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Functional analysis of the *Drosophila* Diaphanous FH protein in early embryonic development

Katayoun Afshar[1], Bridget Stuart[2] and Steven A. Wasserman[1,*]

[1]Department of Biology, Center for Molecular Genetics, University of California, San Diego, La Jolla CA 92093-0634, USA
[2]Department of Molecular Biology and Oncology, University of Texas Southwestern Medical Center, Dallas TX 75235, USA
*Author for correspondence (e-mail: stevenw@ucsd.edu)

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SUMMARY

The Drosophila Formin Homology (FH) protein Diaphanous has an essential role during cytokinesis. To gain insight into the function of Diaphanous during cytokinesis and explore its role in other processes, we generated embryos deficient for Diaphanous and analyzed three cell-cycle-regulated actin-mediated events during embryogenesis: formation of the metaphase furrow, cellularization and formation of the pole cells. In *dia* embryos, all three processes are defective. Actin filaments do not organize properly to the metaphase and cellularization furrows and the actin ring is absent from the base of the presumptive pole cells. Furthermore, plasma membrane invaginations that initiate formation of the metaphase furrow and pole cells are missing. Immunolocalization studies of wild-type embryos reveal that Diaphanous localizes to the site where the metaphase furrow is anticipated to form, to the growing tip of cellularization furrows, and to contractile rings. In addition, the *dia* mutant phenotype reveals a role for Diaphanous in recruitment of myosin II, anillin and Peanut to the cortical region between actin caps. Our findings thus indicate that Diaphanous has a role in actin cytoskeleton organization and is essential for many, if not all, actin-mediated events involving membrane invagination. Based on known biochemical functions of FH proteins, we propose that Diaphanous serves as a mediator between signaling molecules and actin organizers at specific phases of the cell cycle.

Key words: Formin, Cytoskeleton, Cellularization, *Drosophila*, Diaphanous

INTRODUCTION

Reorganization and remodeling of the cortical actin cytoskeleton are essential in animals for many cellular processes, including cell morphogenesis, cell movement and cell division. Cytokinesis is an actin-mediated process required for completion of cell division. During animal cytokinesis, contraction of a membrane-associated actomyosin ring results in equatorial invagination of the cell membrane and subsequent cleavage into two daughter cells. The position of the contractile ring is thought to be determined by the mitotic spindle and by the interaction between the centrosomal microtubules and the cell cortex (for review see Field et al., 1999; Goldberg et al., 1998). Signaling and structural proteins have been identified that localize to the cleavage furrow and play essential roles in formation and stabilization of the contractile ring. In *Drosophila*, these include Rho1, the Rho GTPase exchange factor Pebble, anillin, septins, profilin, actin and myosin II (Fares et al., 1995; Field and Alberts, 1995; Giansanti et al., 1998; Prokopenko et al., 1999). Nevertheless, many questions remain with respect to the mechanisms that are involved in assembly and function of the contractile ring and its coupling to other cell cycle events.

One group of proteins implicated in the regulation of cytokinesis is the Formin-Homology (FH) family. Members of the FH family are involved in a number of actin-mediated cellular processes including alteration of cell polarity and cytokinesis (for review see Frazier and Field, 1997; Wasserman, 1998). Several members of this family function in cytokinesis, including Bni1p and Bnr1p in *Saccharomyces cerevisiae*, Cdc12 in *Schizosaccharomyces pombe*, SepA in *Aspergillus nidulans*, Cyk-1 in *Caenorhabditis elegans* and Diaphanous in *Drosophila melanogaster*. Consistent with this role, a number of these proteins have been shown to colocalize with the actin cytoskeleton, particularly at sites of cell division.

FH proteins contain several conserved sequence elements distributed over the primary sequence (Wasserman, 1998). A centrally located, proline-rich FH1 domain has been demonstrated in fission yeast to serve as a binding site for the actin-binding protein profilin (Chang et al., 1997). Functional domain analysis of a fission yeast FH protein, Fus1, identified an amino-terminal FH3 homology domain required for proper localization (Petersen et al., 1998). In addition, the amino-terminal domain of several FH proteins interacts with Rho-GTPase proteins (Evangelista et al., 1997; Imamura et al., 1997b; Kohno et al., 1996; Watanabe et al., 1997). Much less

is known about the function of the carboxyl-terminal halves of FH proteins, which include the highly conserved FH2 region and, typically, flanking coiled-coil domains.

In *Drosophila*, the FH protein encoded by the *diaphanous* (*dia*) locus has a critical role during cytokinesis in a diverse set of tissues; various combinations of *dia* mutations result in multinucleate spermatids, polyploid larval neuroblasts and adult follicle cells, and pupal lethality (Castrillon and Wasserman, 1994). Subcellular characterization of *dia*-deficient spermatids revealed that inactivation of Diaphanous results in defects in the interzonal microtubules, structures known to be crucial for the execution of cytokinesis, and in the absence of an actomyosin contractile ring (Giansanti et al., 1998).

To gain further insight into the function of Diaphanous, we turned to the cell-cycle-coordinated, actin-mediated events that characterize early embryogenesis in *Drosophila* (Miller and Kiehart, 1995). Embryogenesis starts with thirteen rapid and synchronous nuclear divisions occurring in the absence of cytokinesis (Foe and Alberts, 1983; Zalokar and Erk, 1976). At nuclear cycle 10, the syncytial nuclei migrate to the embryonic cortex and take up positions just below the plasma membrane. During prophase of the subsequent nuclear divisions, a process that is concomitant with the reorganization of actin filaments causes invagination of the plasma membrane between the mitotic spindles (Foe et al., 1993). This results in formation of a structure called the metaphase or pseudocleavage furrow, which controls the spacing between nuclei by providing a barrier between adjacent mitotic apparati (Sullivan and Theurkauf, 1995). In addition, the first nuclei that reach the posterior of the embryo are packaged by cytokinesis events to produce pole cells, the germline progenitors. During interphase of nuclear cycle 14, the rest of the nuclei are packaged into individual cells through a process known as cellularization. During cellularization, the membrane invaginates between each pair of nuclei and is subsequently pinched off below the nuclei by an actin-mediated process to form individual cells (Foe et al., 1993).

For this report, we generated embryos maternally deficient for *dia* function and then analyzed three actin-mediated processes in the syncytial embryo involving membrane invagination: metaphase furrow formation, cellularization and pole cell formation. We found that all three events are defective in the absence of Diaphanous. We also performed immunolocalization studies in wild-type embryos and found that Diaphanous localization during interphase is a harbinger of the site for metaphase furrow formation and it concentrates at the growing tip of metaphase and cellularization furrows. Based on these findings, we propose Diaphanous functions during the cell cycle as a mediator between signaling molecules and actin cytoskeleton organizers at the cortex.

MATERIALS AND METHODS

Generation of germline clones

Germline clones of the dia^9 and dia^5 alleles were generated using the ovo^{D1}, FLP/FRT system (Xu and Rubin, 1993). Mutations in *dia* (38E on the physical map) were recombined onto a chromosome carrying the FLP recombinase target (FRT) at 40A. The resulting *dia*, FRT/CyO females were crossed to hs-FLP; ovo^{D1}, FRT/CyO males and their progeny were heat shocked at 37°C for 2 hours as third-instar larvae or pupae. The heat shock induces the expression of the FLP recombinase, which mediates site-specific recombination between the FRT sequences on the ovo^{D1} and *dia* chromosomes. Since the dominant ovo^{D1} mutation blocks oogenesis, only those recombinant females homozygous for the *dia* mutation have functional ovaries. Such females were mated to *dia*/CyO males and allowed to lay eggs on apple juice agar plates supplied with yeast.

