

Nuclear-fallout, a *Drosophila* protein that cycles from the cytoplasm to the centrosomes, regulates cortical microfilament organization

Wendy F. Rothwell¹, Patrick Fogarty¹, Christine M. Field² and William Sullivan^{1,*}

¹Department of Biology, University of California, Santa Cruz, California, 95064, USA and ²Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, USA

*Author for correspondence (e-mail: sullivan@biology.ucsc.edu)

Accepted 20 January; published on WWW 26 February 1998

SUMMARY

nuclear fallout (*nuf*) is a maternal effect mutation that specifically disrupts the cortical syncytial divisions during *Drosophila* embryogenesis. We show that the *nuf* gene encodes a highly phosphorylated novel protein of 502 amino acids with C-terminal regions predicted to form coiled-coils. During prophase of the late syncytial divisions, Nuf concentrates at the centrosomes and is generally cytoplasmic throughout the rest of the nuclear cycle. In *nuf*-derived embryos, the recruitment of actin from caps to furrows during prophase is disrupted. This results in incomplete metaphase furrows specifically in regions distant from the centrosomes. The *nuf* mutation does not disrupt anillin or peanut recruitment to the metaphase furrows indicating that Nuf is not involved in the signaling

of metaphase furrow formation. These results also suggest that anillin and peanut localization are independent of actin localization to the metaphase furrows. *nuf* also disrupts the initial stages of cellularization and produces disruptions in cellularization furrows similar to those observed in the metaphase furrows. The localization of Nuf to centrosomal regions throughout cellularization suggests that it plays a similar role in the initial formation of both metaphase and cellularization furrows. A model is presented in which Nuf provides a functional link between centrosomes and microfilaments.

Key words: *Drosophila*, Centrosome, Microfilament, Anillin, Peanut, *nuclear fallout* (*nuf*)

INTRODUCTION

Centrosomes are cytoplasmic organelles that nucleate microtubules and play a key role in determining microtubule number and distribution throughout all the phases of the cell cycle (reviewed in Kellogg et al., 1994). Animal centrosomes typically contain a perpendicularly oriented pair of microtubule-based centrioles surrounded by an electron-dense pericentriolar material. Each centriole consists of a ring of microtubule triplets arranged in a short cylinder. Among cytoplasmic organelles, centrosomes are unique because they are precisely duplicated with each division cycle. In addition to organizing microtubules, centrosomes influence actin dynamics. In many instances, the effect of centrosomes on microfilament distribution occurs indirectly through their effect on microtubules. For example, the position of the centrosomes and their associated asters is a key factor determining the location of the microfilament-based contractile ring in many types of animal cells (reviewed in Rappaport, 1986). Several studies also suggest a more direct interaction between centrosomes and microfilaments (Tamm and Tamm, 1988; Clark and Meyer, 1992; Chevrier et al., 1995).

Although a number of centrosomal proteins have been identified, function has been determined for only a few. For this reason, genetic studies are essential for understanding the

relationship between centrosome structure and function. For example, mutational analysis in *Drosophila* has provided insight into the function of the centrosomal component Centrosomin (Cnn). Cnn, originally identified as a target of Antennapedia, is a centrosomal protein involved in microtubule organization and nuclear division (Heuer et al., 1995; Li and Kaufman, 1996). Mutational analysis of the *Drosophila* Asp protein demonstrates that it is required for the production of normal, functional spindles (Gonzalez et al., 1990). Asp localizes to the spindle poles from prophase to anaphase of the syncytial divisions (Saunders et al., 1997). In addition, a *Drosophila* gamma-tubulin mutation confirms previous genetic and in vitro studies demonstrating that it plays a fundamental role in nucleating microtubules (Sunkel et al., 1995; for recent review see Pereira and Schiebel, 1997).

The early *Drosophila* embryo is a particularly useful system to analyze centrosome function because centrosomes mediate dramatic and readily visualized nuclear and cytoskeletal dynamics at this stage. Early *Drosophila* development is characterized by thirteen rapid synchronous nuclear divisions that occur without accompanying cytokinesis (Zalokar and Erk, 1976; Foe and Alberts, 1983). These divisions initially occur in the interior of the embryo and, during nuclear cycles eight and nine, the nuclei migrate toward the cortex. The nuclei with their closely associated pairs of apically localized centrosomes reach

the actin-rich cortex during interphase of nuclear cycle ten. This induces a dramatic reorganization in which the actin concentrates in caps between the centrosome pairs and the plasma membrane (Warn et al., 1984; Karr and Alberts, 1986; Kellogg et al., 1988). The importance of centrosomes in this reorganization is underscored by the observation that free centrosomes, unassociated with a nucleus, induce actin cap formation (Freeman et al., 1986; Raff and Glover, 1989; Yasuda et al., 1991). As the nuclei progress into prophase, the apical pair of sister centrosomes separate toward opposite poles and the actin reorganizes to form an oblong ring outlining the nucleus and its polar centrosomes. During prophase, actin and the tightly associated plasma membrane invaginate to form furrows between the nuclei. At metaphase, these furrows encompass each newly formed spindle in a half-shell. These structures, termed metaphase furrows, prevent inappropriate interactions between neighboring spindles (Sullivan and Theurkauf, 1995). *daughterless-abo-like* (*dal*), a mutation that disrupts the metaphase positioning of centrosomes, also disrupts metaphase furrow formation, demonstrating that the centrosomes play an important role in this process (Sullivan et al., 1990; 1993b).

Centrosome duplication occurs during late anaphase and the newly formed centrosome pairs are again located apically in the next interphase (Callaini and Riparbelli, 1990). In conjunction with this apical repositioning of the centrosomes, actin caps reform. This alteration between interphase actin caps and metaphase actin-based furrows occurs from interphase of nuclear cycle 11 through interphase of nuclear cycle 14, at which time the embryos undergo cellularization (Karr and Alberts, 1986; Foe et al., 1993).

A growing class of *Drosophila* maternal-effect mutations has been identified in which syncytial division abnormalities occur only after the nuclei migrate to the cortex (reviewed in Sullivan and Theurkauf, 1995). This phenotype is similar to that observed in embryos treated with cytochalasin B, an inhibitor of actin polymerization (Zalokar and Erk, 1976). The specific disruption of the cortical divisions in both of these instances is primarily the result of specific disruptions in the actin caps and/or metaphase furrows. For five of these maternal-effect mutations (*daughterless-abo-like*, *nuclear-fallout*, *grapes*, *scrambled* and *sponge*), their effects on the cortical cytoskeleton have been examined in detail (Sullivan et al., 1990; Postner et al., 1992; Sullivan et al., 1993b). Although each of these mutations produces similar nuclear division abnormalities, each exhibits unique cortical cytoskeletal defects.

Here we present a detailed molecular and cellular analysis of *nuclear fallout* (*nuf*). *nuf* encodes a highly phosphorylated coiled-coil protein and, during the cortical nuclear divisions, localizes to the centrosomes during prophase and is cytoplasmic throughout the rest of the cycle. One of the earliest defects in *nuf*-derived embryos is the disruption of actin recruitment during prophase to the metaphase furrows. *nuf* also disrupts the cellularization furrows and the Nuf protein is concentrated at the centrosomes throughout cellularization. This suggests that Nuf plays a similar function in recruiting actin to the metaphase and cellularization furrows.

