A role for non-muscle myosin II function in furrow maturation in the early zebrafish embryo

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Summary
Cytokinesis in early zebrafish embryos involves coordinated changes in the f-actin- and microtubule-based cytoskeleton, and the recruitment of adhesion junction components to the furrow. We show that exposure to inhibitors of non-muscle myosin II function does not affect furrow ingression during the early cleavage cycles but interferes with the recruitment of pericleavage f-actin and cortical β-catenin aggregates to the furrow, as well as the remodeling of the furrow microtubule array. This remodeling is in turn required for the distal aggregation of the zebrafish germ plasm. Embryos with reduced myosin activity also exhibit at late stages of cytokinesis a stabilized contractile ring apparatus that appears as a ladder-like pattern of short f-actin cables, supporting a role for myosin function in the disassembly of the contractile ring after furrow formation. Our studies support a role for myosin function in furrow maturation that is independent of furrow ingression and which is essential for the recruitment of furrow components and the remodeling of the cytoskeleton during cytokinesis.

Key words: Non-muscle myosin, Cytokinesis, Furrow formation, Cell adhesion, Germ plasm, Zebrafish

Introduction
Early development involves the formation of a multicellular embryo from a single fertilized egg, a process that is driven by cell division. Cytokinesis, in which the cytoplasm divides into two daughter cells immediately following chromosome segregation, is a fundamental part of this process. Although decades of analysis in a variety of cell types and species have led to great strides in our understanding of cytokinesis, the precise molecular mechanisms involved in this process remain incompletely understood. Cytokinesis can be divided into two major phases (Rappaport, 1996; Glotzer, 2005). First, there is an initiation phase in which the site of furrow formation is determined, possibly by the interaction between overlapping astral microtubules associated with the mitotic spindle. This leads to the recruitment of furrow components, such as actin and non-muscle myosin as a ring at the prospective furrow site. Activation of contractility of this actomyosin ring is thought to lead to the constriction of the cell at the furrow site. Later, localized exocytosis is thought to contribute to the formation of new, stable membrane between the daughter cells.

In recent years, the zebrafish has become an important model in animal development, as a result of the ability to combine genetic, molecular and embryological approaches in this organism. Previous studies have started to give glimpses of the cytoskeletal involvement in zebrafish cytokinesis. As in other cell types, inhibition of microfilament and microtubule functions blocks cytokinesis (Jesuthasan, 1998; Pelegri et al., 1999). Astral microtubules emanating from both poles of the spindle overlap in the prospective furrow region during anaphase, suggesting that in this system too, they have a role in furrow initiation. Accumulations of f-actin have been observed near the center of the blastodisc coincident with furrow formation, which are absent or abnormal in mutants affecting the initiation of cytokinesis (Kishimoto et al., 2004). Once the furrow becomes specified, the overlapping astral microtubules are substituted by an array of microtubules, parallel to each other and perpendicular to the furrow flanking the maturing furrow (Danilchik et al., 1998; Jesuthasan, 1998). This array, termed the furrow microtubule array (FMA) is thought to be analogous to the midbody and to function in localized exocytosis and concomitant membrane septum formation (Danilchik et al., 1998; Jesuthasan, 1998; Pelegri et al., 1999; Danilchik et al., 2003) (reviewed by Otegui et al., 2005). The FMA undergoes extensive remodeling during cytokinesis: although the FMA microtubules are initially arranged perpendicular to the forming furrow, upon furrow maturation the microtubules acquire an angle with respect to the cleavage plane as they become enriched at the distal end of the furrow, and the FMA eventually disassembles (Jesuthasan, 1998; Pelegri et al., 1999). The process of membrane addition during furrow maturation is also coordinated with the accumulation of molecules involved in cell adhesion, such as the cell adhesion junction molecules β-catenin and cadherins (Jesuthasan, 1998; Pelegri et al., 1999).

In the zebrafish, cytokinesis is intimately linked with the segregation of the zebrafish germ plasm (reviewed by Pelegri, 2003), a specialized cytoplasm consisting of specific maternal mRNAs and proteins that confer the germ cell fate (reviewed by Wylie, 1999) (see also Knauf et al., 2000; Hashimoto et al., 2006; Theusch et al., 2006). Like the FMA itself, the zebrafish germ plasm becomes enriched at the distal end of the first and
second cleavage furrows, a process that requires microtubule function (Pelegri et al., 1999).

By analogy with muscle contraction, non-muscle myosin-II is widely thought to act as a motor protein in constriction of the cleavage furrow (Rappaport, 1996; Reichl et al., 2005). It assembles as a hexameric complex consisting of two heavy chains, two essential light chains (MLC) and two regulatory light chains (RLC) that form two head domains and an intertwined tail domain joined by a flexible hinge region (Sellers, 2000). The head domain contains f-actin-binding sites and an ATPase, and cycles of ATP hydrolysis result in conformational changes that result in movement along f-actin. The tail domain, on the other hand, can assemble into filaments. In higher eukaryotes, activation of non-muscle myosin II is achieved by the phosphorylation of the RLC, allowing myosin to interact with actin and promoting both the assembly of myosin filaments and contractility (reviewed by Matsumura, 2005). Phosphorylation of RLC is promoted by a variety of kinases, each of which is activated by separate upstream regulatory pathways, and inhibited through dephosphorylation by myosin phosphatase.