Antibody generation

A 2966 bp *Bam*HI-*Cla*I DNA fragment from the *diaphanous* cDNA clone (Castrillon and Wasserman, 1994) was subcloned into the pET14b (Novagen) His-tag expression vector to generate plasmid B108. *E. coli* BL21(DE3) cells transformed with this plasmid were grown to log phase, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and lysed. Upon centrifugation of the lysate, the His-tagged Diaphanous protein (~100,000 M_r) was found in the pellet. For preparation of the antigen, His-Diaphanous was incubated in 8 M urea, 0.1 M $NaPO_4$ and 0.01 M Tris-HCl, pH 8.0 overnight at room temperature. The solubilized Diaphanous fusion protein was resolved by SDS-PAGE. Acrylamide strips containing the fusion protein were excised from the gel after staining with cold 0.2 M KCl for 10 minutes.

To prepare the antigen for injection, an acrylamide strip containing approximately 200 μg of His-Dia fusion protein was subjected to three 10-minute washes in PBS. The gel strip was homogenized in PBS, mixed 1:1 with Freund's complete adjuvant and emulsified by vortexing. Subsequent immunizations were performed every 2 to 3 weeks with 200 μg protein in Freund's incomplete adjuvant. Bleeds were performed 10-14 days postinjection.

Immunoblotting and immunocytochemistry

Embryos homozygous for a *dia* mutation or wild type were collected on apple juice agar plates for a period of 2 hours for immunoblot analysis, or 6 hours for immunostaining experiments. The embryos were washed with 0.7% NaCl, 0.05% Triton X-100 solution and were dechorionated in 50% bleach for 2 minutes, followed by 1-minute washes in NaCl-Triton and water.

For immunoblot analysis about 20 embryos were homogenized with a pestle in 20 μl of protein sample buffer, boiled for 3 minutes, spun for 2 minutes at 14000 ***g*** and loaded on a polyacrylamide gel. The SDS-PAGE and immunoblot analyses were performed as described previously (Sambrook et al., 1989). Proteins were transferred to PVDF membrane for blotting. Antibodies generated against the N-terminal domain (a gift from Karen Oegema) and FH2 domains of Diaphanous were used at 1:1000 and 1:60,000 dilution, respectively; anti-tube antibody (Letsou et al., 1993) was used at 1:1000 dilution; and the secondary alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Tropix) was used at 1:1000. The antibody-protein complexes were detected by a chemiluminescence detection system (Tropix).

Except for examination of the microtubule structures during the syncytial nuclear divisions, for which we followed the fixation method described by Warn and Warn (1986), embryo staining was performed as described previously (Karr and Alberts, 1986). In brief, embryos were dechorionated in 50% bleach for 3 minutes, washed with NaCl-Triton solution for 1 minute and washed with distilled water for 5 minutes. The embryos were then fixed for 5 minutes in a 1:1 solution of heptane with 3.5% formaldehyde in PBS. The formaldehyde layer was removed and an equal amount of methanol was added. The embryos were shaken vigorously for 1 minute to remove the vitelline membrane. The heptane layer was removed and an equal volume of methanol was added. After two additional methanol washes, the embryos were rehydrated sequentially in solutions of 70%, 50% and 30% methanol, in 10% methanol in PBS and then in PBS alone. The embryos were incubated in PBT (PBS+1% Triton) for 30 minutes and blocked in PBST (PBT+3%BSA) for 1 hour. The embryos were then

incubated with the primary antibodies in PBST for 3 hours at room temperature. After three 20-minute washes in PBT, the embryos were incubated with the secondary antibodies for 3 hours at room temperature. The embryos were washed three times with PBT, with inclusion of DAPI at 1 μg/ml in the second wash for chromosomal staining. For experiments in which we double-stained embryos for actin and tubulin, we used directly conjugated Cy3-anti-actin and Cy2-anti-tubulin at 1:200 dilution, and incubations with secondary antibodies were omitted.

Primary antibodies were diluted in PBS as follows: monoclonal anti-tubulin (Sigma) 1:500, monoclonal anti-actin (Sigma) 1:100, rabbit anti-tubulin (a gift from Jon Scholey) 1:1000, rabbit anti-dia FH2 domain 1:5000, rabbit anti-myosin heavy chain (a gift from Roger Karess) 1:1000, rabbit anti-anillin (a gift from Chris Field) 1:250, rabbit anti-septin antibody (a gift from John Pringle) 1:500.

Microscopy

Embryos were mounted in Fluoromount solution (Southern Biotechnology Associates) for microscopy. Laser-scanning confocal microscopy was performed using a Nikon Diaphot 300 attached to a Bio-Rad MRC1024 confocal imaging system. Images were collected with Kalman averaging using Lasersharp software and merged in pseudocolor using Adobe Photoshop software. Specific stages of the nuclear cycle were determined based on chromosomal morphology; nuclear cycles were identified on the basis of nuclear density. Light microscopy of live embryos was carried out with a Nikon E800 microscope equipped with a Spot2 CCD digital camera. Images were captured directly into Adobe Photoshop.

RESULTS

Diaphanous is required for embryonic development

To investigate the role of Diaphanous in the actin-mediated processes of the syncytial blastoderm, we analyzed the phenotype of *dia*-deficient embryos during early development. To eliminate the maternal contribution of Diaphanous to the oocyte, we generated homozygous *dia* mutant clones in the female germline using the ovo^{D1}, FLP/FRT recombinase system (see Materials and Methods). We used two alleles of *dia* for generation of these clones. The first, dia^5, is a null allele that causes larval and pupal lethality. The second, dia^9, is weaker, with some homozygous mutant flies surviving to adulthood (Castrillon and Wasserman, 1994).

Fertilized embryos produced by ovarian clones of *dia* mutant tissue exhibited severe developmental defects. Only about 3% of the embryos produced by dia^5 clones hatched, whereas about 20% of those from dia^9 clones survived through the larval and pupal stages. Although we obtained similar results for both mutant alleles, the phenotype of the dia^5 embryos was more severe at all stages of embryogenesis. Therefore, all of the experimental results presented below are from embryos produced by dia^5 clones (hereafter referred to as *dia* mutant embryos).

Defects in *dia* mutant embryos first appeared at nuclear cycle 11; earlier stages, as assessed by nuclear migration, division and organization, appeared wild-type. Abnormalities in nuclear and actin cytoskeletal organization affected almost two-thirds of all fertilized embryos at cycles 11-13 and a higher percentage at later stages (Table 1). Among embryos of a similar stage, the surface area affected varied considerably, ranging from a set of small patches to the entire embryonic surface. This variability did not extend to all phenotypes, however, as 100% of the embryos failed to form any pole cells. Furthermore, greater than 95% of embryos were grossly defective at gastrulation, despite the fact that half received a wild-type copy of *dia* paternally. Cuticle preparations of *dia* mutant embryos revealed a wide range of phenotypes, including failure in head involution, loss of head structures, reduction or absence of denticle bands and incomplete formation of the cuticle (data not shown).