We have also utilized the *nuf*-mutant phenotype to investigate the process by which a furrow is constructed. Metaphase furrows and cytokinesis furrows share many common components including actin, myosin II, Sep1, peanut,

spectrin and anillin (Pesacreta et al., 1989; Young et al., 1991; Neufeld and Rubin, 1994; Thomas and Kiehart, 1994; Fares et al., 1995; Field and Alberts, 1995; Miller and Kiehart, 1995). We find that both anillin and peanut are initially able to localize to regions of the furrow devoid of actin in *nuf*-derived embryos. However, as the embryos progress into metaphase, localization of anillin and peanut is maintained only in those regions in which actin is also present. These results are discussed with respect to the role of the centrosome in microtubule and microfilament organization and the mechanisms by which metaphase and cytokinesis furrows are formed.

MATERIALS AND METHODS

Drosophila stocks

The *nuf* mutation has been previously described (Sullivan et al., 1993b). This mutation originated from a screen of single P-element mutations and was generously provided to us by A. Spradling and S. Wasserman. Oregon-R served as the wild-type control stock (Lindsley and Zimm, 1992). All of the experiments described in this manuscript used the *nuf*^f allele (AS928) unless otherwise noted (Sullivan et al., 1993b). All *nuf* embryos were collected from homozygous *nuf*^f females crossed to wild-type males. The stocks were maintained on a standard corn meal/molasses media.

Fixation and immunofluorescence

Embryos were fixed using formaldehyde (Theurkauf, 1992). Immunofluorescent analysis was performed as described by Karr and Alberts (1986). Centrosomes and microtubules were stained with the anti-centrosomin antibody (Heuer et al., 1995; Li and Kaufman, 1996; kindly provided by K. Li) and an anti- α -tubulin antibody, respectively. Actin was stained with fluorescently labeled phalloidin on formaldehyde-fixed hand-devitellinized embryos (von Dassow and Schubiger, 1995). To view the DNA, the embryos were extensively rinsed in PBS and mounted in a 50% glycerol/PBS solution containing 1 μ g/ml N-N-1-4-phenylenediamine. Immunofluorescent analyses using the rabbit anti-anillin (Field and Alberts, 1995), anti-peanut (Field et al., 1996) and anti-Nuf antibodies were performed on formaldehyde-fixed hand-devitellinized embryos.

Microscopy was performed using an Olympus IMT2 inverted photoscope equipped with a Biorad MRC 600 laser confocal imaging system. The cortical nuclear cycles (10-14) were determined by using the Biorad imaging software to estimate nuclear densities.

In vivo fluorescence analysis

The in vivo analysis of nuclear behavior was accomplished by microinjecting fluorescently labeled histones into embryos during the syncytial cortical divisions as described in Minden et al. (1989). The embryos were prepared for microinjection by hand dechoriation and mounting on a coverslip with a thin film of glue (Minden et al., 1989). Observations and time-lapse recordings were made on an Olympus IMT2 equipped Biorad MRC 600 confocal imaging system.

Isolation of cDNA clones and northern analysis

A 1 kb *Hind*III fragment obtained from genomic sequences immediately flanking the P-element insertion site was utilized as a probe in screening the Tolia ovarian gt22A directional cDNA library (Stroumbakis et al., 1994). Three 2.3 kb cDNAs were identified. One of these cDNAs was then used as a probe for northern analysis. Total RNA from 0-3 hour embryos derived from wild-type and homozygous *nuf*^f-females was isolated as previously described (Tamkun et al., 1992). 22 μ g of this RNA was loaded per lane and ribosomal protein 49 (*rp49*) served as a loading control (O'Connell and Rosbash, 1984).

Sequence analysis

Sequencing was carried out using the Amersham/USB sequenase kit version 1.0 (Amersham Corp., Arlington Heights, IL) and a series of oligonucleotide primers. The sequence of the entire cDNA was determined for both strands. Homology searches were performed using the BLAST electronic mail server with blastn and blastp programs (Altschul et al., 1990; Brutlag et al., 1990). Coiled-coil analysis was performed using the algorithm described in Lupas et al. (1991).

Transformation studies

A 2 kb *HindIII*-*NotI* fragment (containing bases 288-2272 of the *nuf* cDNA) was inserted into the *NheI* and the *NotI* sites in a Germ 10 transformation vector (Serano et al., 1994; kindly provided by Robert Cohen). Blunt ends were first created at the *HindIII* and *NheI* sites. The Germ 10 vector contains a nurse cell promoter to drive expression in the oocyte. This construct was then used for P-element-mediated transformation (Spradling and Rubin, 1982).

Antibody production

A 6-His N-terminal tagged fusion protein was created from the N-terminal 150 amino acids of the Nuf ORF (bases 306-747). This fusion protein was affinity purified, analyzed via SDS-PAGE and sent to Berkeley Antibody Company (Richmond, CA) for the production of rabbit polyclonal antibodies. Electrophoresed Nuf protein bound to nitrocellulose was used to affinity purify anti-Nuf antibody (Tsukiyama et al., 1995). The antibodies were eluted with 0.1 M glycine pH 2.5.

Western immunoblotting, immunoprecipitations and dephosphorylation of the Nuf protein

Protein extracts of 0-3 hour wild-type and *nuf*-derived embryos were prepared as previously described (Dingwall et al., 1995). Extracts were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with Tris-buffered saline, 0.1% Tween-20, 5% BSA, and 5% powdered milk for 15 minutes at room temperature followed by incubation with primary antibody in block solution at a concentration of 2.5 µg/ml overnight at 4°C on a rotator. The membrane was washed in TBS, 0.1% Tween-20 and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000 in block solution for 30 minutes at room temperature. The membrane was washed as above prior to undergoing signal detection using ECL western immunoblot detection reagents (Amersham Corp., Arlington Heights, IL). Affinity-purified polyclonal anti-Nuf antibodies were used to

immunoprecipitate the Nuf protein from 0-3 hour wild-type extracts prepared as described above. Immunoprecipitations and their subsequent phosphatase treatment were performed as described by Kellogg et al. (1995).

RESULTS

Nuclei in *nuf*-derived embryos associate during telophase of nuclear cycle 12

Previous fixed analysis demonstrated that *nuf*-derived embryos exhibit irregularities in the shape, size and spacing of their nuclei specifically during the cortical syncytial divisions (Sullivan et al., 1993b). In order to identify the initial nuclear abnormalities that produced this cortical phenotype, we analyzed the nuclear dynamics in living normal and *nuf*-derived embryos (Minden et al., 1989; Sullivan et al., 1993a). Fig. 1A-D depicts a normal embryo injected with fluorescently labeled histones and followed using confocal microscopy from metaphase to telophase of nuclear cycle 12. Although the syncytial nuclei are closely spaced and divide within a single plane, collisions between neighboring nuclei rarely occur. In *nuf*-derived embryos, fusions occur most frequently between neighboring non-sister nuclei that are close to one another and dividing in parallel. Fig. 1E-H depicts a *nuf*-derived embryo as it progresses from metaphase to telophase of nuclear cycle 12. The arrows follow neighboring non-sister telophase nuclei fusing with one another. As observed for the products of many abnormal divisions, these fused nuclei recede into the interior of the embryo (Sullivan et al., 1993a). Similar fusion events of the cortical syncytial nuclei have been observed in embryos treated with the microfilament-disrupting drug cytochalasin and suggest that the telophase fusions observed in *nuf*-derived embryos may be the result of a disruption in the metaphase furrows that encompass the dividing nuclei (Zalokar and Erk, 1976; Sullivan and Theurkauf, 1995).