Myosin II localizes to the equator of dividing cells in a variety of cell types (Lippincott and Li, 1998; Naqvi et al., 1999; Noguchi and Mabuchi, 2001; Wu et al., 2003). Moreover, MLC-phosphorylated, active myosin has also been shown to accumulate at this location (Matsumura et al., 1998), and inhibition of myosin light chain kinase (MLCK) by treatment with the inhibitor ML7 results in a reduction in myosin accumulation at the furrow (Murthy and Wadsworth, 2005). However, the ATPase activity of myosin appears not to be required for the localization of either f-actin or myosin at the furrow, because enrichment of myosin and formation of the actin ring is initiated normally in embryos treated with the specific myosin ATPase inhibitor blebbistatin (Guha et al., 2005; Murthy and Wadsworth, 2005). Once recruited to the furrow, myosin ATPase activity is thought to power the constriction of the actomyosin ring by promoting the sliding of f-actin filaments. However, there appear to be other functions for myosin activity during furrow formation because blebbistatin treatment interferes with the disassembly of the f-actin contractile band during late cytokinesis (Guha et al., 2005; Murthy and Wadsworth, 2005).

Here, we show that exposure during the first cell cycle to specific inhibitors of non-muscle myosin II activity do not result in an inhibition of furrow ingestion during the early cell cycles but nevertheless result in immediate defects during furrow maturation. Specifically, inhibition of myosin activity interferes with the recruitment of adhesion junction components to the forming furrow, the remodeling of the FMA during furrow maturation, and the disassembly of the FMA and the contractile apparatus during late stages of furrow formation. In addition, our analysis provides further evidence for an involvement of FMA rearrangements in the segregation of the zebrafish germ plasm.

Results

Inhibition of myosin activity affects the completion of cytokinesis

During the early cell cycles, zebrafish embryos show a stereotypical pattern of cleavage in the blastodisc region. As the first cell forms, a result of ooplasmic streaming towards the animal pole region, the blastodisc typically acquires an oblong shape, as observed from an animal view, and the first cleavage plane bisects the blastodisc across its long axis. The second and third cleavage planes form perpendicular and parallel, respectively, to the first cleavage plane. During these cleavage cycles, which occur about 20 minutes apart, furrow formation is not completed before the initiation of the subsequent cell cycle. This results in several stages of furrow maturation being simultaneously present in the same embryo, because the maturation of the earlier furrows continues as furrows for the following cycles are initiated. As seen in lateral view, the forming furrow for the first cell division, which initiates at about 40 minutes post fertilization (p.f.; Fig. 1A, arrowhead), gradually ingresses, spanning most of the depth of the blastodisc (Fig. 1C, arrowhead), and is replaced by a septum of newly formed membrane separating the daughter cells (Fig. 1E,G, arrowheads). The continuation of cell division cycles leads to the formation of a blastula of tightly adhering cells overlying the yolk cell (Fig. 1I).

To understand the role of non-muscle myosin II activity in zebrafish cytokinesis, we tested the effect on early embryos of the non-muscle myosin inhibitor drug blebbistatin, a specific non-muscle myosin II ATPase inhibitor (Straight et al., 2003; Kovacs et al., 2004), and the light-chain kinase inhibitor ML7 (Kaverina et al., 2000). Embryos treated with blebbistatin within 20 minutes p.f. initiate furrow ingression in a manner indistinguishable from untreated or control-treated embryos (Fig. 1B,D, arrowheads). However, blebbistatin-treated embryos fail to form the membrane septum characteristic of mature furrows and their furrows typically regress, leaving behind partially formed furrows (Fig. 1F,H, arrowheads). The initiation and ingestion of the second cleavage furrow also appears normal in blebbistatin-treated embryos (Fig. 1F,H, arrows, compare with Fig. 1E,G, arrows). Like treatment with blebbistatin, exposure to ML7 does not interfere with furrow ingestion in the early cleavage cycles and results in defects in the completion of cytokinesis and membrane septa formation, although ML7-treated cells tend to form deeper and longer-lasting clefts between daughter cells (not shown).

Although inhibition of myosin activity with either blebbistatin or ML7 does not interfere with the ingestion of the first and second cleavage cycles, ingestion is reduced in treated embryos beginning at the third or fourth cellular cleavage cycles (Fig. 1H, right asterisk). As a result of the failure to complete furrows for the first few cycles and defects in furrow initiation in subsequent cycles, blebbistatin- and ML7-treated early blastula embryos appear largely syncytial (Fig. 1J and data not shown). The fact that an effect on furrow ingression occurs only beginning at the third cleavage furrow, but not before that stage, may be caused by a delay in the effect caused by exposure to these drugs. However, injection of myosin-inhibiting drugs at the one-cell stage similarly does not interfere with furrow ingression during the first few cell cycles (data not shown). Alternatively, such a delayed effect could be caused by the entrapment of cytoskeletal elements, which occurs during the defective early cellular cleavage cycles (see below and Discussion). Regardless of the precise role of myosin II in furrow ingression in the early zebrafish embryo, our observations suggest a role for non-muscle myosin II in furrow maturation in this organism.
Rearrangements of the f-actin network during furrow formation and their dependence on myosin function

