RhoGEF2 and the formin Dia control the formation of the furrow canal by directed actin assembly during Drosophila cellularisation

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Summary

The physical interaction of the plasma membrane with the associated cortical cytoskeleton is important in many morphogenetic processes during development. At the end of the syncytial blastoderm of Drosophila the plasma membrane begins to fold in and forms the furrow canals in a regular hexagonal pattern. Every furrow canal leads the invagination of membrane between adjacent nuclei. Concomitantly with furrow canal formation, actin filaments are assembled at the furrow canal. It is not known how the regular pattern of membrane invagination and the morphology of the furrow canal is determined and whether actin filaments are important for furrow canal formation. We show that both the guanyl-nucleotide exchange factor RhoGEF2 and the formin Diaphanous (Dia) are required for furrow canal formation. In embryos from RhoGEF2 or dia germline clones, furrow canals do not form at all or are considerably enlarged and contain cytoplasmic blebs. Both Dia and RhoGEF2 proteins are localised at the invagination site prior to formation of the furrow canal. Whereas they localise independently of F-actin, Dia localisation requires RhoGEF2. The amount of F-actin at the furrow canal is reduced in dia and RhoGEF2 mutants, suggesting that RhoGEF2 and Dia are necessary for the correct assembly of actin filaments at the forming furrow canal. Biochemical analysis shows that RhoI interacts with both RhoGEF2 and Dia, and that Dia nucleates actin filaments. Our results support a model in which RhoGEF2 and dia control position, shape and stability of the forming furrow canal by spatially restricted assembly of actin filaments required for the proper infolding of the plasma membrane.

Key words: formin, blastoderm, furrow canal, morphogenesis, Drosophila melanogaster

Introduction

Many morphogenetic processes during development involve precise changes in the shape of the plasma membrane. Migrating cells form extensions at their leading edge, while during cytokinesis the plasma membrane constricts between the future daughter cells (Glotzer, 2001). The curvature of the plasma membrane is determined to a large degree by the cortical cytoskeleton and actin filaments being an integral part of it (Revenu et al., 2004). Shape changes and internalisation events require a reorganisation of the cell cortex and the actin cytoskeleton, which is controlled by GTPases of the Rho family (Etienne-Manneville and Hall, 2002; Lu and Settleman, 1999a).

The enclosure of the nuclei into cells during the cellular blastoderm of Drosophila embryos is achieved by a specialised process of membrane invagination and reorganisation of the actin cytoskeleton (Foe et al., 1993; Schejter and Wieschaus, 1993; Mazumdar and Mazumdar, 2002). Following the exit from the last mitosis of the cleavage stage, the plasma membrane folds in between adjacent nuclei to form the furrow canals that are visible by light microscopy as the cellularisation front after about 10 to 20 minutes when the shape of the nuclei is already ellipsoid. The furrow canals have a diameter of about 0.2 µm, are coated with actin filaments and remain connected with the surface plasma membrane. Apical to the furrow canal the so-called basal junction tethers the two adjacent membranes and thus stabilises the furrow (Hunter and Wieschaus, 2000). The mechanism of the spatially restricted assembly of F-actin at the furrow canal, as well as the factors that nucleate F-actin at this site, have not yet been identified. Furthermore, it is unclear whether actin filaments have an instructive function for the initial formation and shape of the furrow canal.

A few genes are known to be involved in furrow canal formation. Embryos mutant for nullo, sry-α, nuf, Rab11, Abl, dah or dia lack furrow canals between adjacent nuclei to a variable extent, which leads to the formation of multinuclear cells (Schweisguth et al., 1990; Postner and Wieschaus, 1994; Zhang et al., 1996; Rothwell et al., 1998; Afshar et al., 2000; Riggs et al., 2003; Grevengoed et al., 2003). Among this group of genes, nullo and sry-α are particularly interesting because they are early markers for the furrow canal and affect the formation of the basal junction (Hunter and Wieschaus, 2000). Another early marker for the furrow canal is the novel protein...
Slam, which is required for timed invagination of the furrow. Localised to the furrow canal and the basal junction, Slam recruits MyoII to the furrow canal and affects the accumulation of Arm at the basal junction (Lecuit et al., 2001; Stein et al., 2002). However, a direct link to furrow canal formation and F-actin polymerisation has not been established for any of the genes in this group.

To identify additional components required for proper cell morphology in the blastoderm we have screened a large collection of female-sterile mutants derived from germline clones (Luschnig et al., 2004) (our unpublished data). We found two allelic mutations that affect the cellulolarisation front. Mapping and complementation analysis identified RhoGEF2 as the mutated gene.

RhoGEF2 is required during gastrulation for apical constriction of the cells undergoing mesoderm invagination (Barrett et al., 1997; Häcker and Perrimon, 1998). Although there is evidence that RhoGEF2 genetically interacts with Rho1 and is controlled by Folded gastrulation during Drosophila gastrulation, the mechanism of how RhoGEF2 controls cell shape at this stage and whether this involves spatially restricted control of F-actin is not understood.