During the cortical divisions, *nuf*-derived embryos form normal interphase actin caps, but incomplete metaphase and cellularization furrows

To determine the basis of these abnormal divisions, normal and *nuf*-derived embryos were double stained with phalloidin and

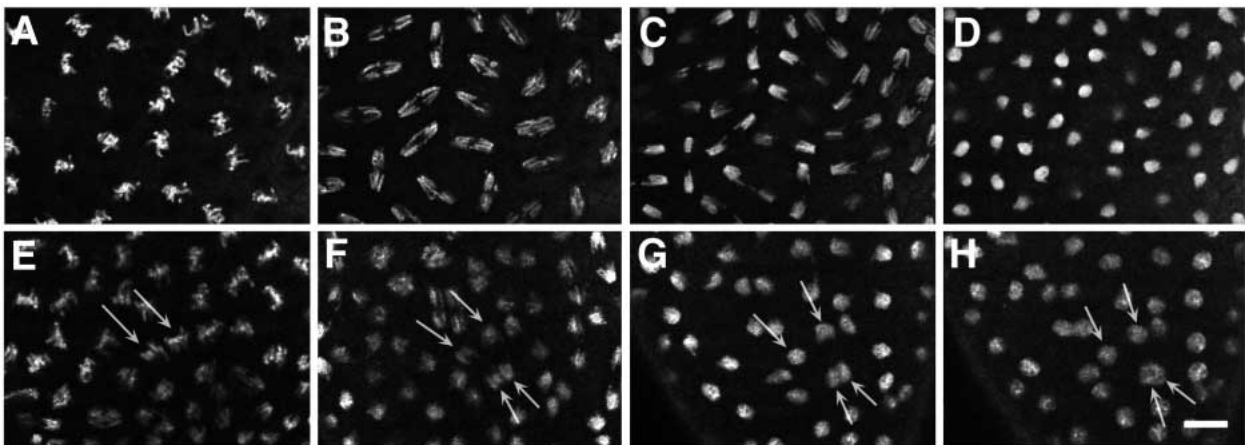


Fig. 1. Nuclear dynamics in *nuf*. Images of living wild-type (A-D) and *nuf*-derived (E-H) embryos injected with fluorescently labeled histones. The nuclear dynamics are followed from metaphase of nuclear cycle 12 (A,E) to interphase of nuclear cycle 13 (D,H). In *nuf*-derived embryos, non-sister telophase nuclei dividing in parallel with each other fuse in the following interphase (E-H, arrows). Bar, 10 µm

propidium iodide to view the microfilaments and nuclei respectively. Fig. 2A depicts a normal embryo during interphase of nuclear cycle 13. The actin is concentrated in an apical cap between each nucleus and the plasma membrane. Fig. 2E depicts a similarly prepared *nuf*-derived embryo at an equivalent stage and demonstrates that the *nuf* mutation does not disrupt actin cap formation during the cortical syncytial divisions.

As shown in Fig. 2B, during metaphase of the cortical divisions, furrows encompass each of the nuclei. In *nuf*-derived embryos, the network of metaphase furrows exhibit extensive gaps (Fig. 2F). These gaps often occur in regions of the furrow that are in line with the metaphase plate (Fig. 2F, arrows). As revealed by embryos double stained for microfilaments and centrosomes (Heuer et al., 1995; Li and Kaufman, 1996), the failure of microfilaments to accumulate at the regions farthest from the spindle poles in *nuf* occurs as early as nuclear cycle 11 (Fig. 2D,H, arrows). In the areas in which the furrows do occur, the depth of the furrows is equivalent to that found in normal embryos. In *nuf*-derived embryos, the furrows are usually intact at the spindle poles. Consequently, directly opposing telophase nuclei rarely collide. The tendency of the gaps to occur in regions farthest from the poles and near the metaphase plates readily account for the observed pattern of telophase fusions: neighboring non-sister nuclei dividing in parallel fuse with one another.

Cellularization occurs during interphase of nuclear cycle 14. At this stage, furrows form around each nucleus to produce the honeycomb pattern depicted in Fig. 2C. In *nuf*-derived embryos, cellularization is severely disrupted (Sullivan et al., 1993b). The furrows are incomplete and often encompass multiple nuclei (Fig. 2H). This cellularization phenotype is similar to that observed in the zygotic mutations *nullo* and *serendipity* (Schweisguth et al., 1990; Simpson and Wieschaus, 1990; Rose and Wieschaus, 1992).

During prophase, in *nuf*-derived embryos, anillin and peanut localize to regions of the furrows lacking actin

We employed immunofluorescent analysis to determine the localization of the furrow components anillin and peanut with respect to actin during furrow formation in normal and *nuf*-derived embryos. Anillin was originally identified through actin-affinity chromatography and localizes to the metaphase and cellularization furrows as well as the cleavage furrows observed later in development (Miller et al., 1989; Field and Alberts, 1995). Peanut is a member of the conserved family of septins and exhibits a localization pattern similar to anillin (Neufeld and Rubin, 1994; Fares et al., 1995). In wild-type embryos, actin and anillin exhibit extensive colocalization to the furrows as they initiate at prophase (Fig. 3A,B). Similarly prepared *nuf*-derived embryos reveal regions

in which anillin, but not actin, is localized properly during prophase (Fig. 3C,D, arrow). At metaphase in *nuf*-derived embryos, actin and anillin colocalize; regions of the furrow in which actin is absent are also regions in which anillin is absent (Fig. 3E,F).

An equivalent set of experiments were performed with peanut. As with anillin, actin and peanut exhibit extensive colocalization during prophase in wild-type embryos (Fig. 3A',B'). In *nuf*-derived embryos, however, there are many regions in which peanut, but not actin is properly localized (Fig. 3C',D'). As these embryos progress into metaphase, actin and peanut colocalize (Fig. 3E',F').

nuf encodes a maternally supplied major 2.4 kb and minor 3.5 kb transcript

Mapping and reversion studies demonstrate that the *nuf* mutation is caused by the insertion of a modified transposable P-element (Sullivan et al., 1993b). This facilitated the isolation of flanking genomic DNA that was used to isolate three cDNA clones, all of which contained an insert of approximately 2.3 kb. One of these cDNAs was used as a probe for northern analysis and detected a major 2.4 kb and a minor 3.5 kb