Table 1. Defects in actin-mediated events in *diaphanous* mutant embryos

Affected process	% Normal	% Abnormal	No. scored
Fertilization	75	25	434
Somatic nuclear and actin cytoskeletal organization			
Cycle 2-11	100	0	47
Cycle 11-13	36	64	70
Cellularization	13	87	61
Postcellularization	3	97	153
Germline nuclear and actin cytoskeletal organization			
Pole cell formation	0	100	284

Diaphanous is required for organization of the metaphase furrow

To explore the nature of the defects seen in the absence of *dia* function, we stained wild-type and *dia* mutant embryos at nuclear cycles 11-13 with the DNA dye DAPI and with an antibody directed against F-actin. In the wild type, nuclei are positioned at the embryo cortex at interphase of nuclear cycles 11-13; a structure referred to as the actin cap is situated between each nucleus and the plasma membrane (Fig. 1A,B). During the transition to prophase, filament reorganization results in a concentration of actin at the edge of the caps. At metaphase, the resulting rings of cortical actin, together with associated plasma membrane, invaginate to form metaphase furrows (Foe et al., 1993). As viewed from above, actin staining at these furrows appears as a hexagonal array over the embryonic surface (Fig. 1C). In the sagittal view, actin staining at the metaphase furrow appears as a line between the metaphase nuclei (Fig. 1G,H).

In *dia*-deficient embryos, severe structural changes in the actin cytoskeleton are manifested after nuclear cycle 11. Formation of the hexagonal actin arrays is disrupted during prophase and metaphase (Fig. 1E) and there is an absence of actin staining between the metaphase nuclei (Fig. 1I,J). Similar patterns of staining were obtained when we stained *dia* embryos with antibodies directed against anillin and Peanut, other components of the metaphase furrow (see below). There is thus a failure in formation of the metaphase furrow.

Consistent with the known role of metaphase furrows in maintaining nuclear organization, the nuclei in *dia* mutant embryos frequently exhibit abnormal spacing and, in some cases, fuse in subsequent nuclear cycles. These irregularities are readily apparent in contrast to the uniform pattern observed in the wild type (compare Fig. 2A,C). In regions in which cortical actin staining is weak or absent, nuclei are frequently found displaced into the interior of the embryo (Fig. 2D), although the centrosomes remain at the surface (data not shown).

To investigate whether the absence of metaphase furrows

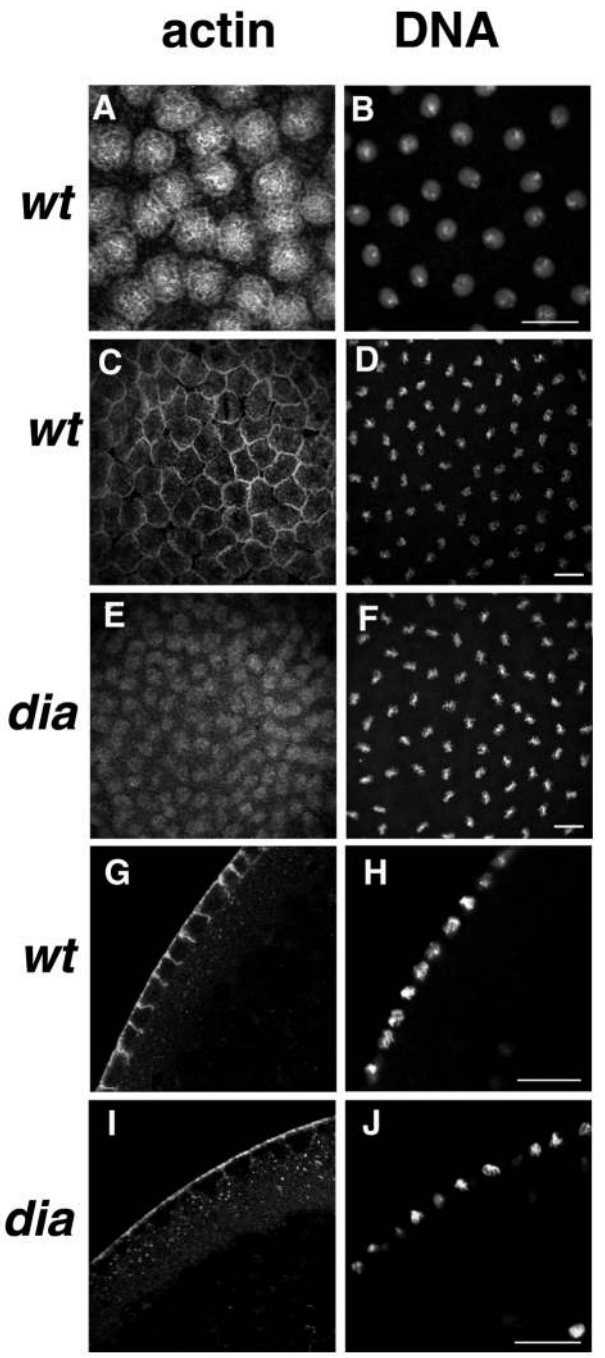


Fig. 1. Requirement for Diaphanous in metaphase furrow formation. Paired confocal images of wild-type (A-D,G,H) and *dia* mutant (E,F,I,J) embryos stained with anti-actin antibody (left column) and DAPI (right column) to detect actin and DNA. (A,B) Actin caps are apparent during wild-type interphase. (C,D) En face view of metaphase embryo, revealing hexagonal actin arrays associated with metaphase furrows. (E,F) En face view of *dia* mutant embryo at metaphase, revealing the absence of hexagonal actin arrays. (G,H) Sagittal view of a metaphase wild-type embryo, revealing actin lining the metaphase furrow. (I,J) Sagittal view of a *dia* mutant embryo, revealing the failure of metaphase furrow formation. Bars, 20 μm.

results from a failure in membrane invagination, we stained *dia* embryos with antibodies directed against myosin. In wild-type embryos, myosin localizes to the embryonic cortex between

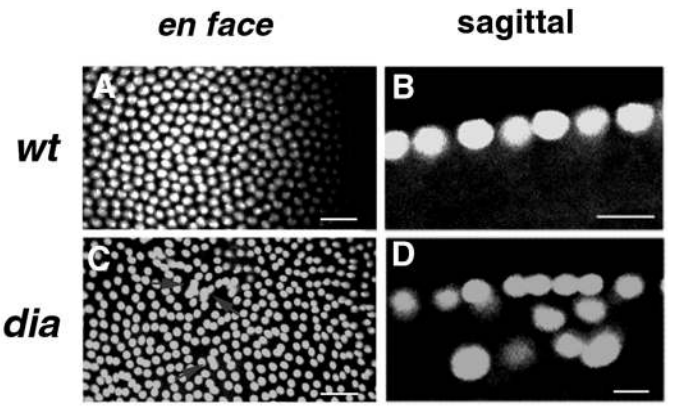


Fig. 2. Abnormalities in nuclear spacing in *dia* mutant embryos. Confocal micrographs of interphase embryos stained with DAPI. (A,B) Wild-type; (C,D) *dia*-deficient. Arrows indicate regions where nuclei exhibit abnormalities in size, shape, or spacing. Some nuclei have detached from the cortex and fallen to the interior of the *dia* mutant embryo, as is evident in the sagittal view. Bar, 10 μm in A,C and 20 μm in B,D.

the actin caps at each interphase (Fig. 3A-D), appears at the tip of the invaginating membrane at prophase (Fig. 3E-H) and disappears at metaphase. In *dia* embryos, we detect myosin staining, albeit very weak and irregular, between the actin caps at the cortex during interphase (Fig. 3I-L). At prophase, myosin, where detectable, remains at the cortex, with no detectable membrane pinching or invagination (Fig. 3M-P). Therefore, despite the presence of myosin at the cortex between actin caps, the membrane invagination that precedes metaphase furrowing is absent in *dia* embryos.