Because myosin is widely thought to interact with f-actin in the contractile ring apparatus (Rappaport, 1996; Robinson and Spudic, 2000), we analysed changes in f-actin organization during the first three cell cycles, which span the time involved in the completion of the first furrow. Embryos were fixed at various stages of cellularization and labeled for f-actin with fluorescent phalloidin. As the furrow forms, several regions rich in f-actin become apparent (Fig. 2). During furrow initiation, an enrichment of f-actin appears along the length of the furrow (Fig. 2A,B, arrowhead in B). As the furrow matures, this medial structure develops into a contractile band at the cleavage site composed of several long cables oriented in the direction of the furrow (Fig. 2E,F, arrowhead in F). At the same stage, flanking the medial contractile band, regions of intense f-actin staining appear, which develop into lamella-like structures (Fig. 2E,F, short arrows in F). Further from the furrow, relatively long f-actin cables can often be observed (Fig. 2F,J, long arrows), which probably correspond to centrifugally moving actin cables recently described (Theusch et al., 2006). During furrow maturation the lamella-like f-actin enrichments appear to gradually converge towards the center of the furrow and coalesce into a single medial structure (Fig. 2I,J, arrowhead in J), probably reflecting f-actin at the cytoplasmic side of the tightly apposed daughter membranes. At first, this membrane septum contains many larger f-actin cables that do not necessarily have identical paths through the depth of the furrow (Fig. 2J, short arrows), although it appears to become more uniform and compact at later stages (Fig. 2M,N). In addition, occasional bubble-like structures can be observed in the septum (not shown), indicating the presence of some regions where new membranes are not tightly apposed. Thus, the f-actin-based structures we observe appear to serve at least two purposes: the long cables along the furrow probably correspond to the contractile band, whereas the flanking f-actin regions converge at the furrow and contribute to the formation of the new adhesive septum between daughter cells.

Embryos treated with blebbistatin exhibit reproducible defects in the organization of f-actin at the forming furrow. In these embryos, f-actin accumulates in the presumptive contractile ring region (Fig. 2C,D, arrowhead in D), where they form an f-actin band aligned along the direction of the furrow (Fig. 2H,L, arrowheads) in a configuration similar to that observed in control embryos. However, such embryos can exhibit abnormal f-actin cables perpendicular to the cleavage plane (Fig. 2D,H, long arrows). In blebbistatin-treated embryos, enrichments of f-actin appear on both sides of the furrow (Fig. 2G,H; short arrows in H), although these regions do not form normal lamella-like structures. As furrow formation progresses, pericleavage f-actin regions in blebbistatin-treated embryos do not coalesce into a single f-actin band (Fig. 2K,L). Instead, these flanking regions tend to be located at a greater distance from each other than at earlier stages of furrow formation, because a conspicuous f-actin-free region appears between the contractile ring and these regions (Fig. 2L, brackets). At later stages of furrow development, the pericleavage f-actin enrichments are no longer apparent (Fig. 2O,P).
Notably, at this late stage in furrow formation, f-actin cables of the contractile ring apparatus remain at the center of the furrow (Fig. 2P, arrowhead). However, whereas during earlier stages these cables appear contiguous and aligned in the direction of the furrow, those present at late stages in blebbistatin-treated embryos appear as a ladder-like pattern of short fragments arranged perpendicular to the furrow. These observations are consistent with a recently proposed role for myosin function in the disassembly of the contractile ring apparatus (Guha et al., 2005; Murthy and Wadsworth, 2005) and suggest that such disassembly may require an intermediate stage involving the severing of the f-actin in the contractile ring into short f-actin cables.

Treatment of embryos with ML7 results in f-actin defects similar to those caused by blebbistatin, namely a reduction in f-actin accumulation at the furrow, as well as the presence of short f-actin cables in the furrow region late in cellularization (Fig. 2Q,R, arrowheads).

The defects in f-actin dynamics do not appear to be caused by a general slowing in the cell cycle, as indicated by a lack in delay of furrow ingression (above) and in the nuclear divisions, as shown by labeling of DNA (not shown, see also Fig. 5). Thus, myosin function is important for rearrangements of the f-actin network during furrow maturation, which include the recruitment of pericleavage f-actin to form the intercellular septum at the furrow and the disassembly of the contractile ring apparatus.
Inhibition of myosin function leads to defects in the recruitment of cell adhesion molecules to the furrow