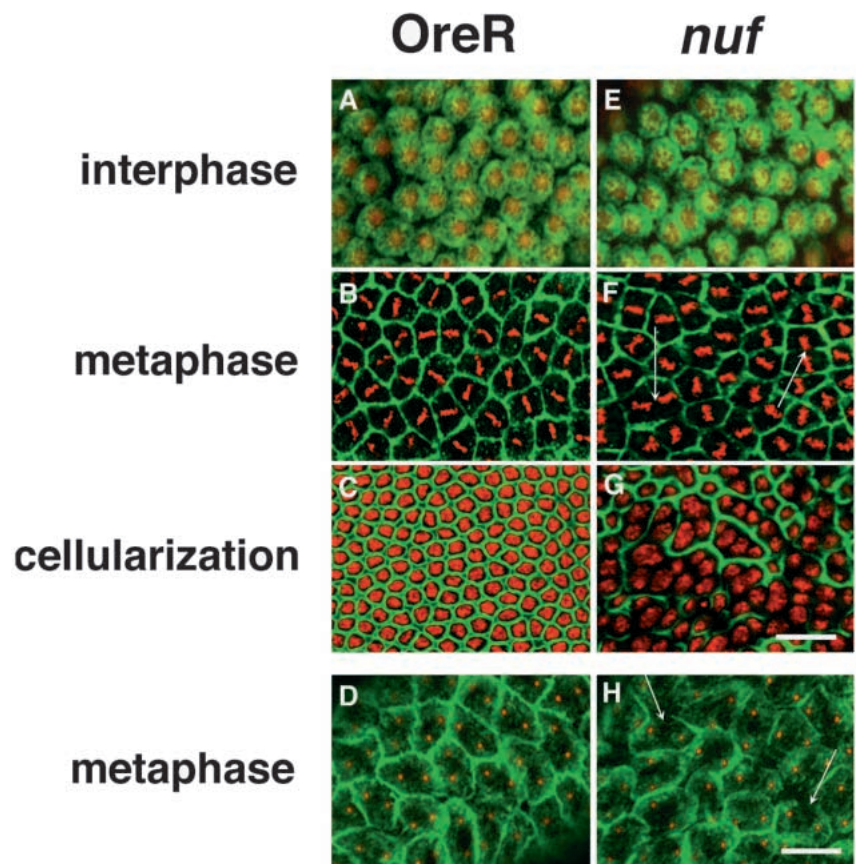


Fig. 2. The actin phenotype for *nuf*. Surface views of wild-type (A-D) and *nuf*-derived (E-H) embryos double stained for actin (green) and either DNA (red, A-C and E-G) or centrosomin (orange, D and H) during the syncytial and cellular blastoderm. Embryos shown are in interphase (A,E) and metaphase (B,F) of nuclear cycle 13, metaphase of nuclear cycle 11 (D,H) and at cellularization (C,G). Breaks in the metaphase furrows of *nuf*-derived embryos correlate with the region nearest the metaphase plate and farthest from the centrosomes (F and H, arrows). Bars, 10 μ m. Bar in G refers to A-C and E-G; bar in H refers to D and H.

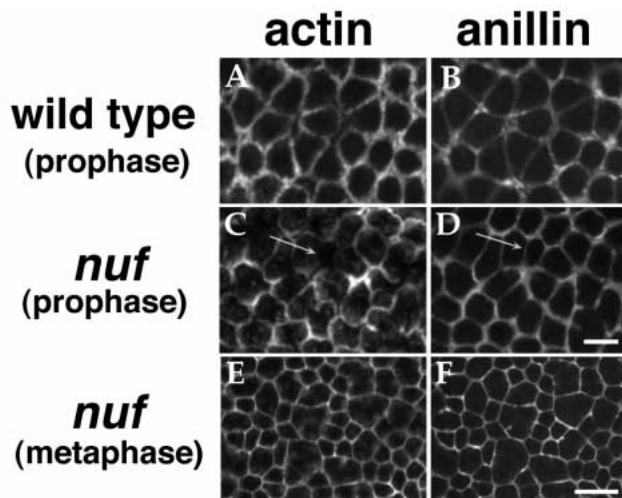


Fig. 3. Localization of furrow components in wild-type and *nuf*-mutant backgrounds. Surface views of wild-type (A,B,A',B') and *nuf*-derived (C-F, C'-F') embryos double stained for actin (A,C,E, A',C',E') and either anillin (B,D,F) or peanut (B',D',F'). Anillin and peanut are able to localize to actin-free regions in *nuf*-derived embryos at prophase (C,D,C',D' arrows) but not at metaphase (E,F,E',F'). Bars, 10 μ m. Bars in D and D' refer to A-D and A'-D'; bars in F and F' refer to E,F and E',F'.

transcript present in RNA preparations from 0-3 hour wild-type embryos. Both of these transcripts are greatly reduced in RNA preparations from similar collections of *nuf*-derived embryos (Fig. 4A).

***nuf* encodes a highly phosphorylated novel protein with coiled-coil domains at its C-terminal end**

Sequence analysis of the 2.3 kb *nuf* cDNA reveals a putative open reading frame of 1506 bp immediately preceded by the sequence AAAC(ATG), which agrees well with the consensus translation start sequence in *Drosophila* (Cavener, 1987). The predicted Nuf protein contains 502 amino acids with a predicted molecular mass of 57 kDa (Fig. 4B). The Nuf sequence does not exhibit significant homologies to other

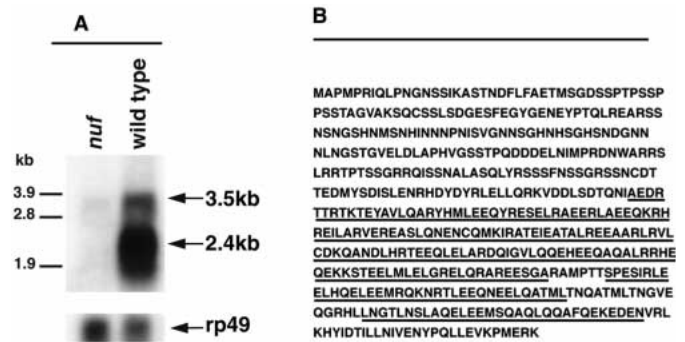


Fig. 4. Molecular analysis of Nuf. (A) Northern analysis. Total RNA from 0-3 hour collections of wild-type and *nuf*-derived embryos was probed with a 2.3 kb *nuf* cDNA. 22 μ g of RNA was loaded per lane and *rp* 49 served as a loading control. Two transcripts (2.4 kb and 3.5 kb) were identified in the wild-type preparations. These transcripts are greatly reduced in RNA preparations from *nuf*-derived embryos. (B) The predicted amino acid sequence of the Nuf protein contains 502 amino acids with a pI of 4.8. Three regions with a high probability of forming coiled-coils are underlined (Lupas et al., 1991). The nucleotide sequence for the entire cDNA is available from EMBL/GenBank/DDBJ under accession number AF045015.

sequences currently in the database. Further analysis utilizing the algorithm described by Lupas et al. (1991) reveals three regions near the Nuf C terminus strongly predicted to form coiled-coils. The probability of forming coiled-coils is greater than 95% for residues 231-382 and 389-431 and greater than 90% for residues 442-472. The first two coiled-coil domains are separated by a potential 6 amino acid 'hinge segment' containing proline.

Germline transformants were constructed with the *nuf* cDNA sequence spanning the translation start site and the 3' end of the gene fused to the Germ 10 vector (Spradling and Rubin, 1982; Serano et al., 1994) thereby driving expression of Nuf during oogenesis. Two transformant lines were recovered. These transformants increased the hatch rate of embryos derived from *nuf* females from 0% to 33% and 38%. This demonstrates that mutations in this sequence are responsible for the *nuf* maternally induced lethality.