We also used immunolocalization to determine whether Diaphanous plays a role in the recruitment of two other furrow components, anillin and Peanut, a *Drosophila* septin. In wild-type embryos both anillin and Peanut localize to the embryonic cortex, between the actin caps at interphase (Fig. 4A,I; Fares et al., 1995; Field and Alberts, 1995). During prophase and metaphase, they localize to the metaphase furrow and their pattern of stainings are similar to that of actin (Fig. 4E,M). In *dia* embryos, the staining patterns of both anillin and Peanut are very weak during interphase (Fig. 4C,K). Similarly, in *dia* embryos the localization of both of these proteins is disrupted during prophase and metaphase, when the metaphase furrow is being formed in wild-type embryos (Fig. 4G,O). Diaphanous is thus required for recruitment and proper localization of anillin and Peanut as well as myosin to the regions of membrane invagination.

Diaphanous is required for proper cellularization of the syncytial embryo

Following the 13 syncytial nuclear division cycles, wild-type

Fig. 4. Defects in localization of Peanut and anillin in *dia*-deficient embryos. Surface view of wild-type (A,B,E,F,I,J,M,N) and *dia* (C,D,G,H,K,L,O,P) embryos double stained for DNA (B,D,F,H,J,L,N,P) and either Peanut (Left) or anillin (Right) during syncytial nuclear divisions. Embryos shown are at interphase (A-D,I-L) or prophase (E-H,M-P). In wild-type embryos, Peanut and anillin localize between the nuclei at interphase and mark the position of the metaphase furrow at prophase. In the absence of *Diaphanous*, the localization pattern of these proteins is disrupted. Bars,10 μm.

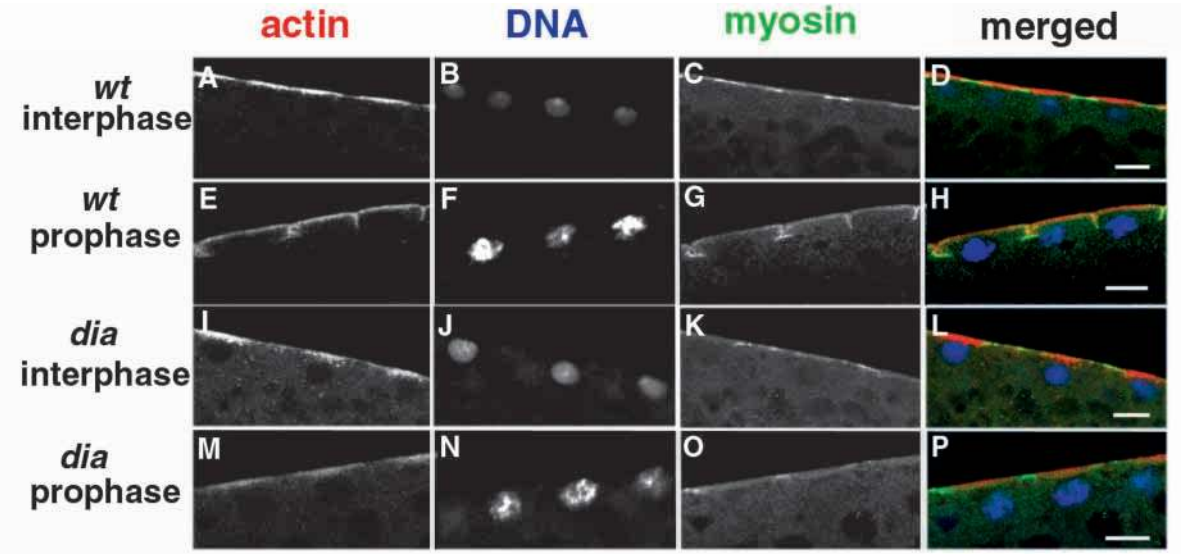
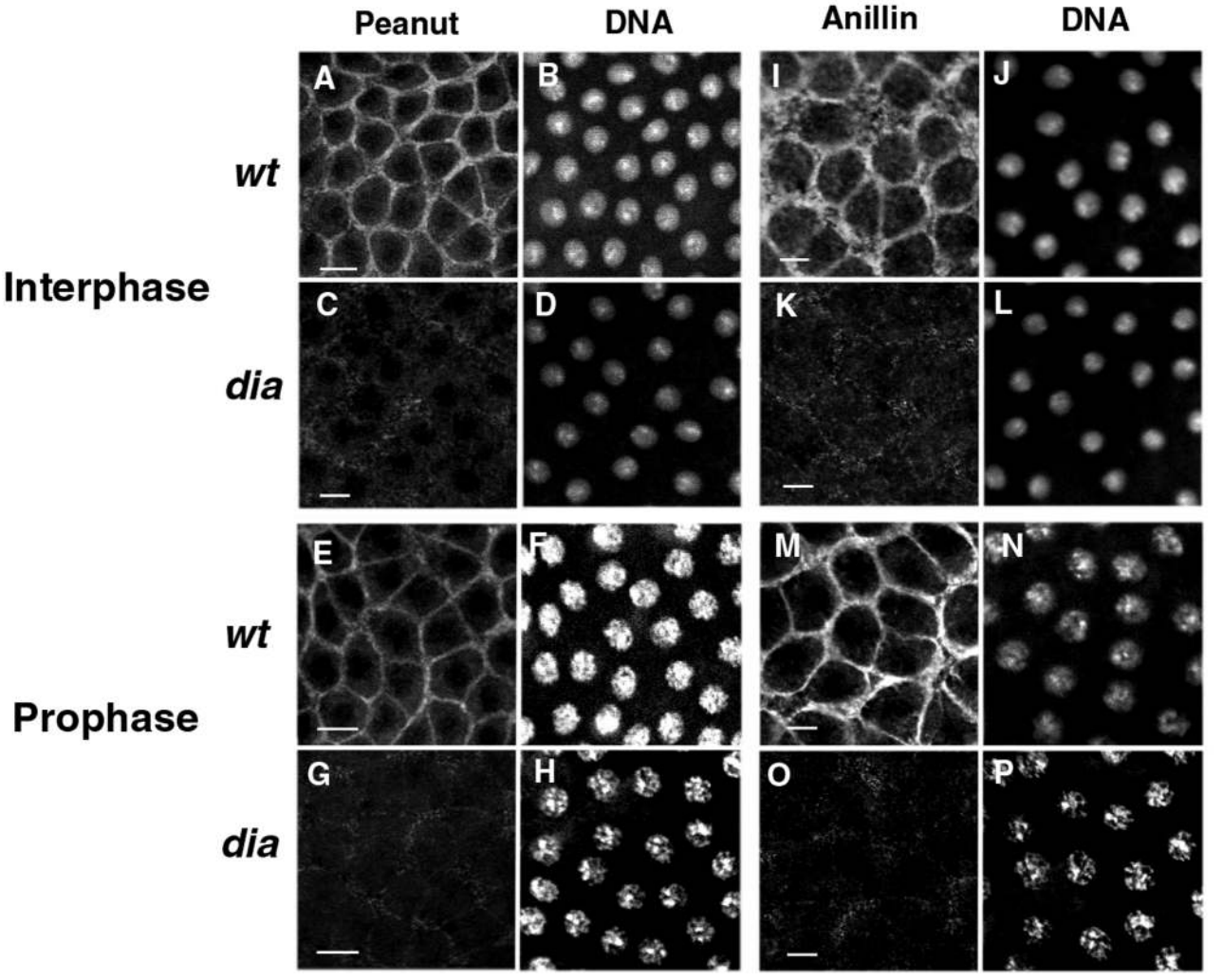