To study the role of myosin in furrow formation further, we tested the effect of treatment with myosin-inhibiting drugs on the recruitment of β-catenin, a protein known to localize to cell adhesion junctions (Kemler, 1993) and which has been previously shown to localize to furrows during late stages of maturation (Jesuthasan, 1998; Pelegri et al., 1999). Embryos were fixed at various times of furrow formation, spanning the initiation and completion of the first furrow, and labeled to detect β-catenin protein. During the early stages of furrow initiation, we observe β-catenin in discrete cortical aggregates enriched in a broad region centered on the prospective second cleavage furrow (shown in detail in Fig. 3I). These aggregates will also coalesce into a contiguous line as the second furrow develops (arrow in I). In blebbistatin-treated embryos, the enrichment of aggregates centered on the prospective furrow occurs (bracket in C) and some β-catenin punctae align at the forming furrow (arrowhead in C,D), but there is no further recruitment of β-catenin at the furrow as a contiguous structure (arrowheads and arrows in G,H,K,L). Instead, β-catenin cortical aggregates remain dispersed. A β-catenin-free region often develops flanking the furrow center (brackets in G), which may correspond to the region where internal membrane has undergone localized exocytosis. Microtubule labeling shows the lack of FMA remodeling in myosin-inhibited embryos (shown in detail in Fig. 4). (M,N) Embryos treated with ML7 also exhibit defects β-catenin recruitment to the furrow and in FMA remodeling. Arrowheads, arrows and asterisks indicate first, second and third cleavage furrows, respectively. Bar, 50 μm (A,C,E,G,I,K,M) and 16 μm (B,D,F,H,J,L,N).

In blebbistatin- or ML7-treated embryos, β-catenin cortical aggregates can also be observed enriched in a region centered on the prospective furrow (Fig. 3C, bracket). Treatment of embryos with blebbistatin or ML7 results in strong defects in the subsequent recruitment of β-catenin to the maturing furrow (Fig. 3G,H,K,L; arrowheads, first furrow; arrows, second furrow). As in the case of pericleavage f-actin, mature furrows in such treated embryos often show areas free of β-catenin aggregates immediately flanking their centers (Fig. 3G, brackets). Of interest, in the β-catenin-containing region flanking these aggregate-free areas, the cortical β-catenin aggregates appear to maintain a relatively wide distribution similar to that observed in the initiating furrow. These observations are consistent with a role for myosin function in the recruitment of β-catenin aggregates along the plane of the cortex towards the furrow.
Myosin activity is required for the remodeling and disassembly of the FMA

Previous reports have shown a role for the FMA (Danilchik et al., 1998; Danilchik et al., 2003) in the localized exocytosis of membrane vesicles at the furrow (Otegui et al., 2005). In zebrafish embryos, the FMA has been shown to exhibit a dynamic pattern during furrow formation (Jesuthasan, 1998; Pelegri et al., 1999). This dynamic pattern is also observed in embryos treated with carrier solvent. In the incipient furrow, parallel microtubules appear uniformly distributed along the furrow and form at a 90° angle to the plane of cleavage (Fig. 4A). However, as development of the furrow proceeds, microtubules acquire an oblique angle with respect to the furrow, such that sets of microtubules on both sides of the furrow form v-shaped structures centered on the furrow and with their pointed end oriented towards the distal end of the furrow (Fig. 4C). At the same time, microtubules of the FMA become enriched at the distal ends of the furrow. Both the distal enrichment and the tilting of microtubules appear to occur gradually during cellularization, becoming progressively more conspicuous in mature furrows. Towards furrow completion, microtubules in control embryos are no longer detectable flanking the furrow, form a compact mass at the distal ends of the furrows (Fig. 4E, arrowhead), and ultimately disappear. Thus, dynamic changes in the FMA during furrow maturation result in the remodeling of this structure, which eventually disassembles.

In embryos exposed to blebbistatin and ML7, microtubules arrange at the incipient furrow parallel to each other and perpendicular to the furrow as in control embryos (Fig. 4B). However, maturing furrows of drug-treated embryos show a strong reduction in both the enrichment of FMA microtubules to the distal end of the furrow and their tilting with respect to the plane of the furrow (Fig. 4D). Instead, during furrow maturation, drug-treated embryos exhibit arrays of microtubules that persist in their original arrangement, perpendicular to the plane of the furrow. At the beginning of the eight-cell stage, for example, when the first furrow FMA has largely disassembled in control embryos (Fig. 4E), persistent FMA arrays can be observed perpendicular to these furrows in drug-treated embryos (Fig. 4F, see also Fig. 3L,N). Similar persistent FMA arrays can be observed in blebbistatin-treated embryos at the 16-cell stage (90 minutes p.f., not shown), the latest stage we have examined under these conditions. Concomitant with the lack of FMA rearrangement, the distal microtubule aggregates do not form in treated embryos. Thus, inhibition of myosin activity appears to interfere with the distal enrichment and tilting of microtubules of the FMA during furrow formation, as well as the eventual disassembly of the FMA.