Potential effectors of RhoGEF2 and Rho1 are Rho kinase/sqh/myoII (Royou et al., 2004), citron kinase (Shandala et al., 2004; Naim et al., 2004; D’Avino et al., 2004), protein kinase N (Lu and Higgs, 2003; Xu et al., 2004; Shimada et al., 2004; Higashida et al., 2004; Romero et al., 2004). mDia1 is assumed to be activated by binding of Rho1 that releases an inhibitory intramolecular interaction of the C-and N-terminal domains of mDia1 (Alberts, 2001; Watanabe et al., 1997; Watanabe et al., 2004). Biochemical and structural studies of the yeast (BN1) and mouse (mDia; also known as Diap1 – Mouse Genome Informatics) homologues have shown how actin filaments are nucleated (Pruyne et al., 2002; Sagot et al., 2002; Li and Higgs, 2003; Xu et al., 2004; Shimada et al., 2004; Higashida et al., 2004; Romero et al., 2004). mDia1 is assumed to be activated by binding of Rho1 that releases an inhibitory intramolecular interaction of the C-and N-terminal domains of mDia1 (Alberts, 2001; Watanabe et al., 1997; Watanabe et al., 1999). However, this activation mechanism could only partially be reconstituted in vitro (Li and Higgs, 2003). Besides controlling actin fibres, Dia may also regulate microtubules (Ishizaki et al., 2001; Palazzo et al., 2002; Li and Higgs, 2003; Xu et al., 2004; Shimada et al., 2004; Higashida et al., 2004; Romero et al., 2004). mDia1 is assumed to be activated by binding of Rho1 that releases an inhibitory intramolecular interaction of the C-and N-terminal domains of mDia1 (Alberts, 2001; Watanabe et al., 1997; Watanabe et al., 1999).

We describe here a new function of RhoGEF2 and dia in the formation of the furrow canal. Mutant embryos have strongly enlarged furrow canals containing cytoplasmic blebs. Double immunolabelling studies show that RhoGEF2 and Dia are specifically concentrated between adjacent nuclei before the cellulolarisation front becomes visible and thus may serve as a template for the hexagonal pattern of membrane invagination. The amount of F-actin at the furrow canal is reduced in RhoGEF2 and dia mutants, suggesting that RhoGEF2 and Dia assemble actin filaments at the site of invagination and thus control the location, size and stability of the furrow canal. This further implies that spatially restricted F-actin polymerisation plays an important role for the initial infolding of the plasma membrane. Since many furrow canals still form in the absence of RhoGEF2 or dia, we tested whether nullo has a redundant function. Embryos lacking both RhoGEF2 and nullo, as well as embryos lacking both dia and nullo, do not form any furrow canals. This additive effect indicates that nullo functions in a genetic pathway separate from RhoGEF2 and dia. We propose that these pathways work in parallel to control two distinct but complementary aspects of furrow canal formation: actin polymerisation at the site of the infolding membrane and adherens junction formation.

Materials and methods

Genetics

Commonly used procedures were applied according to standard protocols (Roberts, 1998) and genetic material and fly strains were as described by FlyBase (http://www.flybase.net). The following chromosomes and alleles were used: Frt2R RhoGEF21 (Barrett et al., 1997), Frt2R RhoGEF24 (Barrett et al., 1997), Frt2R RhoGEF29 (Häcker and Perrimon, 1998), dia RhoGEF2 (Afshar et al., 2000), slam (Stein et al., 2002), Df(3R)X3F (sry-α), Df(16F) (nullo), nullo-HA [nullo fused to a haemagglutinin tag and expressed with the nullo promoter (Hunter and Wieschaus, 2000)], CyO, hh-lacZ, moesin-GFP [line sGMCa-n2 (Kiehart et al., 2000)]. slam embryos from slam germline clones were identified by absence of the hh-lacZ reporter. Germline clones were produced with Flippase-induced mitotic recombination and counter-selection by ovO-.

The lethality on chromosome fs(2R)201 was fine-mapped to 53EF by meiotic recombination with the w* of P-element insertions (l(2)k03609, l(2)k04222b, l(2)k07805b). The lethality of the two mutations from our screen, fs(2R)201 and fs(2R)350 was not complemented by the previously identified RhoGEF21 (Barrett et al., 1997). As the transcript is missing in the allele RhoGEF25, the described defects most probably represent a complete loss-of-function phenotype (Häcker and Perrimon, 1988). The RhoGEF2 phenotype (allele fs(2R)201), as well as the dia phenotype depend only on the maternal genotype, because zygotically homozygous and heterozygous embryos, as marked with a hh-lacZ reporter gene showed the same range of defects (data not shown). Molecular characterisation of the dia locus showed that about 3 kb of the original P element remained at the original insertion site (data not shown).