Nuf is concentrated around the centrosomes during the syncytial divisions

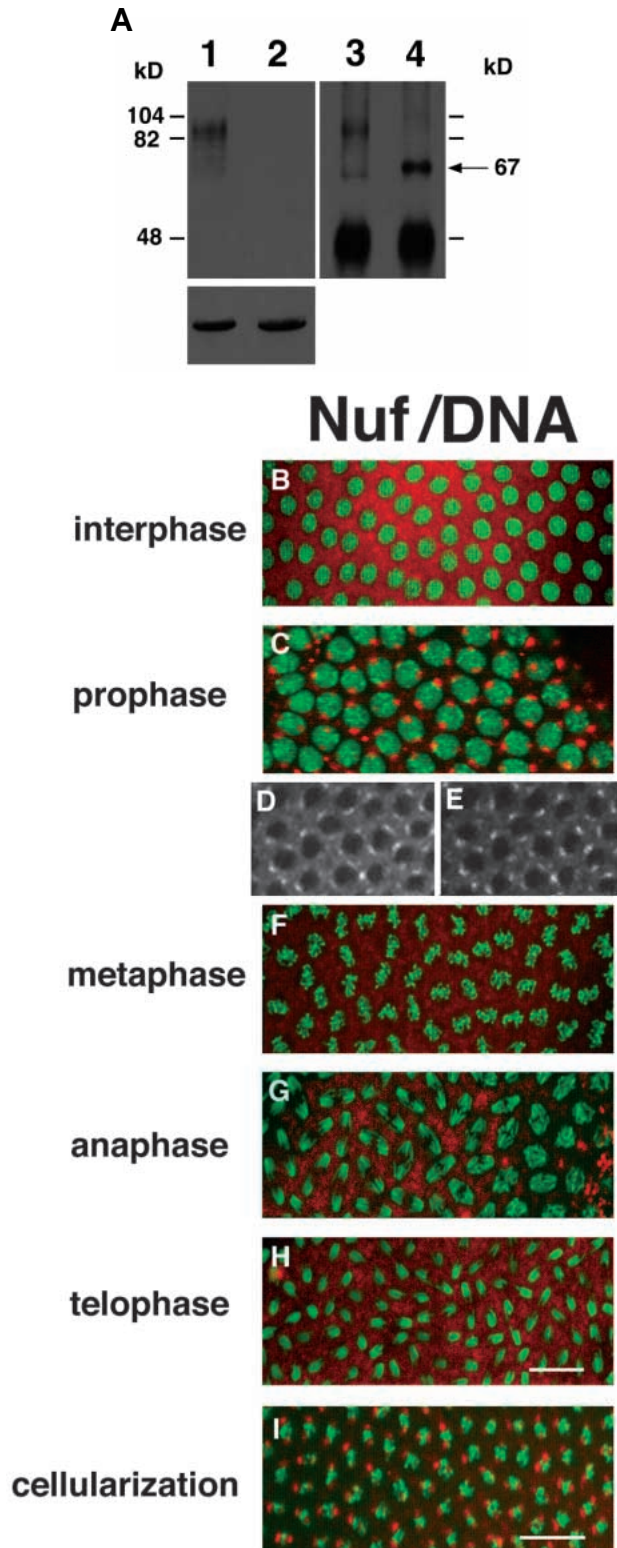
Anti-rabbit polyclonal antibodies were generated against an N-terminal Nuf/6-His fusion protein. Western analysis with this antibody reveals a major band at 95 kDa and a set of minor bands ranging from 82 kDa to 67 kDa in 0-3 hour protein preparations from wild-type but not *nuf*-derived embryos (Fig. 5A, lanes 1,2). That the predicted molecular mass of the Nuf protein is smaller than these resulting bands suggests the existence of post-translational modifications. To test this idea, we performed western analysis on Nuf immunoprecipitates treated with phosphatase. These immunoprecipitates reveal a range of bands similar to those from the wild-type extracts when not treated with phosphatase and a single 67 kDa band upon phosphatase treatment (Fig. 5A, lanes 3,4). This result indicates that Nuf is heavily phosphorylated *in vivo*.

Immunofluorescent analysis with this antibody reveals that, during the late syncytial divisions, Nuf is highly concentrated at the centrosomes during prophase and is cytoplasmic during

the other stages of the nuclear cycle (Fig. 5B-H). Nuf is also concentrated at the centrosomes during interphase of nuclear cycle 14 and throughout cellularization (Fig. 5I). Double stains of wild-type embryos with Nuf and tubulin verify the centrosomal localization of Nuf (Fig. 5D,E). The Nuf antibody does not stain centrosomes in equivalently staged *nuf*-derived

embryos (data not shown). To show that this lack of staining was not due to an inability of the antibodies to access the centrosomes in the *nuf*-derived embryos, we stained normal and *nuf*-derived embryos with anti-centrosomin, a *Drosophila* centrosomal antibody (Heuer et al., 1995; Li and Kaufman, 1996). Both normal and *nuf*-derived embryos reacted strongly with this antibody (Fig. 2D,H). This demonstrates that *nuf* embryos contain centrosomes.

Wild-type embryos were double stained with phalloidin and anti-Nuf antibodies to correlate actin dynamics with Nuf centrosomal localization (Fig. 6). This analysis reveals that the localization of Nuf to the centrosomes during prophase of the syncytial divisions occurs during the most dramatic reorganization of actin from caps to furrows (Fig. 6C,D,I,J). Nuf centrosome staining during cellularization occurs as the actin enters cellularization furrows and as the furrows extend (Fig. 6F,L).



DISCUSSION

Nuf is a member of a growing class of proteins that concentrate at the centrosome in a cell-cycle-specific manner (reviewed in Kalt and Schliwa, 1993). Immunofluorescent analysis demonstrates that Nuf is diffusely localized throughout the cortical cytoplasm from early metaphase through interphase. During prophase, Nuf concentrates at the centrosomes. Other *Drosophila* centrosomal proteins that exhibit a cell-cycle-specific centrosomal localization include CP60 and CP190. These proteins localize to the nuclei during interphase and the centrosome at mitosis and are members of a larger protein complex (Kellogg et al., 1989, 1995; Kellogg and Alberts, 1992; Whitfield et al., 1995). Separable domains within these proteins have been identified that are responsible for their nuclear and cytoplasmic localization (Oegema et al., 1995). Given the difference in the cell cycle timing of the centrosomal localization between Nuf and the CP60/190 complex, it is likely that they are involved in different cellular functions. The recently characterized *Drosophila* Asp protein localizes to regions near the centrosome during prophase through anaphase and concentrates at the midbody during telophase (Saunders et al., 1997). Mutations in Asp disrupt microtubule organization (Gonzalez et al., 1990). As with many proteins that localize to the centrosome, Asp and Nuf both include C-terminal regions with a high probability of forming coiled-coils.

Fig. 5. Immunological analysis of Nuf. (A) When used as a probe on a western blot of total protein from 0-3 hour wild-type and *nuf*-derived embryos, the anti-Nuf antibody recognizes a major 95 kDa band and a set of smaller minor bands present in preparations from wild-type (lane 1) but not *nuf*-derived (lane 2) embryos. In the panel below lanes 1 and 2, the western was probed with a control anti-actin antibody. Lanes 3 and 4 depict untreated and phosphatase-treated Nuf immunoprecipitations, respectively. Phosphatase treatment converts the major 95 kDa band and the smaller, minor bands to a single band of 67 kDa. The colored panels depict the localization of Nuf (red) with respect to the nuclei (green) throughout nuclear cycle 13 (B,C,F-H) and cellularization (I) in wild-type embryos. Tubulin (D) and Nuf (E) colocalize during prophase. Nuf concentrates at the centrosomes specifically at prophase and at cellularization. Bars, 10 μ m. Bar in H refers to B-H.