Distal aggregation of germ plasm components along the furrow depends on the rearrangement of the FMA

Segregation of the zebrafish germ plasm, a specialized cytoplasm that confers the germ cell fate, is intimately linked to the process of cytokinesis (Pelegri, 2003). During the first cellular cleavages cortical aggregates containing germ plasm components are recruited as rod-like structures along about two-thirds of the distal-most span of the forming furrow (Yoon et al., 1997; Braat et al., 1999; Pelegri et al., 1999; Theusch et al., 2006), where they appear to be in physical contact with the ends of the FMA microtubules (Pelegri et al., 1999; Knaut et al., 2000). Upon furrow maturation, the recruited germ plasm material, as is the case for the FMA microtubules, becomes enriched at the distal end of the furrow, where it forms a compact mass of tightly aggregated germ plasm (Pelegri et al., 1999). Genetic analysis and drug inhibition studies have shown that the distal aggregation of the germ plasm along the forming furrow is dependent on an intact FMA (Pelegri et al., 1999). The inhibition of FMA remodeling by blebbistatin allows us to specifically test the hypothesis that the distal compaction of the germ plasm is dependent on the distal enrichment of FMA microtubules. Embryos were exposed to either blebbistatin or carrier solvent, fixed at the late four-cell stage (65 minutes p.f.), and the localization of vasa RNA was analyzed using whole-

![Fig. 4. Inhibition of myosin activity interferes with FMA remodeling during furrow formation. Control (A,C,E) and blebbistatin-treated (B,D,F) embryos labeled with an anti-α-tubulin antibody. In control embryos, the FMA forms as a parallel array of microtubules perpendicular to the furrow (A). Upon furrow maturation, FMA tubules end closer to the furrow center appear at progressively more distal locations along the furrow, forming an oblique angle with respect to the plane of the furrow and resulting in v-shaped structures flanking the furrow (C). By the eight-cell stage (E), when the first furrow is nearly completed, the distally enriched FMA microtubules form a compact mass (arrowhead in E) that eventually disappears. The inset in E shows an intermediate stage of furrow maturation, where FMA microtubules appear enriched in the distal region of the furrow (arrowhead in inset). Treatment of embryos with blebbistatin does not interfere with the initial formation of the FMA (B), but inhibits tubule distal enrichment and tilting during furrow maturation (D), as well as the formation of the distally located residual body and the disassembly of the FMA (F). Images are animal views. Bar, 16 μm for all panels.](image-url)
mount in situ hybridization. At this developmental stage, control embryos show the presence of distally located compact aggregates in cleavage furrows for both the first and second cell divisions. At this stage, the germ plasm has already undergone a distal aggregation during furrow formation, and appears as compact aggregates at the distal ends of furrows of the first two cellular cycles (Fig. 5A,B). Blebbistatin treatment does not interfere with the initial localization of vasa mRNA at the furrow, suggesting that the process of germ plasm recruitment to the forming furrow is not dependent on myosin function (Fig. 5C,D). However, in blebbistatin-treated embryos the vasa mRNA-containing aggregates remain as elongated structures (brackets in Fig. 5C indicate recruited rod-like aggregates) and do not become enriched at the distal end of the furrow. Thus, the absence of FMA tubule distal enrichment is associated with a failure in the distal aggregation of the germ plasm. These data support the idea that the enrichment of FMA microtubules to the distal end of the furrow, which is affected by interference with myosin activity, is involved in the aggregation of recruited germ plasm to this region.

Localization of myosin in early zebrafish embryos
To confirm a role for myosin in various events in zebrafish cytokinesis, we used whole mount immunolabeling to detect non-muscle myosin II in untreated embryos during the early cleavage stages. Embryos were fixed at regular intervals from the time of furrow initiation for the first cellular division (40 minutes p.f.) until the eight-cell stage (75 minutes p.f.), when the first furrow is fully mature, and labeled using an antibody previously shown to recognize non-muscle myosin II in zebrafish oocytes (Becker and Hart, 1999). Myosin protein can be detected throughout the cortex of the blastodisc (Fig. 6), compared with mock-labeled control embryos (not shown). During furrow initiation, little or no labeling can be observed

Fig. 5. Myosin activity is required for the distal aggregation of the zebrafish germ plasm. Control (A,B) and blebbistatin-treated (C,D) embryos labeled for the vasa RNA, a component of the zebrafish germ plasm, using in situ hybridization and for DNA using DAPI. B and D show the same embryo as in A and C, respectively, under the DAPI fluorescence channel, revealing that the embryos are at a similar stage in the cell cycle, immediately before the third cell division. At this stage, the germ plasm has already undergone a distal aggregation during furrow formation, and appears as compact aggregates at the distal ends of furrows of the first two cellular cycles (A). Inhibition of myosin function does not interfere with the recruitment of the germ plasm to the furrow (C), but the recruited germ plasm aggregate maintains its original rod-like structure and does not undergo further aggregation to the distal end of the furrow. Brackets in C delineate the extent of the germ plasm aggregate at the cleavage furrows. Images are animal views. Arrowheads and arrows indicate the first and second cleavage furrows, respectively, which are recognizable by the pattern of nuclear division.