Histology

Embryos were heat-fixed in 0.4% NaCl, 0.03% Triton X-100 or fixed with 4% formaldehyde in PBS (except for phalloidin staining when 8% formaldehyde was used) and stored in methanol. Fixed embryos were stained in PBS with 0.2% Triton X-100 consecutively with solutions of primary antibody, fluorescent secondary antibodies (4 µg/ml, Alexa488, Alexa546, Alexa647; Molecular Probes), DNA dyes (DAPI, Hoechst, Oligreen or propidium iodide) and mounted in
either Mowiol/DABCO or Aquapolymount (Polyscience). Antibodies to the following proteins were used: RhoGEF2 (0.1 µg/ml), Dia (1:4000; J. G. and S. Wasserman), Slam (1:5000 Stein et al., 2002), Mel (B. Mechler), Arm (1:50), Sry–α (1:10), Dlg (1:20; Hybridoma Center), HA (12CA5, 1 µg/ml; Roche), β-gal (0.1 µg/ml; Roche), γ-tubulin (0.2 µg/ml; Sigma), phallolidin coupled to Alexa dyes (6 nM; Molecular Probes). To compare Dia and F-actin distribution in wild-type, RhoGEF2 and dia embryos, embryos of the two genotypes to be compared were mixed prior to fixation and processed as a mixture. Wild-type embryos were marked with a nullo-HA transgene, by the presence of RhoGEF2 staining or recognised by a proper F-actin array. For quantification of the RhoGEF2 or dia mutant phenotype nuclei in fields of 238×238 µm were counted in embryos stained with either Arm or Dlg antibodies or phallolidin.

**Microscopy**

Fluorescent images were recorded with a Leica confocal microscope (DMI2RE, 20× NA 0.7 water, HCX PL APO 63× NA1.2 corr, HCX PL APO 63× NA1.4-0.6 oil, laser at 405, 488, 543, 633 nm). Development of live embryos was recorded using an inverted microscope with differential interference contrast optics and a computer controlled stage (Leica DMI2RE, PL APO 63× NA1.4-0.6 oil; Hamamatsu ORCA-ER, Openlab software, Improvision). Digital photographs were processed with Photoshop (Adobe). For the analysis of the ultrastructure, embryos were staged in halocarbon oil (27S, Sigma) with a dissecting microscope. After removing the chorion with hypochlorite and rinsing, embryos were transferred to hexadecane, mounted in 100 µm deep aluminium plates and fixed by rapid high pressure freezing using a Balzer HPFM 10 machine. Fixed embryos were collected in liquid nitrogen and freeze-substitution was carried out with acetone containing 2% OsO4 at −90°C for 24 hours, −60°C for 6 hours and −40°C for 3 to 9 hours. Following embedding in Epon, sectioning and staining with Pt(II)citrate and U(II)acetate, specimens were examined on a Zeiss 109 or Philips CM10 transmission electron microscope.

**Microinjection, drug treatment**

Eggs were dechorionated in 50% bleach, dried in a desiccation chamber, covered with halocarbon oil and subsequently injected posteriorly with 50-100 pl of aqueous dsRNA at 1 µg/µl except for sry-α dsRNA, which was injected into wild-type embryos at 6 µg/µl. Embryos were fixed with 4% formaldehyde for 30 minutes. The vitelline membrane was removed manually. For the latrunculin A treatment, embryos of the two genotypes to be compared were mixed prior to fixation and processed as a mixture. For radioactive labelling, proteins were expressed from template plasmids carrying a modified β-globin leader sequence (pc2S derivatives) with SP6 RNA polymerase in a coupled in vitro transcription-translation system. Following SDS-PAGE the gel was visualised by phosphoimager (Fuji BAS1000).

**Actin polymerisation assay**

The indicated protein solution (60 µl) in G-buffer (20 µM CaCl2, 20 µM ATP, 0.5 mM Tris-HCl pH 8) and 10 µl of polymerisation buffer (12.5 mM KCl, 0.5 mM MgCl2, 25 µM ATP) were added to 30 µl of G-actin (10% labelled with pyrene, final concentration 3 µM; Cytoskeleton, USA). Fluorescence was excited at 365 nm and recorded at 407 nm.
Results

RhoGEF2 and dia are involved in furrow canal formation

The lethality of the mutation fs(2R)201 from our screen of germline clone mutations was mapped to the RhoGEF2 region by meiotic recombination. Complementation analysis with previously identified alleles showed that it was a new allele of RhoGEF2 (Barrett et al., 1997; Häcker and Perrimon, 1998).

In living embryos from RhoGEF2 germline clones the cellularisation front was invisible by light microscopy and the nuclei became irregularly arranged during the second half of cellularisation (see Movies 1 and 2 in the supplementary material). Similarly, we observed that in about one half of the embryos from dia germline clones (8 out of 14) the cellularisation front was invisible as well (see Movie 3 in the supplementary material). Thus the mutant phenotypes revealed a novel function of RhoGEF2 and dia during cellularisation that had not been described previously.