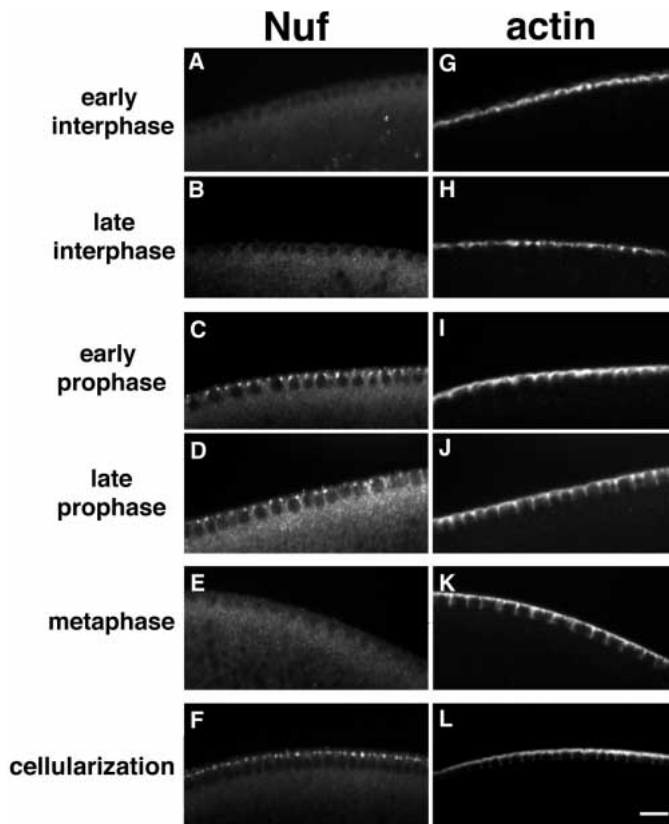


Fig. 6. Nuf localization and the corresponding actin dynamics. Cross-sectional views of wild-type embryos double stained for Nuf (A-F) and actin (G-L) during nuclear cycle 13 (A-E and G-K) and at cellularization (F and L). Nuf concentrates at centrosomes specifically during furrow initiation and extension. Bar, 10 μ m.

In mutations that lack Centrosomin (Cnn), a protein that localizes to the centrosome throughout the *Drosophila* syncytial nuclear cycles, Nuf no longer localizes to the centrosome (Heuer et al., 1995; Li and Kaufman, 1996; K. Li and T. Kaufman, personal communication). Conversely, Cnn localization is not disrupted in *nuf*-derived embryos. These results suggest that Nuf is not a core component of the centrosome. In *nuf*-derived embryos, prior to migration, the nuclear divisions are normal. This phenotype is also in accord with the idea that Nuf is not a core component of the centrosome.

In *nuf*-derived embryos, there are no obvious defects in the interphase actin caps (Fig. 2E). During metaphase, however, the furrows are incomplete in regions near the metaphase plate (Fig. 2F,H). Through both live (data not shown) and fixed analysis (Fig. 3C,C'), we observe this defect during the earliest stages of furrow formation at prophase. This suggests a defect in the recruitment of actin to these regions of the furrow rather than the stabilization of actin already present in these furrow regions. It is not known whether or not Nuf interacts directly with actin. Insight into the mechanism of Nuf action will require identifying interacting proteins and other components involved in the process. A potential interactor is Dah, a *Drosophila* protein with some homology to dystrophin (Zhang et al., 1996). *dah*-derived embryos have syncytial and cellularization phenotypes similar to *nuf*, indicating that they may be involved in a common pathway.

Nuf is concentrated at the centrosomes during prophase and is cytoplasmic throughout the rest of the cell cycle. We do not know when, in the division cycle, Nuf is required for actin recruitment to the furrows. For example, cytoplasmic Nuf may regulate actin dynamics and the localization of Nuf to the centrosome may serve to sequester Nuf in an inactive state. Alternatively, Nuf may influence actin dynamics while it is localized to the centrosomes. Nuf localizes to the centrosomes during prophase, specifically when the most extensive reorganization of actin from caps to furrows is occurring. In addition, Nuf is concentrated at the centrosomes from early interphase of nuclear cycle 14 through to the completion of cellularization. *nuf*-derived embryos produce a dramatic cellularization phenotype with extensive gaps in the furrows and it is likely that, as found for the metaphase furrows, this is a result of failed actin recruitment. These results suggest that, at least during cellularization, the centrosomal localization of Nuf is important for its effect on actin localization.

The prophase centrosomal localization of Nuf coincides with the timing of actin reorganization and supports a model in which Nuf is acting at the centrosomes to organize cortical actin. Nuf may influence microfilament organization by organizing microtubules. Although previous analysis failed to reveal disruption in microtubule organization in the *nuf* mutant, it remains possible that subtle microtubule defects were overlooked. Alternatively, Nuf may act more directly to influence microfilament organization. For example, centrosomal Nuf may facilitate the cap-to-furrow transition by stimulating severing or depolymerization of the microfilaments. Another possibility is that the cap-to-furrow transition involves loading and transport of components onto and along the microtubules and Nuf may be involved in this process.

Although *nuf*¹ only partially disrupts the formation of metaphase furrows, molecular analysis indicates that it is a null allele. One explanation for the fact that the *nuf*¹ phenotype is not more severe is that Nuf may be a member of a protein complex that is involved in the recruitment of actin to the metaphase furrows. The absence of Nuf compromises, but does not eliminate, function in this complex. During cellularization, the furrow defect is much more severe in *nuf*¹-derived embryos and extensive regions of the embryo lack cellularization furrows. It may be that, during cellularization, greater demands are placed on the functioning of this complex. Alternatively, the partially disrupted metaphase furrows of *nuf*¹-derived embryos may reflect the fact that this process involves redundant mechanisms. Null alleles of another maternal effect mutation, *dah*, also produce slight defects in the metaphase furrows and severe cellularization defects (Zhang et al., 1996).