Fig. 6. Localization of non-muscle myosin II during zebrafish cytokinesis. (A-F) Wild-type embryos labeled with an anti-non-muscle myosin II antibody during the early cellular cleavages. B,D,F are higher magnification images of the boxed regions of the embryos shown in A,C,E, respectively. During furrow initiation (A,B), myosin appears to be present at the cortex of the embryo but does not show a significant enrichment at the forming furrow (arrowheads in A,B). At the four-cell stage (C,D), when the first furrow is undergoing furrow maturation, myosin accumulates at this furrow (arrowheads in C,D) but is not yet observable at the initiating first furrow (arrow in C). At the eight-cell stage (E,F), myosin remains at the site of the first furrow (arrowheads in E,F) and has become localized to the now mature second furrow (arrow in E). (H,I) Blebbistatin-treated embryos at the late four-cell stage (65 minutes p.f.). I is a higher magnification of the first furrow in the embryo shown in H. Myosin does not accumulate at the first furrow at this late stage of furrow maturation, when it is normally accumulated at the furrow in control embryos. Images are animal views. In panels with the lower magnification images arrowheads, arrows and asterisks indicate the first, second and third cleavage furrows, respectively. Bar, 50 μm (A,C,E,H); 16 μm (B,D,F,I).
at the forming furrow (Fig. 6A,B, arrowhead in B). However, at later stages intense fluorescent labeling appears at the maturing furrow (Fig. 6C,D, arrowhead in D), a pattern that is maintained at later stages of furrow maturation (Fig. 6E,F, arrowhead in F). Thus, myosin protein appears to be present in the zebrafish embryo cortex and to accumulate at the furrow during furrow maturation. The localization of myosin II in mature furrows is significantly reduced in blebbistatin-treated embryos, as observed in the first furrow at the end of the second cleavage cycle (65 minutes p.f.; Fig. 6H,I, arrowhead in I). These localization patterns are consistent with the proposed roles of myosin in the recruitment of cortical furrow components and in cytoskeletal rearrangements at the furrow.

Discussion

This report describes the dependency on non-muscle myosin II function of cytoskeletal rearrangements and furrow component recruitment during cytokinesis in the early zebrafish embryo. Our analysis contributes to the understanding of the molecular events involved in cytokinesis in this organism and indicates a role for non-muscle myosin function during furrow formation that appears to be independent of furrow ingression.

Recruitment of f-actin to the contractile ring and furrow ingression may occur independently of myosin function

One of the earliest cytoskeletal events that can be observed during cellularization in the zebrafish embryo is the appearance of long, parallel f-actin cables aligned along the plane of the furrow. The position of this band at the center of the furrow and in deeper regions of the ingressing furrow indicates that this band corresponds to the contractile ring as defined in other organisms (Perry et al., 1971; Mabuchi et al., 1988; Noguchi and Mabuchi, 2001). Our data show that a contractile-ring-like structure at the zebrafish furrow and furrow ingression can form in the presence of myosin inhibitors. Similar conclusions have been recently reported in mammalian cells in culture (Guha et al., 2005; Murthy and Wadsworth, 2005). In such cells, myosin activation (although not its ATPase activity) appears to be essential for contractile ring formation, because inhibition of myosin phosphorylation with the myosin light chain kinase (MLCK) inhibitor ML7 interferes with this process (Murthy and Wadsworth, 2005). We find that, in the early zebrafish embryo, inhibition of myosin II activity also allows for the recruitment of f-actin to the prospective contractile band. Thus, there appears to be a mechanism independent of myosin activity involved in the recruitment of f-actin to the furrow. The direct recruitment of G-actin to the contractile ring has been detected in Xenopus embryos (Noguchi and Mabuchi, 2001), and this probably constitutes a driving force for contractile ring formation. Further studies will be required to test the molecular requirements for contractile ring formation during zebrafish cytokinesis.

It is intriguing that exposure to myosin-inhibiting reagents does not interfere with furrow initiation and ingression during the first few cycles, and only affects these processes starting at the third or fourth cellular cleavage cycles. It is possible that this lack of an effect in the earliest cell division cycles results from a time lag in which initial exposure to these membrane-permeable drugs does not have an effect in the embryo. However, similar drug exposures do lead to defects in other processes that occur at the same time as furrow ingression, such as the remodeling of the cytoskeleton and the recruitment of furrow components (see below), indicating that the drugs have an immediate effect on at least some aspects of furrow formation. On the other hand, a time lag in the effect on furrow initiation caused by these reagents can be explained by the stabilization of the cytoskeleton caused by such treatment, which would be expected to lead to a reduction in the pool of cytoskeletal components available for subsequent cellular cycles.

It is also possible that the myosin-inhibiting reagents blebbistatin and ML7 used in this study affect targets other than non-muscle myosin II in the zebrafish. However, the fact that two reagents that affect myosin function by different mechanisms result in similar defects in the zebrafish is consistent with their having an effect on non-muscle myosin II function in this organism. This is further supported by our observation that blebbistatin interferes with contractile ring disassembly in the zebrafish, as has been reported in other cell types (see below). Similarly, we cannot rule out the fact that these reagents result in gain-of-function effects. This possibility, however, is not supported by the observation that exposure to blebbistatin does not result in ectopic myosin II localization and results in a predictable loss of myosin accumulation at the mature furrow. The presence of maternal myosin II protein in the zebrafish mature oocyte (Becker and Hart, 1999) precludes morpholino-mediated functional knockdown of myosin II during the early cleavage cycles. Moreover, expression of dominant-negative or constitutively active mutant forms of myosin II through mRNA injection is not expected to be effective during the early cellular cycles, because translated product from mRNA injected at the one-cell stage does not appear to be produced before the eight-cell stage (T.Y. and F.P., unpublished data). Thus, the development of reverse-genetic strategies for maternally derived factors will be necessary to test these possibilities further.