To investigate possible morphological defects, we analysed the ultrastructure of the furrow canals in embryos during mid-cellularisation by transmission electron microscopy. In wild-type embryos, the furrow canal can be seen as a hairpin loop with a diameter of about 0.2 µm in specimens fixed by high pressure freezing (Fig. 1A,B). Sometimes the diameter of furrow canals appears enlarged because of the plane of the section, in particular at the intersections of furrow canals. In six different embryos from two preparations, we found that 90% of furrow canals had the typical compact loop structure. In contrast, in embryos from RhoGEF2 germline clones, the furrow canal diameters were enlarged up to three fold and in most cases (78% in 6 embryos examined) did not show the typical hairpin loop (Fig. 1C,D). Instead, cytoplasmic blebs of variable size were present in the furrow canal, which we did not observe in wild-type embryos, suggesting that the enlarged mutant furrow canals are less stable, possibly because of their larger size or changed properties of the membrane. Defects in the morphology of the furrow canals were also observed in embryos from dia germline clones. The furrow canals in these embryos were more variable than in RhoGEF2 mutants ranging from about threefold dilated to diameters of more than 1 µm (Fig. 1E,F). Based on the analysis of the ultrastructure we conclude that RhoGEF2 and dia are required for compact and stable furrow canals during cellularisation.

Furthermore, in embryos from RhoGEF2 and dia germline clones furrow canals are not only enlarged, but are often missing between adjacent nuclei. We visualised this aspect of the phenotype by staining for F-actin, which marks the furrow canal and thus a regular hexagonal array is evident in surface views (Fig. 2A). In embryos from RhoGEF2 and dia germline clones these hexagons were frequently interrupted (Fig. 2B,D) and a variable proportion of the forming cells contained multiple nuclei (Fig. 2F,G). In contrast, the hexagonal array was complete in embryos mutant for slam (Fig. 2E), suggesting that slam controls different aspects of furrow canal formation than RhoGEF2, dia and nullo. The proportion of multinuclear cells was not different in embryos early or late in cellularisation, indicating that the defect occurs during the formation of the furrow canal. In about half of the embryos from dia germline clones almost all cells contained multiple nuclei, which is consistent with the variable and often severely disturbed morphology of these embryos (Fig. 2G).

To confirm the specificity of the defects in furrow canal formation, we checked whether other aspects of cellularisation, membrane invagination, nuclear extension and basal closure are affected. Membrane invagination and nuclear extension were compared in embryos from RhoGEF2 germline clones (n=10) and wild-type (n=5) embryos by time-lapse recordings (see Movies 1 and 2 in the supplementary material). For both genotypes, the nuclei extended at the same rate and cellularisation was completed in about 60 minutes (T=20–22°C). Staining of the furrow canal with Slam antibodies (Stein et al., 2002) showed a widening and pinching off of the base of the furrow canal, which indicates that basal closure occurs (see Fig. S1 in the supplementary material).
These data show that RhoGEF2 does not play a significant role in membrane invagination, basal closure and nuclear elongation and that even the dilated and abnormal furrow canals can lead the invagination of the plasma membrane at normal speed. The morphology in embryos from dia germline clones was more disturbed and variable (Afshar et al., 2000), especially in later celluarisation, the plasma membrane invaginates, possibly with a delay and indications of basal closure were clearly observed at least in some embryos (see Movie 3 and Fig. S1 in the supplementary material).

Distinct functions of RhoGEF2 and nullo in furrow canal formation

nullo and sry-α are also required for correct furrow canal formation (Postner and Wieschaus, 1994; Schweisguth et al., 1990). nullo and sry-α mutant embryos contain multinucleate cells because furrow canals are frequently absent (Fig. 2C). sry-α probably acts downstream of nullo, because Sry-α protein localisation depends on nullo (Postner and Wieschaus, 1994). To test whether they act in the same genetic pathway as RhoGEF2 or dia, we analysed the formation of the furrow canals, marked by F-actin in embryos lacking both gene functions by producing RNAi-induced phenocopies of nullo or sry-α in embryos from RhoGEF2 or dia germline clones (Fig. 3; see Fig. S2 in the supplementary material). In embryos from RhoGEF2 germline clones treated with RNAi for nullo (73%, n=33, Fig. 3B,E) or sry-α (63%, n=16, Fig. 3C,D), F-actin at the furrow canal was almost absent and only a few singular rings and irregular patches remained. The strength of the ‘double mutant’ phenotype directly correlated with the amount of Sry-α protein as seen in embryos with locally deposited sry-α dsRNA at the posterior pole (Fig. 3C). In transversal sections, F-actin staining at the furrow canal was observed only in anterior regions. This staining was gradually lost towards posterior regions in the ‘double mutant’ situation, which suggests that furrow canals are completely absent (Fig. 3D,E). Following nullo or sry-α RNAi injection into embryos from dia germline clones we observed, in both cases, an almost complete absence of furrow canals (data not shown).

The observed defects are specific because injection of nullo or sry-α dsRNA into wild-type embryos induced their respective phenocopies (see Fig. S2 in the supplementary material), whereas injection of an unrelated dsRNA did not change the RhoGEF2 and dia mutant phenotypes (Fig. 3A). In contrast to the strongly enhanced phenotype of the RhoGEF2–nullo ‘double mutant’, we observed an only slightly enhanced phenotype, when sry-α dsRNA was injected into nullo embryos (data not shown), which is consistent with the model that nullo and sry-α act in the same genetic pathway. Since the related phenotypes of RhoGEF2 and nullo/sry-α are additive and since the RhoGEF2 allele does not form a transcript and since the RhoGEF2 allele is amorphic.