In addition to actin, we have examined the effect of the *nuf* mutation on anillin and peanut localization. Anillin, peanut and many other proteins are common to both metaphase and more conventional cleavage furrows (Pesacreta et al., 1989; Young et al., 1991; Neufeld and Rubin, 1994; Thomas and Kiehart, 1994; Fares et al., 1995; Field and Alberts, 1995; Miller and Kiehart, 1995). Therefore, many of the lessons learned in one system will likely apply to the other. During the initial stages of furrow formation in *nuf*-derived embryos, anillin and peanut localize to furrow regions in which actin fails to localize (Fig. 3C-D, 3C'-D'). These results demonstrate that, during the initial stages of furrow formation, proper localization of anillin

and peanut to the furrow is independent of the proper localization of actin to the furrow. These results also suggest that Nuf functions in a pathway that is downstream or independent of anillin and peanut localization. Because these initial events of furrow formation occur normally in *nuf*-derived embryos, the signals for positioning and timing of furrow formation are probably intact. Although during prophase the localization of anillin and peanut is independent of actin localization, as the embryos progress into metaphase, anillin and peanut fail to maintain their localization in regions of the furrows in which actin fails to localize (Fig. 3E,F,E',F'). It is likely that one of the furrow components that fail to be recruited in *nuf*-derived embryos, possibly actin, is required for stabilization of the furrow during the prophase to metaphase transition.

nuf-derived embryos produce a dramatic cellularization phenotype in which the gaps in the furrows are so extensive that the furrows usually encompass multiple nuclei (Fig. 2G). It is likely that the origin of this phenotype is equivalent to that of the metaphase furrows. This phenotype is strikingly similar to that observed for the zygotic mutations *nullo* and *serendipity* (Schweisguth et al., 1990; Simpson and Wieschaus, 1990; Rose and Wieschaus, 1992). Analysis of these genes demonstrates that they encode novel proteins that localize to the invaginating cellularization furrow (Schweisguth et al., 1990; Postner and Wieschaus, 1994). The *nullo* mutation disrupts the localization of Serendipity, but the *serendipity* mutation does not disrupt the localization of Nullo. This indicates that Serendipity functions downstream of Nullo. As the cellularization furrows initiate normally in the *nullo* mutation, the Nullo protein apparently is not required for the initial stages of cellularization but is required for the stabilization of the growing cellularization furrow. Serendipity is also probably not required for the initial stages of cellularization as it functions downstream of Nullo. In contrast, *nuf* disrupts the initial formation of the cellularization furrows. This indicates that the maternally supplied Nuf acts upstream to the zygotic genes *nullo* and *serendipity* in initiating and establishing the cellularization furrow.

We thank H. Francis-Lang, R. Abu-Shumays, V. Foe, G. Odell and J. Cunniff for their critical reading of the manuscript and helpful discussions. We also thank J. Tamkun and D. Kellogg for sharing their laboratories and valuable advice, R. Cohen for providing the Germ 10 vector, K. Yu for assisting in the transformation studies, and K. Li and T. Kaufman for kindly supplying us with the anti-centrosomin antibody and communicating unpublished results. We especially thank B. Alberts for his generous support. Finally, a hearty thanks to C. Cramer for precious reagents. This work was supported by grants to W. Sullivan from the National Institutes of Health (R29GM46409) and from the American Cancer Society (JFRA-366), a grant supporting C. M. Field from the National Institutes of Health (GM23928), and by a GAANN Fellowship to W. Rothwell.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.
- Brutlag, D. L., Dautricourt, J. P., Maulik, S. and Relph, J. (1990). Improved sensitivity of biological sequence database searches. *Comput. Appl. Biosci.* **6**, 237-245.
- Callaini, G. and Riparbelli, M. G. (1990). Centriole and centrosome cycle in the early *Drosophila* embryo. *J. Cell Sci.* **97**, 539-543.
- Cavener, D. R. (1987). Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Res.* **15**, 1353-1361.
- Chevrier, V., Paintrand, M., Koteliansky, V., Block, M. R. and Job, D. (1995). Identification of vinculin as a pericentriolar component in mammalian cells. *Exp. Cell Res.* **219**, 399-406.
- Clark, S. W. and Meyer, D. I. (1992). Centractin is an actin homologue associated with the centrosome. *Nature* **359**, 246-50.
- Dingwall, A. K., Beek, S. J., McCallum, C. M., Tamkun, J. W., Kalpana, G. V., Goff, S. P. and Scott, M. P. (1995). The *Drosophila* *snr1* and *brm* proteins are related to yeast SWI/SNF proteins and are components of a large protein complex. *Mol. Biol. Cell* **6**, 777-791.
- Fares, H., Peifer, M. and Pringle, J. R. (1995). Localization and possible functions of *Drosophila* septins. *Mol. Biol. Cell* **6**, 1843-1859.
- Field, C. and Alberts, B. M. (1995). Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. *J. Cell Biol.* **131**, 165-178.
- Field, C. M., Omayma, A., Rosenblatt, J., Wong, M. L., Alberts, B. and Mitchison, T. J. (1996). A purified *Drosophila* septin complex forms filaments and exhibits GTPase activity. *J. Cell Biol.* **133**, 605-616.
- Foe, V. E. and Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behavior during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* **61**, 31-70.
- Foe, V. E., Odell, G. and Edgar, B. A. (1993). Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 149-300. Plainview, New York: Cold Spring Harbor Laboratory Press.
- Freeman, M., Nusslein-Volhard, C. and Glover, D. M. (1986). The dissociation of nuclear and centrosomal division in *gnu*, a mutation causing giant nuclei in *Drosophila*. *Cell* **46**, 457-468.
- Gonzalez, C., Saunders, R. D., Casal, J., Molina, I., Carmena, M., Ripoll, P. and Glover, D. M. (1990). Mutations at the *asp* locus of *Drosophila* lead to multiple free centrosomes in syncytial embryos, but restrict centrosome duplication in larval neuroblasts. *J. Cell Sci.* **96**, 605-16.
- Heuer, J. G., Li, K. and Kaufman, T. C. (1995). The *Drosophila* homeotic target gene *centrosomin* (*cnn*) encodes a novel centrosomal protein with leucine zippers and maps to a genomic region required for midgut morphogenesis. *Development* **121**, 3861-3876.
- Kalt, A. and Schliwa, M. (1993). Molecular components of the centrosome. *Trends Cell Biol.* **3**, 118-128.
- Karr, T. L. and Alberts, B. M. (1986). Organization of the cytoskeleton in early *Drosophila* embryos. *J. Cell Biol.* **102**, 1494-1509.
- Kellogg, D. R., Mitchison, T. J. and Alberts, B. M. (1988). Behavior of microtubules and actin filaments in living *Drosophila* embryos. *Development* **103**, 675-686.
- Kellogg, D. R., Field, C. M. and Alberts, B. M. (1989). Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early *Drosophila* embryo. *J. Cell Biol.* **109**, 2977-2991.
- Kellogg, D. R. and Alberts, B. M. (1992). Purification of a multiprotein complex containing centrosomal proteins from the *Drosophila* embryo by chromatography with low-affinity polyclonal antibodies. *Mol. Biol. Cell* **3**, 1-11.
- Kellogg, D. R., Moritz, M. and Alberts, B. M. (1994). The centrosome and cellular organization. *Annu. Rev. Biochem.* **63**, 639-74.
- Kellogg, D. R., Oegema, K., Raff, J., Schneider, K. and Alberts, B. M. (1995). CP60: a microtubule-associated protein that is localized to the centrosome in a cell cycle-specific manner. *Mol. Biol. Cell* **6**, 1673-1684.
- Li, K. and Kaufman, T. C. (1996). The homeotic target gene *centrosomin* encodes an essential centrosomal component. *Cell* **85**, 585-596.
- Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. San Diego: Academic Press.
- Lupas, A., Van Dyke, M. and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* **252**, 1162-1164.
- Miller, K. G., Field, C. M. and Alberts, B. M. (1989). Actin-binding proteins from *Drosophila* embryos: a complex network of interacting proteins detected by F-actin affinity chromatography. *J. Cell Biol.* **109**, 2963-75.
- Miller, K. G. and Kiehart, D. P. (1995). Fly Division. *J. Cell Biol.* **131**, 1-5.
- Minden, J. S., Agard, D. A., Sedat, J. W. and Alberts, B. M. (1989). Direct cell lineage analysis in *Drosophila melanogaster* by time lapse three dimensional optical microscopy of living embryos. *J. Cell Biol.* **109**, 505-516.
- Neufeld, T. P. and Rubin, G. M. (1994). The *Drosophila* *peanut* gene is