Fig. 3. Behavior of myosin at the cortex during the interphase to prophase transition in syncytial embryos. Confocal images of wild type (A-H) and *dia* (I-P) embryos at nuclear cycle 11, stained for actin (A,E,I,M), DNA (B,F,J,N), and myosin II (C,G,K,O). The stages of the cell cycle were determined by morphology of the chromosomes. In the wild-type embryos, myosin localizes to the cortex between the actin caps above the interphase nuclei (A-D), then extends from the embryonic cortex to the tip of metaphase furrows growing between the prophase nuclei (E-H). In *dia* mutant embryos, weak and irregular myosin II staining is observed between the actin caps at interphase (I-L) and no movement of myosin into the embryo is observed at prophase (M-P). Bars, 10 μm.



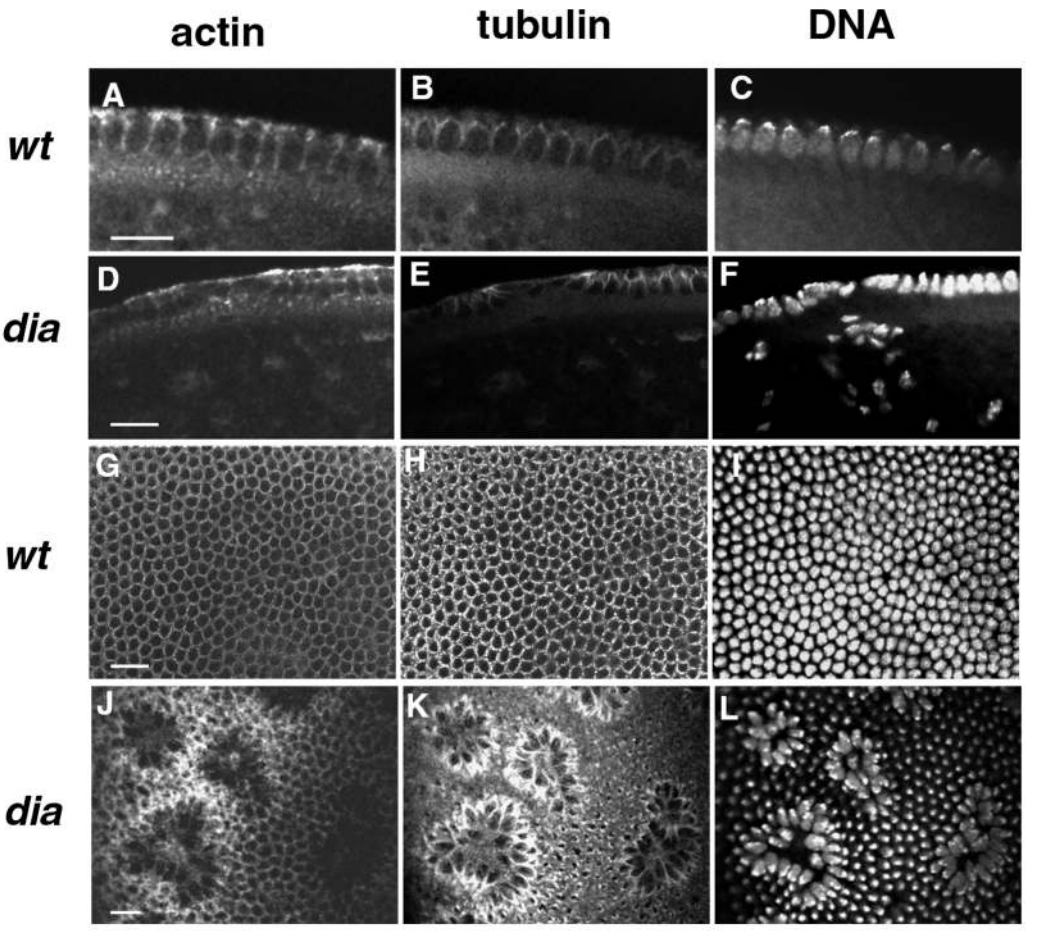


Fig. 5. Cellularization defects in *dia*-deficient embryos. Confocal micrographs of cellularizing wild-type and *dia* mutant embryos, stained for actin (left), microtubules (middle) and nuclei (right). (A-C) Sagittal view of a wild-type embryo. Actin and microtubule structures have grown inward, perpendicular to the surface of the embryo. (D-F) Sagittal view of a *dia* embryo. The cortical actin distribution is nonuniform and, for some nuclei the furrow canal is improperly oriented or absent. The microtubules do not project at right angles to the surface and nuclei have fallen into the yolky region of the embryo. (G-I) En face view of a wild-type embryo. Both microtubules and actin form hexagonal structures and nuclei are regularly spaced. (J-L) En face view of a *dia* embryo. The hexagonal array of actin and microtubules is disrupted. In patches devoid of organized actin, nuclei are misoriented and microtubules project parallel to the surface, forming a flower-shaped structure. Bars, 20 μm.

cellularization initiates. Actin-associated membrane invaginates between neighboring nuclei (Fig. 5A). This invagination is accompanied by the growth of microtubules, which extend from the pair of centrosomes above each nucleus, to form a basket-shaped structure that eventually surrounds each nucleus (Fig. 5B,C). The subsequent formation of individual cells involves further growth of the membrane and an actomyosin-mediated contractile event that pinches off the membrane at the bottom of each nucleus.

In *dia* embryos, there is a variable defect in the organization of both actin- and microtubule-based structures during cellularization. In the least severe cases, the cellularization furrow is absent between some nuclei, without any noticeable