RhoGEF2, Dia and F-actin colocalise at the furrow canal

Dia protein is localised at the furrow (Afshar et al., 2000), whereas the distribution of RhoGEF2 protein has not yet been

Fig. 2. Multinuclear cells in embryos from RhoGEF2 and dia germline clones. (A-E) Embryos of the following genetic constitution were stained for DNA (blue), γ-tubulin (green, added from a higher optical section) and F-actin (red). (A) Wild type, (B) from RhoGEF2Δ1/2 germline clones, (C) nullo, (D) from dia germline clones and (E) slam embryos from slam germline clones. Number of embryos with indicated proportion of nuclei in multinuclear cells plotted for (F) RhoGEF2 alleles (1.1, 4.1, 04291) and for (G) dia. Scale bars: 10 μm.
determined. We raised antibodies against an N-terminal RhoGEF2 fragment to investigate the intracellular distribution of RhoGEF2. The antibody specifically detects RhoGEF2 because in western blots a band migrating at more than 200 kDa was detected in extracts of wild-type but not of mutant embryos (Fig. 4G). In whole-mount staining of germline clone embryos from four alleles of RhoGEF2 (RhoGEF2201, RhoGEF21.1, RhoGEF24.1, RhoGEF204291) only a low and uniform signal was detected (Fig. 4D), whereas a locally restricted staining was observed at the tip of the furrow throughout cellularisation in wild-type embryos (Fig. 4A-C). The specific staining was visible when the nuclei were still spherical, that is before the cellularisation front appeared. In surface view RhoGEF2 formed a hexagonal array enclosing the nuclei (Fig. 4E). This distribution changed at the onset of gastrulation when RhoGEF2 staining was lost basally in the invaginating ventral cells and simultaneously appeared apically (Fig. 4F). These data show that RhoGEF2 localisation precedes furrow canal formation at the site of invagination, suggesting that it may determine the hexagonal pattern of membrane invagination.

To compare the localisation of RhoGEF2 with Dia, F-actin and Arm and to define the localisation of Dia in detail, we performed multiple immunolabelling. Although the actual formation of the furrow canals occurs within the first phase of cellularisation, we analysed the protein distribution slightly later, when membrane domains can be better distinguished, assuming that the later localisation pattern reflects an intrinsic property of the proteins. RhoGEF2 primarily localises to the furrow canal and not to the basal junction, marked by Arm, since we observed no significant overlap of RhoGEF2 and Arm staining (Fig. 5A). In contrast, RhoGEF2, Dia and F-actin staining clearly matched each other, and therefore colocalise at the furrow canal (Fig. 5C-E). As in the case of RhoGEF2, Dia localisation appears to precede furrow canal formation, since the specific staining was already observed when the nuclei were spherical (Fig. 5B). Thus the timing and pattern of RhoGEF2 and Dia localisation together with the specific defects in furrow canal formation strongly suggest that RhoGEF2 and Dia have an instructive function for the pattern and mechanism of membrane invagination.

Dia localisation at the furrow canal depends on RhoGEF2, but not on F-actin
Dia, RhoGEF2 and F-actin localisation may functionally depend on each other. We first tested whether F-actin is required for the localisation of Dia and RhoGEF2 at the furrow canal by treating permeabilised embryos with the drug latrunculin A (Coue et al., 1987) to depolymerise F-actin (Fig. 6). We observed that the distribution of the actin binding protein moesin (Polescello and Payre, 2004) depends on the presence of actin filaments (Fig. 6C), whereas Dia and RhoGEF2 localisation was not affected by the latrunculin treatment (Fig. 6A,B). These data show that Dia and RhoGEF2 localise at the furrow canal independently of F-actin and thus may act upstream of F-actin polymerisation.

Second, we tested whether F-actin localisation at the furrow canal depends on RhoGEF2 and dia, comparing phalloidin staining in wild-type, RhoGEF2 and dia mutant embryos in the early phase of cellularisation and observed a consistent reduction of F-actin at the furrow canal in RhoGEF2 embryos, which was even more prominent in embryos from dia germline clones (n>20, Fig. 7A-D). Consistent with the reduction of F-actin at the furrow canal, levels of MyoII were also reduced in the mutant embryos (see Fig. S3 in the supplementary material). In contrast to the reduction at the furrow canal, cortical F-actin appeared to be increased in some embryos from dia germline clones. This increase was variable and not observed in all of the experiments, however. These data suggest...
that RhoGEF2 and dia are involved in controlling a subpopulation of actin filaments at the furrow canal.