- required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins. *Cell* **77**, 371-379.
- O'Connell, P. O. and Rosbash, M.** (1984). Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12**, 5495-513.
- Oegema, K., Whitfield, W. G. F. and Alberts, B.** (1995). The cell-cycle dependent localization of the CP190 centrosomal protein is determined by the coordinate action of two separable domains. *J. Cell Biol.* **131**, 1261-1273.
- Pereira, G. and Schiebel, E.** (1997). Centrosome-microtubule nucleation. *J. Cell Sci.* **110**, 295-300.
- Pesacreta, T. C., Byers, T. J., Dubreuil, R., Kiehart, D. P. and Branton, D.** (1989). *Drosophila* spectrin: the membrane skeleton during embryogenesis. *J. Cell Biol.* **108**, 1697-709.
- Postner, M. A., Miller, K. G. and Wieschaus, E.** (1992). Maternal-effect mutations of the *sponge* locus affect cytoskeletal rearrangements in *Drosophila melanogaster* embryos. *J. Cell Biol.* **119**, 1205-1218.
- Postner, M. A. and Wieschaus, E. F.** (1994). The *nullo* protein is a component of the actin-myosin network that mediates cellularization in *Drosophila melanogaster* embryos. *J. Cell Sci.* **107**, 1863-1873.
- Raff, J. W. and Glover, D. M.** (1989). Centrosomes, not nuclei, initiate pole cell formation in *Drosophila* embryos. *Cell* **57**, 611-619.
- Rappaport, R.** (1986). Establishment of the mechanism of cytokinesis in animal cells. *Int. Rev. Cytol.* **105**, 245-281.
- Rose, L. S. and Wieschaus, E.** (1992). The *Drosophila* cellularization gene *nullo* produces a blastoderm-specific transcript whose levels respond to the nucleocytoplasmic ratio. *Genes Dev.* **6**, 1255-1268.
- Saunders, R. D. C., do Carmo Avides, M., Howard, T., Gonzalez, C. and Glover, D. M.** (1997). The *Drosophila* gene *abnormal spindle* encodes a novel microtubule-associated protein that associates with the polar regions of the mitotic spindle. *J. Cell Biol.* **137**, 881-890.
- Schweisguth, F., Lepesant, J. and Vincent, A.** (1990). The *serendipity alpha* gene encodes a membrane-associated protein required for the cellularization of the *Drosophila* embryo. *Genes Dev.* **4**, 922-931.
- Serano, T. L., Cheung, H. K., Frank, L. H. and Cohen, R. S.** (1994). P element transformation vectors for studying *Drosophila melanogaster* oogenesis and early embryogenesis. *Gene* **138**, 181-186.
- Simpson, L. and Wieschaus, E.** (1990). Zygotic activity of the *nullo* locus is required to stabilize the actin-myosin network during cellularization in *Drosophila*. *Development* **110**, 851-863.
- Spradling, A. C. and Rubin, G. M.** (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341-347.
- Stroumbakis, N. D., Li, Z. and Tolias, P. P.** (1994). RNA- and single stranded DNA-binding (SSB) proteins expressed during *Drosophila melanogaster* oogenesis: a homolog of bacterial and eukaryotic mitochondrial SSBs. *Gene* **143**, 171-177.
- Sullivan, W., Minden, J. S. and Alberts, B. M.** (1990). *daughterless-abo-like*, a *Drosophila* maternal-effect mutation that exhibits abnormal centrosome separation during the late blastoderm divisions. *Development* **110**, 311-323.
- Sullivan, W., Daily, D. R., Fogarty, P., Yook, K. and Pinpinelli, S.** (1993a). Delays in anaphase initiation occur in individual nuclei of the syncytial *Drosophila* embryo. *Mol. Biol. Cell* **4**, 885-896.
- Sullivan, W., Fogarty, P. and Theurkauf, W.** (1993b). Mutations affecting the cytoskeletal organization of syncytial *Drosophila* embryos. *Development* **118**, 1245-1254.
- Sullivan, W. and Theurkauf, W. E.** (1995). The cytoskeleton and morphogenesis of the early *Drosophila* embryo. *Curr. Opin. Cell Biol.* **7**, 18-22.
- Sunkel, C. E., Gomes, R., Sampaio, P., Perdigo, J. and Gonzalez, C.** (1995). gamma-tubulin is required for the structure and function of the microtubule organising centre in *Drosophila* neuroblasts. *EMBO J.* **14**, 28-36.
- Tamkun, J. W., Dearing, R., Scott, M. P., Kissinger, M., Pattatucci, A. M., Kaufman, T. C. and Kennison, J. A.** (1992). brahma: A regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell* **68**, 561-572.
- Tamm, S. and Tamm, S. L.** (1988). Development of macrociliary cells in *Beroe* 1. Actin bundles and centriole migration. *J. Cell Sci.* **89**, 67-80.
- Theurkauf, W. E.** (1992). Behavior of structurally divergent alpha-tubulin isotypes during *Drosophila* embryogenesis: evidence for post-translational regulation of isotype abundance. *Dev. Biol.* **154**, 205-217.
- Thomas, G. H. and Kiehart, D. P.** (1994). Beta heavy-spectrin has a restricted tissue and subcellular distribution during *Drosophila* embryogenesis. *Development* **120**, 2039-2050.
- Tsukiyama, T., Daniel, C., Tamkun, J. and Wu, C.** (1995). ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell* **83**, 1021-1026.
- von Dassow, G. and Schubiger, G.** (1995). How an actin network might cause fountain streaming and nuclear migration in the syncytial *Drosophila* embryos. *J. Cell Biol.* **127**, 1637-1653.
- Warn, R. M., Magrath, R. and Webb, S.** (1984). Distribution of F-actin during cleavage of the *Drosophila* syncytial blastoderm. *J. Cell Biol.* **98**, 156-162.
- Whitfield, W. G. F., Chaplin, M. A., Oegema, K., Parry, H. and Glover, D. M.** (1995). The 190 kDa centrosome-associated protein of *Drosophila melanogaster* contains four zinc finger motifs and binds to specific sites on polytene chromosomes. *J. Cell Sci.* **108**, 3377-3387.
- Yasuda, G. K., Baker, J. and Schubiger, G.** (1991). Independent roles of centrosomes and DNA in organizing the *Drosophila* cytoskeleton. *Development* **111**, 379-391.
- Young, P. E., Pesacreta, T. C. and Kiehart, D. P.** (1991). Dynamic changes in the distribution of cytoplasmic myosin during *Drosophila* embryogenesis. *Development* **111**, 1-14.
- Zalokar, M. and Erk, I.** (1976). Division and migration of nuclei during early embryogenesis of *Drosophila melanogaster*. *J. Micro. Cell* **25**, 97-106.
- Zhang, C. X., Lee, M. P., Chen, A. D., Brown, S. D. and Hsieh, T.** (1996). Isolation and characterization of a *Drosophila* gene essential for early embryonic development and formation of cortical cleavage furrows. *J. Cell Biol.* **134**, 923-934.