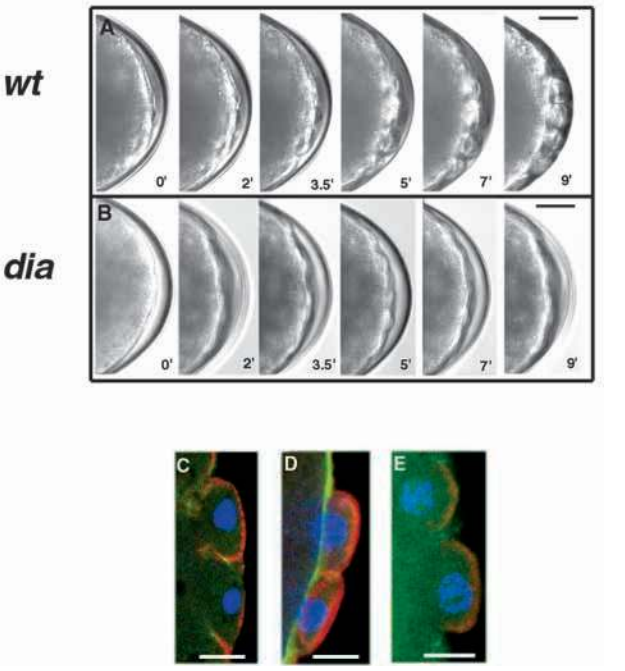


Fig. 6. Absence of pole cell formation in *dia* embryos. (A,B) Light microscopy of live embryos at their posterior pole. (C-E) Posterior poles of embryos stained with actin (red), myosin (green) and DAPI (blue). The stages of the cell cycle were determined by chromosomal morphology. (A,B) The series represent a course of pole cell formation during nuclear cycles 9-10 in wild-type (A) and *dia* mutant embryos (B) recorded by time-lapse microscopy. t=0 minutes is the beginning of observation of cytoplasmic buds. In *dia* embryos, although the cytoplasmic buds form normally, they will not grow and undergo cytokinesis to form pole cells. (C) Posterior pole of a wild-type embryo at interphase of nuclear cycle 10. The cytokinesis process is started, being apparent by an inward growth of the membrane which is marked by a concentration of myosin II at the tip of the invaginating membrane. At prophase (D), membrane invagination has progressed further and an actomyosin contractile ring is apparent at the base of the presumptive cells. (E) Posterior pole of a *dia* embryo at prophase of nuclear cycle 10. Note the absence of membrane invagination and an actomyosin contractile ring. Bars, 30 μm in A,B and 10 μm in C-E.

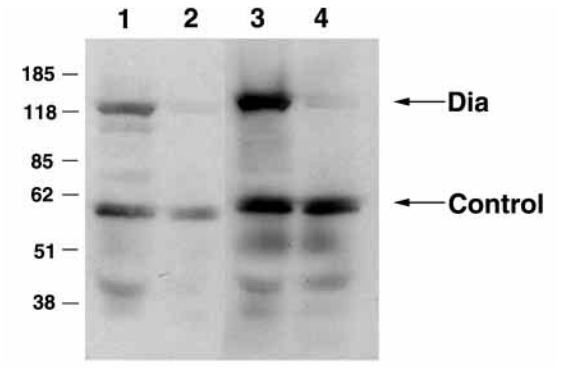


Fig. 7. Immunoblot analysis of the Diaphanous antibodies. Samples prepared from wild-type embryos (lanes 1 and 3) or from embryos derived from *dia* germline clones (lanes 2 and 4). Lanes 1 and 2 were probed by an affinity-purified antibody made against the N-terminal domain of Diaphanous. Lanes 3 and 4 were probed with sera made against the FH2 domain of Diaphanous. Anti-Tube antibody (Letsou et al., 1993) was used as a loading control.

defect in morphology or positioning of nuclei or microtubule structure (data not shown). In more severely affected embryos, actin staining is absent at the furrow canals and irregular at some regions of the cortex (Fig. 5D). Surface regions that lack any organized actin display abnormalities in the positioning of both nuclei and microtubule baskets (Fig. 5E,F). Viewed from *en face*, such embryos display readily apparent irregularities in the hexagonal actin and microtubule arrays and nuclear positioning (Fig. 5J-L). These abnormalities are manifested as starburst arrays of nuclei and associated microtubules (Fig. 5K,L), with the nuclei tilted outward (Fig. 5L). In such regions, actin staining is faint or absent at the cortex (Fig. 5J), although some patchy staining is detected deeper in the embryo (data not shown). In addition, γ-tubulin staining reveals that centrosomal behavior is abnormal in these regions (data not shown). This phenotype is similar to that observed in embryos treated with Cytochalasin D, in which the disruption of the cortical actin results in misorganization of the nuclei and mislocalization of microtubule baskets (Edgar et al., 1987). In the most severely affected *dia* embryos the defective cortical actin and arrays of misoriented nuclei were observed over the entire embryonic surface (data not shown).

The localization of both anillin and Peanut in *dia* embryos was abnormal in regions of nuclear misorientation, but was wild-type elsewhere (data not shown). For example, anillin, which can be detected in the nuclei of wild-type embryos only after cellularization, was present in the tilted nuclei in *dia* embryos during cellularization.

Diaphanous is necessary for pole cell formation

During interphase of the wild-type cortical nuclear divisions, actin cap formation causes protrusion of the plasma membrane and cytoplasm around each nucleus, forming a cytoplasmic bud. At the posterior pole, in contrast to the rest of the embryo, these cytoplasmic buds grow extensively during nuclear cycle 10. Cytokinesis at the base of each bud results in the formation of a set of pole cells, progenitors of the adult germline (Foe and Alberts, 1983; Swanson and Poodry, 1980).

In *dia* mutant embryos, formation and growth of the cytoplasmic buds at the posterior pole is wild-type (Fig. 6A,B). Such buds, however, never cleave to produce pole cells. Rather, they regress in synchrony with the buds covering the rest of the embryonic cortex. Buds reform at the posterior pole at each nuclear cycle, but do not undergo cytokinesis. In some embryos, the number and size of the somatic buds are abnormal compared to wild-type embryos. Moreover, unlike the somatic nuclei, the posterior pole nuclei fail to initiate cellularization.

To investigate the basis for the failure in pole cell formation, we assayed for the presence of an actomyosin contractile ring at the base of the posterior cytoplasmic buds. In contrast to wild-type embryos (Fig. 6C,D), *dia* embryos lack any concentration of actin or myosin at the base of the cytoplasmic buds, suggesting the contractile process does not start in the absence of Diaphanous (Fig. 6E).

Diaphanous localizes to sites of membrane invagination in the syncytial embryo

The phenotypic analysis of *dia* embryos indicated a requirement for Diaphanous for nuclear organization, metaphase furrow formation, cellularization and pole cell formation. To investigate the function of Diaphanous in these actin-mediated events, we used anti-Diaphanous sera to assay the spatial and temporal pattern of localization of Diaphanous during early embryonic development. The specificity of the

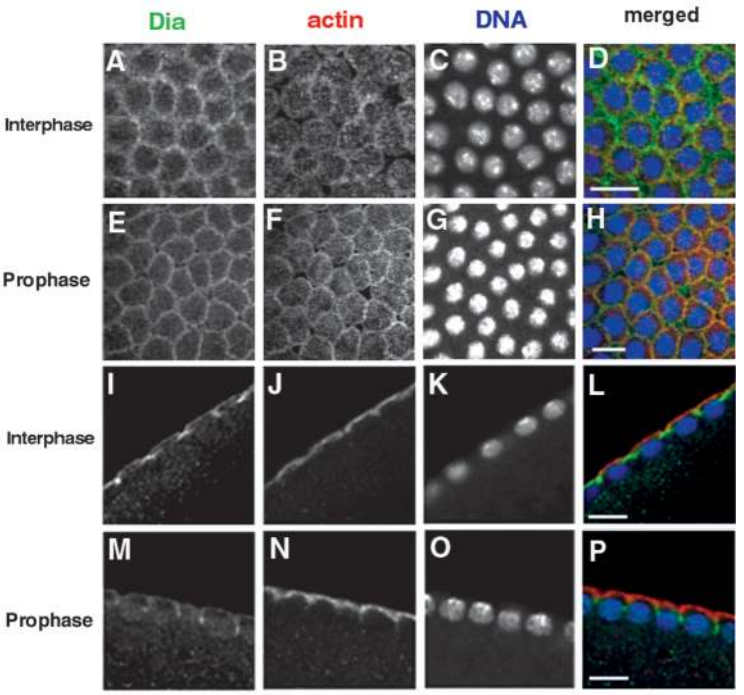


Fig. 8. Diaphanous localization at the cortex during interphase and prophase of nuclear cycles. Embryos are stained with anti-Diaphanous antibody (A,E,I,M,), anti-actin antibody (B,F,J,N,) and DAPI (C,G,K,O). (A-D) Diaphanous localization between the actin cap structures from an en face view during interphase. The same embryo is shown from a sagittal view in I-L. En face (E-H) and sagittal views (M-P) of Diaphanous localization during prophase. Note the abundance of Diaphanous at the tip of the actin filaments at the invaginating metaphase furrow (M-P). Bar, 10 μm.

sera was confirmed by demonstrating that: (1) two different sera generated against distinct portions of the Diaphanous protein detect the same species in an immunoblot and (2) this species is absent in an extract prepared from 0- to 2-hour embryos derived from a *dia* germline clone (Fig. 7). Furthermore, none of the patterns of Diaphanous localization described below is observed in *dia* null embryos (data not shown). For subsequent immunolocalization experiments, we used the antibody raised against the FH2 domain of Diaphanous.