In a third step we assayed whether Dia localisation depends on RhoGEF2. In contrast to the striking localisation of Dia in wild-type embryos (Fig. 7E, \( n=56 \)), in embryos from RhoGEF2 germline clones Dia staining was dispersed during the first phase of cellularisation when the nuclei are still spherical (Fig. 7F, \( n=48 \)). Later Dia became weakly enriched at the furrow canal (data not shown). To exclude the possibility that RhoGEF2 affects Dia protein levels, we compared the total amount of Dia protein in wild-type and RhoGEF2 mutant embryos by western blotting. We detected comparable amounts of Dia protein in both extracts (Fig. 7G), suggesting that RhoGEF2 does not affect the synthesis or stability but rather the intracellular distribution of Dia. In contrast, RhoGEF2 localisation at the furrow canal is normal in embryos from dia germline clones (see Fig. S4 in the supplementary material). Consistent with the double mutant analysis, RhoGEF2 and Dia localise normally in nullo mutants and Sry-\( \alpha \) distribution is unchanged in embryos from RhoGEF2 and dia germline clones when compared to wild-type embryos (see Fig. S4 in the supplementary material). These data support a model in which RhoGEF2 localises to the site of membrane invagination and then directly or indirectly recruits Dia to this site, which in turn nucleates actin filaments in the hexagonal pattern instructed by RhoGEF2.

**Does the small GTPase Rho1 provide a link among RhoGEF2, Dia and F-actin?**

The proposed biochemical activities of the guanyl nucleotide exchange factor RhoGEF2 and the formin Dia suggest that

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### Fig. 4.
RhoGEF2 is enriched at the furrow canal. Embryos (heatfixed) stained with RhoGEF2 antibody (green, white), Dlg (red) and DNA (blue). Right half of A-D shows only the RhoGEF2 staining. (A-C,E,F) Wild type, (D) from RhoGEF2\(^{292291}\) germline clone. (E) Surface view; wild type. (F) Cross section of an embryo undergoing ventral furrow formation. Scale bar: (A-E) 10 \( \mu \)m, (F) 20 \( \mu \)m. (G) Western blot for RhoGEF2 or Dlg of extracts from wild-type embryos and embryos from RhoGEF2\(^{292291}\) germline clones.

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### Fig. 5.
RhoGEF2 colocalises with Dia and F-actin, but not Arm. Double labelling of wild-type embryos in green for (A,C,D) RhoGEF2, (B,E) Dia and in red for (A,B) Arm, (C) Dia and (E,F) F-actin. (A-C) heat-fixed, (D,E) fixed with formaldehyde. Scale bar: 10 \( \mu \)m.
RhoGEF2 activates a Rho type GTPase and that actin polymerisation by Dia is activated by a Rho type GTPase. To critically test such a model we reconstituted the interactions in vitro with purified components (see Fig. S5 in the supplementary material). First, to identify the substrates of the RhoGEF2 guanyl-nucleotide exchange activity we sampled all six Drosophila Rho GTPases (Newsome et al., 2000) in an in vitro GDP-GTP exchange assay (Fig. 8A,B). We loaded GST fusion proteins of Rho GTPases with 3H-labelled GDP and measured the release of GDP catalysed by the GEF domain of RhoGEF2. We found that GDP was significantly released only from the Rho1 protein, but not from the RhoL, Rac or Cdc42 GTPases, whereas the GEF domain of Trio was specific for the three Rac GTPases (Newsome et al., 2000). The GEF domain of RhoGEF2 with a single point mutation (T1544A) (Zhu et al., 2000) and GST had no activity. Consistent with the reported genetic interaction of RhoGEF2 and Rho1 (Barrett et al., 1997), these data show that Rho1 is the only GEF substrate of RhoGEF2.

Second, we tested the physical interaction of Rho1 and Dia with an in vitro binding assay (Fig. 8C). We found GST-Rho1 bound to the N-terminal part of Dia (DiaΔC464), but not to a fragment of Dia lacking the putative Rho binding domain (DiaAN318). The association of Rho1 and Dia was stronger with the activated form of Rho1 (Rho1Q63L) than with the GDP-loaded form.

In a third step, we tested whether Dia can induce actin polymerisation in a Rho1-dependent manner (Li and Higgs, 2003). We purified two fragments of Dia: an N-terminal fragment (DiaΔC518, containing the Rho-binding site) and a C-terminal fragment (DiaΔN519, containing the FH1, FH2 and autoinhibitory domains) and tested their activity on actin polymerisation in vitro. The C-terminal part alone induced actin polymerisation at submicromolar concentrations (see Fig. S6 in the supplementary material) similar to BN1 fragments from yeast (Pruyne et al., 2002). This activity was inhibited by equimolar amounts of the N-terminal part, but was partially restored by a 10-fold molar excess of Rho1 (Fig. 8D). However, no difference in activity was observed between activated forms of Rho1 (loaded with GTPγS, GMP-PNP or Rho1Q63L) and Rho1 loaded with GDP (data not shown). Our data show that the C-terminal part of Dia is sufficient for efficiently polymerising actin filaments. The polymerisation activity is inhibited by the N-terminal part, suggesting that Dia activity is controlled by an intramolecular inhibition. However, since it was not possible to fully reconstitute the release of Dia autoinhibition by Rho1, a behaviour also described for mDia1 (Li and Higgs, 2003), the mechanisms for Dia activation remains elusive. Together with the weak binding of Rho and Dia that only slightly depended on the activation state of Rho1 and consistent with the properties of mDia1, these data indicate, that Rho1-independent mechanisms appear to be involved (Wallar and Alberts, 2003).