However, our data raise the possibility that myosin function may be dispensable for membrane contraction in the zebrafish embryo. Such myosin-independent mechanisms have been found to occur in Dictyostelium, where a pathway dependent on membrane attachment allows myosin-null cells to divide (Neujahr et al., 1997; Zang et al., 1997; Nagasaki et al., 2002). A similar myosin-independent cell furrowing process has been recently reported in dividing mammalian cells (Kanada et al., 2005). In large embryonic cells such as those of zebrafish, myosin-independent membrane contraction could in principle be caused by localized membrane secretion at the site of furrow initiation and/or local differences in membrane rigidity. Further studies will be required to test these possibilities. In spite of these uncertainties, our studies suggest a requirement(s) for myosin function during furrow maturation that is independent of furrow ingression.

Myosin-dependent recruitment of furrow components and formation of adhesion junctions

We show that, in addition to the contractile ring, there is an accumulation of f-actin in the region immediately flanking the furrow. This f-actin-rich region develops into lamella-like structures, which themselves subsequently converge into a
septum separating the daughter cells. These lamella-like structures may be analogous to bleb-like structures described at the furrow in *Xenopus* (Noguchi and Mabuchi, 2001). In those studies, it was shown that the slope of f-actin accumulation in the medial regions of the contractile ring exceeds that of the accumulation of cortical particles labeled with fluorescent wheatgerm agglutinin (WGA), which suggested that the increase in f-actin recruitment at the furrow is derived from such bleb-like structures. Moreover, the formation of bleb-like structures in *Xenopus* is affected in the presence of BDM, an inhibitor of myosin function (Noguchi and Mabuchi, 2001). Similarly, we find that in the zebrafish furrow, the amount of f-actin that eventually is recruited to the furrow is greatly increased above the levels present in the contractile ring by the joining of pericleavage lamella-like structures, and that inhibition of myosin activity interferes with their convergence towards the furrow (Fig. 7A,A’). Thus, a myosin-dependent process involving the joining of lamella-like structures may be a conserved mechanism during cytokinesis in early animal embryos.

Similar to the case of pericleavage f-actin, we find that the recruitment of β-catenin aggregates to the furrow is also dependent on myosin activity (Fig. 7B,B’). Previous studies have suggested that β-catenin is provided at the forming furrow through the localized exocytosis of internal membrane vesicles at the furrow site, primarily based on the observation of this protein on vesicles immediately adjacent to the furrow using immuno-electron microscopy (Jesuthasan, 1998). We find, however, that before furrow initiation and during the early stages of furrow formation, most detectable β-catenin is present as cortical aggregates distributed on a broad band centered on the prospective furrow, although we do not yet know whether these aggregates are small membrane-bound vesicles near the cortex. As the furrow matures, such β-catenin cortical aggregates are recruited to the furrow, eventually accumulating as a wall-like structure in the mature furrow, in a pattern reminiscent of that of recruited pericleavage f-actin. The fact that in blebbistatin-treated embryos both pericleavage f-actin and β-catenin cortical aggregates remain in a distribution similar to that observed in incipient furrows suggests that myosin may be important for the movement of adhesion junction components towards the furrow. Several studies have implicated a requirement for myosin function in the cell cortex in a process that may be related to cortical flow (Canman and Bement, 1997; Rosenblatt et al., 2004), and it is possible that recruitment of adhesion junction components involves myosin-dependent flow of the cortex towards the furrow. We are currently testing this hypothesis through the live imaging of cortical components.

Previous studies have revealed localized exocytosis of internally derived membrane vesicles at the site of the forming furrow (reviewed by Albertson et al., 2005; Otegui et al., 2005). Our observations in blebbistatin-treated embryos are consistent with this idea, as we observe during furrow maturation an increase in the distance between pericleavage f-actin-rich regions and the appearance of β-catenin-free zones flanking the mature furrow. The appearance of regions of presumed membrane addition at the furrow in spite of defects in β-catenin recruitment further supports the idea that, during cytokinesis, exocytosis of the bulk of internally-derived membrane occurs independently of the recruitment of cortical β-catenin to the furrow.