During the first ten nuclear division cycles, we do not detect Diaphanous localization to specific structures. Once the nuclei migrate to the cortex, however, Diaphanous staining appears as a hexagonal array at the surface of interphase and prophase embryos (Fig. 8A,E). At interphase, actin filaments remain in cap structures (Fig. 8B) and Diaphanous localizes to the site of formation of the metaphase furrow prior to any significant redistribution of actin, which occurs at prophase (Fig. 8F). This is readily apparent in a sagittal view, revealing intense Diaphanous staining between the interphase actin caps (Fig. 8I,L). By prophase, Diaphanous is abundant at the tip of the metaphase furrow (Fig. 8M,P); this intense localization continues through metaphase (data not shown).

During cellularization, Diaphanous is enriched at the tip of the cellularization front and at the site of membrane invagination (Fig. 9A-F). As cellularization ends, Diaphanous localizes to the basal surface of each newly formed cell, where a contractile event pinches off the membrane at the base of the nuclei to produce individual cells (Fig. 9G-I).

DISCUSSION

Diaphanous has a broad role in organization of actin into contractile structures

Metaphase furrow formation and cellularization share fundamental features with each other and with cytokinesis; studies involving identification and characterization of molecules in each of these processes have improved our general understanding of the mechanistic basis of actin-dependent cortical events. Nevertheless, these processes differ in many aspects and each has unique characteristics and component molecules. Several mutagenesis screens have identified genes that are involved in early nuclear and cytoskeletal organizations in *Drosophila*. Among those genes, the products of *sponge*, *daughterless abo-like* (*dal*) and *scrambled* are specifically required for formation of the metaphase furrow, whereas mutations in *nullo*, *serendipity-α* and *bottleneck* only affect cellularization (Postner et al., 1992; Schejter and Wieschaus, 1993; Sullivan et al., 1990, 1993). The functions of *nuclear-fallout* (*nuf*) and *discontinous actin hexagon* (*dah*) are required for proper formation of both metaphase and cellularization furrows (Sullivan et al., 1993; Zhang et al., 1996). Lastly, mutations in *pebble* disrupt cytokinesis in all cells but have no effect on early development (Hime and Saint, 1992). Our finding that Diaphanous is required for metaphase furrow formation, pole cell formation and cellularization indicates that Diaphanous, like myosin, plays an essential role in all actin-mediated processes involving membrane invagination.

Function of Diaphanous

In the absence of Diaphanous, actin filaments do not reorganize from actin caps to metaphase furrows and the actin contractile ring fails to form at the base of the polar nuclei. As a consequence, events that lead to the formation of metaphase

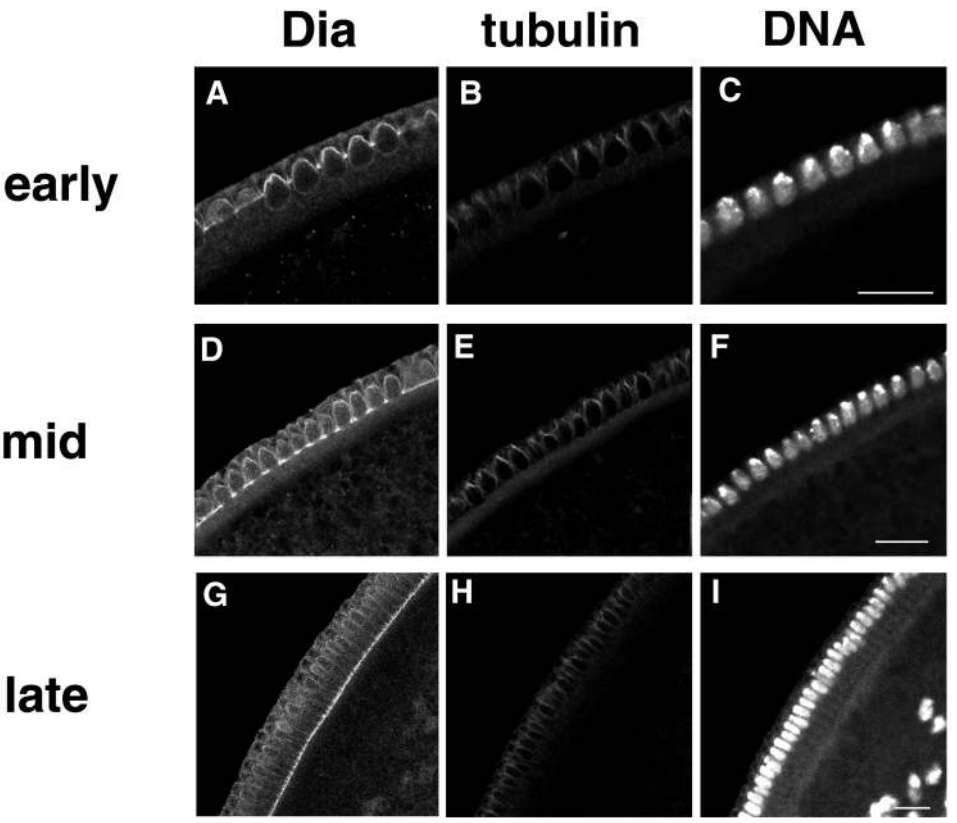


Fig. 9. Immunolocalization of the Diaphanous protein to cellularization furrows, sagittal view. (A,D,G) Diaphanous staining; (B,E,H) microtubule staining; (C,F,I) chromosomal staining. Different phases of the cellularization were determined according to Young and Kiehart (Young et al., 1993). (A-C) Slow phase of cellularization: the membrane grows inward between the nuclei until it reaches the bottom of the nuclei, which are changing their shape from round to oval structures (B). Diaphanous staining is concentrated at the tip of the growing furrow (A). (D-F) The end of the slow phase: the neighboring nuclei are surrounded and separated by the lateral membrane, but they are still open at the base. Nuclei have adopted an oval shape (F). Diaphanous still localizes to the tip of the growing membrane and has surrounded the nuclei, except at their base (D). (G-I) End of the cellularization. Diaphanous is localized to the base of the nuclei between the cytoplasm and the yolk boundary, where a contractile event pinches the membrane and packages the nuclei into individual cells (G). Bar, 10 μm.

furrows and pole cells are absent. Similarly, in *dia*-deficient spermatocytes, the actin contractile ring is missing and there is no membrane constriction at the mid-body during anaphase (Giansanti et al., 1998). Therefore, different FH proteins may be involved at different steps of cytokinesis.