Discussion

Changes of cell shape and curvature of the plasma membrane depend on the reorganisation of actin filaments. During cellularisation, actin fibres are assembled in a hexagonal array that prefigures the invagination of the plasma membrane. We have presented evidence supporting the model that spatially restricted assembly of actin filaments by the formin Dia at the site of invagination is involved in bending the plasma membrane and in furrow canal formation. These actin filaments may form a coat that determines the shape and stability of the furrow canal.

Our morphological analysis of the mutant phenotypes reveals a new function of RhoGEF2 and dia in the formation of the furrow canal. This function is consistent with the co-localisation of both proteins with F-actin at the furrow canal and the reduced amounts of F-actin in RhoGEF2 and dia mutants. Biochemical analysis demonstrates actin polymerisation by Dia and thus supports the model that RhoGEF2 and Dia organise actin filaments to control the formation of the furrow canals. Furthermore, we provide evidence that the previously characterised genes nullo and sry-

Fig. 6. RhoGEF2 and Dia localisation at the furrow canal do not depend on microfilaments. Permeabilized wild-type (A,B) embryos or (C) embryos with a GFP-moesin transgene were incubated with (A) buffer or (B,C) latrunculin A (LatA) to inhibit actin polymerisation, and stained for DNA (blue), F-actin (green); (A,B) RhoGEF2 and Dia; (C) GFP fluorescence. Scale bar: 50 µm.
Development directly or indirectly involved in the assembly of F-actin since proteins does not depend on F-actin (Fig. 6). However, they are of the cellularisation front (Fig. 5). The localisation of both localised at the furrow canal and both precede the appearance filled with cytoplasmic blebs (Fig. 1). Both proteins are disrupted. The furrow canals are much larger than normal and RhoGEF2 mutants the morphology of the furrow canal is spatially restricted assembly of actin filaments. In both and dia act in a genetic pathway in parallel to RhoGEF2 and dia, suggesting that they control two distinct aspects of furrow canal formation. This conclusion is based on the assumption that we used amorphic situations in our experiment. We cannot exclude that RhoGEF2 and dia stabilise the furrow canal rather than control its initial formation. A function in the formation is supported by our observation that the proportion of nuclei in multinuclear cells does not increase in the course of cellularisation.

The following arguments support the hypothesis that RhoGEF2 and dia act in the same genetic pathway that controls spatially restricted assembly of actin filaments. In both dia and RhoGEF2 mutants the morphology of the furrow canal is disrupted. The furrow canals are much larger than normal and filled with cytoplasmic blebs (Fig. 1). Both proteins are localised at the furrow canal and both precede the appearance of the cellularisation front (Fig. 5). The localisation of both proteins does not depend on F-actin (Fig. 6). However, they are directly or indirectly involved in the assembly of F-actin since the amount of F-actin is reduced at the furrow canal of the mutant embryos (Fig. 7). The strongest argument for a functional connection is that Dia localisation at the furrow canal depends on RhoGEF2 during the early phase of cellularisation (Fig. 7). Rh1 may mediate this functional link by direct interactions with RhoGEF2 and Dia (Fig. 8). However, our findings do not show that RhoGEF2 exclusively functions via dia. Other targets of Rh1-GTP, like citron kinase, protein kinase N or Rh kinase (Lu and Settleman, 1999b; Shandala et al., 2004; Naim et al., 2004; D’Avino et al., 2004; Royou et al., 2004) may be activated in parallel to Dia. Although we observe a reduction of MyoII at the furrow canal during the first half of cellularisation in embryos from RhoGEF2 germline clones, correspondingly lower MyoII levels are also observed in embryos from dia germline clones, which indicates that the reduction of MyoII may be a consequence of reduced F-actin levels. Consistent with the reduction of F-actin at the furrow canal, levels of MyoII were also reduced in the mutant embryos (see Fig. S3 in the supplementary material). In contrast to the reduction at the furrow canal, cortical F-actin appeared to be increased in some embryos from dia germline clones. This increase was variable and not observed in all of the experiments, however.

The difference in the RhoGEF2 and dia mutant phenotypes clearly shows that dia has additional functions and may be controlled by other not yet identified factors besides RhoGEF2. Whereas RhoGEF2 mutants pass through the cleavage cycles without obvious defects (data not shown), dia is involved in formation of pole cells and pseudo cleavage furrows (Afshar et al., 2000). As a possible consequence of these additional functions, dia mutants in contrast to RhoGEF2 mutants often have a more disrupted F-actin array, larger furrow canals and a more disturbed cellularisation than RhoGEF2 mutants (Figs 1, 2). Furthermore in the early phase of cellularisation Dia localisation depends on RhoGEF2, whereas later, after the furrow has formed, Dia becomes enriched to a certain degree at the cellularisation front independently of RhoGEF2. One gene that may act in parallel to RhoGEF2 to control Dia localisation is Abl. Embryos from Abl germline clones have reduced amounts of Dia at the furrow canal and show a disrupted F-actin array similar to that observed in dia and RhoGEF2 mutants (Grevengoed et al., 2003). However, the molecular link between Abl and Dia is elusive and no abnormalities in the morphology of the furrow canal in Abl mutants have been described. Thus Dia may be controlled and activated by multiple pathways including RhoGEF2 among others.

