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Note Added in Proof

In a recent article by Stevens et al. (Stevens, N.R., Raposo, A.A.S.F., Basto, R., St Johnston, D., and Raff, J.W. [2007]. From stem cell to embryo without centrioles. Curr. Biol. *17*, 1498–1503), it was shown that DSas-4 mutant embryos, which lack centrosomes, also fail to develop significantly, thus adding further evidence to our conclusion that centrosomes are essential for early embryonic development.