Diaphanous appears dispensible for functions of the actin cytoskeleton that do not involve membrane invagination. Thus an absence of Diaphanous does not disrupt organization of cortical actin into cap structures or formation of cytoplasmic (somatic) buds during interphase in early embryogenesis. Furthermore, our success in generating female germline clones indicates that Diaphanous is dispensable for the germline cell divisions that lead to formation of the nurse cells and oocytes. Indeed, nurse cells are interconnected by ring canals, structures that are produced by incomplete cytokinesis and are considered non-contractile (Knowles and Cooley, 1994). Nevertheless, we cannot rule out the possibility that Diaphanous participates in these processes but has a redundant function.

Our phenotypic analysis suggests that the function of Diaphanous is necessary for proper cellularization. Lack of the Diaphanous protein results in defects in centrosomal and nuclear organization at the onset of cellularization. Indeed, the nuclear patterning and cellularization defects that we detect in *dia* mutant embryos resemble those observed in wild-type embryos injected with cytochalasin D (Edgar et al., 1987; Foe and Alberts, 1983; Zalokar and Erk, 1976), indicating a requirement for *dia* in organization of cortical actin at cellularization. These defects do not simply reflect earlier failure in formation of metaphase furrows, as indicated by the greater number of embryos affected during cellularization than in early stages. In addition, previous studies have shown that cellularization is independent from early nuclear divisions, since mutations in *scrambled* and *sponge* affect early nuclear divisions but do not disrupt cellularization (Postner et al., 1992; Sullivan et al., 1993).

It is interesting that some of phenotypes observed in *dia* mutant embryos are similar to those observed in embryos derived from females defective for *cappuccino* (*capu*), a second *Drosophila* FH gene (Emmons et al., 1995). Such embryos, like those defective for *dia*, lack pole cells, have variable defects in cellularization and generate larval cuticles with defects affecting both the dorsoventral and the anteroposterior axes (Manseau and Schüpbach, 1989). Thus, although *cappuccino* plays an essential role in determining cell polarity and *diaphanous* is required for cytokinesis, the function of these genes may overlap in embryogenesis.

Consistent with the model that Diaphanous is necessary for assembly of actin filaments at sites of membrane invagination, we find that Diaphanous localizes to such sites prior to recruitment of actin. In particular, we find Diaphanous at the site of metaphase furrow formation prior to actin localization to this site and at the growing tip of the cellularization furrow, ahead of the actin front. Furthermore, Diaphanous localization precedes that of actin at the cleavage furrow during cytokinesis (K. A. and S. A. W., unpublished data). The timing and position of Diaphanous localization at contractile structures are very similar to those of myosin II, anillin and Peanut (Field and Alberts, 1995; Fares et al., 1995).

Our studies indicate that Diaphanous localization is both a marker for and a determinant of actin filament assembly at sites of membrane invagination. A similar role was proposed previously for the *S. pombe* FH protein Cdc12 (Chang et al., 1997). Consistent with this idea, we find that the localization of myosin II, anillin and Peanut is disrupted in *dia* embryos, suggesting that Diaphanous is necessary for recruitment of the components of the metaphase furrow and possibly acts in a pathway upstream to localization of these proteins. Alternatively, the localization of these proteins and Diaphanous to the position of the metaphase furrow can be interdependent. It is possible that the function of Diaphanous in reorganization of the actin filaments from actin cap to the metaphase furrow is separate from its function in recruitment of furrow components. Nevertheless, the primary function of the Diaphanous might be recruitment of anillin and Peanut, which in turn mediate reorganization of the actin filaments. Further investigations involving the phenotypic analysis of embryos deficient for Peanut and anillin is necessary to distinguish among these possibilities.

Role of Diaphanous in signaling to the actin cytoskeleton

During nuclear cycle 11 in the syncytial *Drosophila* embryo, distinct actin-mediated events take place simultaneously in different regions of a common cytoplasm. At the posterior pole, cytokinesis affects pole cell division; in the remainder of the embryo, metaphase furrow formation maintains nuclear spacing and integrity. Furthermore, whereas the cytokinesis event that generates a pole cell begins during interphase, cytokinesis in other tissues has an onset in anaphase. There is thus a requirement for diverse developmental and cell-cycle cues to be channeled into a common pathway for actin-mediated contraction.

Diaphanous is well suited to serve as a bridge between components of signal transduction pathways that govern development and the cell cycle and components of the cytoskeleton that mediate assembly of actin-based structures. In particular, there is accumulating evidence from a variety of experimental systems that FH proteins exert their effect on actin organization by mediating signal transduction between the Rho GTPases and the actin-binding protein profilin (Evangelista et al., 1997; Imamura et al., 1997a; Kohno et al., 1996; Watanabe et al., 1997). Consistent with the requirement for Diaphanous, Rho proteins and profilin have essential roles in the *Drosophila* germline and cellularization (Crawford et al., 1998; Verheyen and Cooley, 1994).

We imagine that specific signals, acting through Diaphanous, could assign actin cytoskeletal function for a specific cortical event. Different sets of Rho proteins and their regulators could recruit and/or activate Diaphanous in particular locations and at particular times. Diaphanous has a genetic interaction with Rho1 and the RhoGTPase exchange factor encoded by the *Drosophila pebble* gene. Pebble is strictly required for cytokinesis, but not for any membrane invagination events in the syncytial blastoderm (Hime and Saint, 1992; Prokopenko et al., 1999). In addition, the immunolocalization of Diaphanous in dividing cells closely overlaps that of Pebble, being nuclear during telophase and interphase and at the cleavage furrow at anaphase (K. A. and S. A. W., unpublished data). Thus, one can imagine that Diaphanous function is controlled by Pebble at anaphase during cytokinesis, whereas other factors regulate Diaphanous function during early embryogenesis.

We postulate that Diaphanous mediates crosstalk between microtubule and actin filaments. It has been suggested previously that centrosomes dictate the behavior of cortical actin during the syncytial nuclear cycles (Foe et al., 1993). In particular, it has been argued that interactions between the astral microtubules and the embryonic cortex mediate cycling of actin filaments from caps to the metaphase furrow during prophase and metaphase. In *dia* mutant embryos, centrosomal separation and formation of the bipolar spindle proceed normally during nuclear divisions, and actin caps are formed and divide in conjunction with the spindle structure. Nevertheless, there is a failure in reorganization of actin for metaphase furrow formation. Based on this phenotype and localization of the Diaphanous, we suspect that Diaphanous is a good candidate for a protein at the cortex through which microtubules signal actin organization. Indeed, recent studies in budding yeast provide a clue to the potential position of Diaphanous in positioning and assembly of cytoskeletal structures. The FH proteins Bni1p and Bnr1p mediate cross-talk between cortical actin and the spindle structure and this cross-talk is necessary for correct positioning of the spindle in the bud neck (Lee et al., 1999; Miller et al., 1999). In addition, the role of Rho GTPases signaling molecules, putative interactors with Diaphanous, in interaction between microtubules and actin is well documented (For review see Waterman-Storer and Salmon, 1999). As an interaction between the mitotic spindle and the cell cortex is also thought to determine the position of the animal cell contractile ring, Diaphanous could similarly act in a pathway linking microtubule and microfilament organization.

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