It is not known how the position of the invaginating plasma membrane is determined. RhoGEF2 and Dia are not likely to be part of a pattern formation process, but their localisation reflects an early readout of this pattern, since the nuclei and centrosomes are properly arranged in RhoGEF2 and dia mutants (Fig. 2). RhoGEF2 and Dia proteins are early markers for these sites and precede furrow canal formation because we detected specific staining for both Dia and RhoGEF2 when the nuclei were still spherical and when the cellularisation front was not yet visible (Fig. 4A, Fig. 5B). Other factors beside RhoGEF2 and Dia are also involved in furrow canal formation, because many furrow canals still form in RhoGEF2 and dia mutants, which indicates that there is genetic redundancy.

At present we can only speculate about which factors and
mechanisms are responsible for RhoGEF2 localisation. Candidates may be among the group of genes involved in furrow canal formation. However, for all of these mutations no ultrastructural analysis has been reported that would allow us to define the morphological defect and compare their function for furrow canal formation with the function of RhoGEF2 and dia. Among this group are Rab11 and nuf, which encode a GTPase of the recycling endosome and its putative effector (Riggs et al., 2003). Considering the assumed biochemical activities, it is conceivable that vesicle targeting is important for transporting factors to the site of membrane invagination (Riggs et al., 2003). This raises the possibility that RhoGEF2 is transported by such vesicles to the sites of membrane infolding. Analysis of RhoGEF2 protein distribution in nuf and Rab11 mutants and the phenotype of double mutants may address this hypothesis. Alternatively, RhoGEF2 may be transported to the site of the future furrow canal along microtubules that form open baskets around the nuclei, or other recruiting factors may precede at the site of membrane invagination.

Furthermore, slam is required for timed formation of the furrow and invagination of the membrane in the first half of cellularisation. Like Dia and RhoGEF2 Slam protein localises to the furrow canal and localisation precedes furrow canal formation. Slam may act by recruiting MyoII to the furrow canal, but the biochemical activities of Slam have not been defined (Lecuit et al., 2002; Stein et al., 2002). Although the membrane does not invaginate initially in slam mutants, a complete F-actin array is visible (Fig. 2). Thus despite the overlapping localisation of Slam, RhoGEF2 and Dia their functions are clearly distinguishable.

How do RhoGEF2 and Dia act in furrow canal formation? If we consider the biochemical activity of Dia to nucleate actin filaments (Fig. 8) and the enlarged and labile furrow canals in the dia mutants (Fig. 1), it is conceivable that Dia organises and assembles a coat of F-actin at the site of membrane invagination and furrow canal formation. The coat of F-actin may be important for the compactness and stability of the furrow canal to prevent infoldings of the cytoplasm. Such a function may be related to the function of F-actin in endocytic events (Engqvist-Goldstein and Drubin, 2003). The subset of actin filaments controlled by RhoGEF2 would not significantly contribute to pulling in the plasma membrane, since membrane invagination proceeds with normal speed in RhoGEF2 mutants. Alternatively, RhoGEF2 and Dia may perform their function independently of actin polymerisation. Although we have shown that the amount of F-actin is reduced in the mutants (Fig. 7), we do not exclude the possibility that the polymerisation activity of Dia is not required for all or part of its function. Dia may also influence the organisation of microtubules, as interactions of mDia1 with microtubules and EB1, a microtubule-associated protein, have been described (Ishizaki et al., 2001; Palazzo et al., 2001; Wen et al., 2004).

The differences in protein localisation and mutant phenotypes of RhoGEF2 and nullo suggest that they have distinct activities. In contrast to the frequently missing furrow canals in single mutants, their complete absence in embryos lacking both gene functions (Fig. 3) clearly implies, however, that their functions are redundant from a genetic point of view.
As proposed by Hunter and Wieschaus (Hunter and Wieschaus, 2000) the basal junction that tethers the two membranes of the furrow and that is located apically to the furrow canal is controlled by nullo, whereas our results show that RhogEF2 and dia are required for the formation of a compact and stable furrow canal. If one of the two pathways is disturbed, the furrow canal can still form, albeit with a lower and variable efficiency that depends on the conditions. For example the nullo phenotype is strongly temperature sensitive (Hunter et al., 2002). However, if both pathways are affected, furrow canals do not form at all. Future studies will resolve how the actin filaments are involved in bending the plasma membrane that leads to the furrow canal and will further demonstrate how RhogEF2 protein is expressed in the hexagonal array to serve as a template for local actin polymerisation.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/5/1009/DC1

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