**Fig. 7.** Summary of myosin-dependent processes during zebrafish cytokinesis. A-C and A’-C’ represent animal views of embryos centered on the first furrow, immediately after furrow initiation (A-C) and towards furrow completion (A’-C’). (A,A’) During early furrow formation, the contractile ring apparatus is formed at the center of the furrow, as well as f-actin enrichments in pericleavage regions of the furrow (A). Upon furrow maturation, pericleavage f-actin forms lamella-like structures that converge towards a septum at the center of the furrow (A’). (B,B’). Cortical β-catenin aggregates appear distributed in a broad band centered on the initiating furrow (B), and these aggregates are recruited to the furrow during furrow maturation (B’). (C,C’) FMA microtubules are recruited to the forming furrow as a parallel array perpendicular to the furrow (red lines in C), and the germ plasm components become recruited to the furrow as rod-like aggregates (blue rectangles in C) that are in physical contact with FMA tubule ends. Upon furrow maturation, FMA tubule ends closest to the center of the furrow become enriched at the distal end of the furrow, exhibiting a characteristic tilting of the microtubules (red lines in C’), and the germ plasm aggregate becomes compacted at the distal end of the furrow (blue triangle in C’). Events that occur during furrow maturation and completion (A’-C’) are myosin dependent. Analysis of blebbistatin-treated embryos also reveals a ladder-like f-actin arrangement of the contractile ring towards furrow completion (not shown in this figure, see Fig. 2P), which is consistent with a role for myosin in contractile ring disassembly. See text for details.
Requirement for myosin function for dynamic changes in the FMA

As the furrow begins to form, the FMA assembles at the incipient furrow. The origin of these microtubules is not completely understood, although they are thought to be derived from overlapping astral microtubules, which themselves, are important for furrow initiation (Otegui et al., 2005). The precise function of the FMA has not been conclusively proven, although they have been proposed to act as tracks along which vesicles containing new membrane can be added to the forming furrow through localized exocytosis (Otegui et al., 2005).

The microtubules of the FMA are initially arranged in an orientation perpendicular to the furrow, reflecting their presumed astral microtubule origin. As the furrow matures, however, they progressively become enriched at the distal end of the furrow and acquire a characteristic oblique angle with respect to the plane of the furrow. We have found that inhibition of myosin activity leads to the absence of both distal tubule enrichment and tilting (Fig. 7C,C' ). It is possible that this effect is a consequence of defects in the cortical events involved in the recruitment of adhesive components to the forming furrow. Indeed, recent studies have implicated a role for myosin II function in the connections of microtubules to the moving cell cortex (Rosenblatt et al., 2004). An alternative scenario is that non-muscle myosin may have a direct role on FMA dynamics, for example by moving along the contractile ring while bound to FMA tubule ends. Further data, including the live imaging of the cytoskeleton and myosin II should help shed light on the role of myosin on FMA remodeling.

Function of FMA remodeling in germ plasm aggregation and the completion of cytokinesis

One potential function of the FMA rearrangements during furrow maturation could be the transport of components that associate at the furrow towards its distal end. This may be the case for the zebrafish germ plasm, which is recruited to the furrow as a rod-like structure and which becomes enriched at the distal end of the furrow during maturation to form a large, compact germ plasm aggregate (reviewed by Pelegri, 2003).

We have previously shown that FMA microtubules are embedded in the zebrafish germ plasm and that microtubule function is important for the enrichment of germ plasm to the distal end of the furrow (Pelegri et al., 1999). Here, we show that in blebbistatin-treated embryos the germ plasm aggregate remains as a rod-like structure and does not become enriched at the distal end of the furrow. Thus, myosin-dependent rearrangements of FMA microtubules appear to mediate the distal aggregation of the germ plasm. This in turn may provide the appropriate level of aggregation for the germ plasm to confer its effect as a germ cell fate determinant.

The peripheral accumulation and tilting of FMA microtubules may also be important for the remodeling and eventual disassembly of the FMA after furrow completion. Previous studies (Jesuthasan, 1998; Pelegri et al., 1999) have shown that, in untreated embryos, FMA microtubules are substituted during furrow maturation by a mass of microtubules at the distal end of the furrow. Once the furrow is completed, this distal mass is itself disassembled. The absence of FMA rearrangements caused by inhibition of myosin activity, where FMA microtubules appear “trapped” in their initial orientation (perpendicular to the furrow), is associated with a reduction in the formation of the distal microtubule aggregates and a lack of FMA disassembly. These observations suggest that FMA remodeling is essential for its eventual disassembly.

A role for myosin function in the disassembly of f-actin during late cytokinesis

Inhibition of myosin activity leads to the appearance during late stages of furrow formation of an array of short f-actin filaments, aligned perpendicular to the furrow, which form a ladder-like arrangement along the length of the furrow. As described above, in blebbistatin-treated embryos only f-actin corresponding to the contractile ring accumulates at the furrow, whereas f-actin regions flanking the furrow do not. Thus, the ladder-like arrangement of short f-actin probably originates from the contractile ring apparatus and, because such structures are not observed in untreated embryos, myosin activity may be required for the remodeling of the contractile ring towards the end of cytokinesis. Structural studies have shown that actin in the contractile ring disassembles as it slides during cytokinesis (Schroeder, 1972), and the actin depolymerization factor ADF/cofilin is essential for cytokinesis in various cell types (Robinson and Spudic, 2000). Thus, disassembly of the contractile ring is important for the completion of cytokinesis. Moreover, recent studies using fluorescent recovery analysis after photobleaching have shown that myosin II activity is required for the dynamic turnover of f-actin in the contractile ring of mammalian cells and have suggested a role for this motor protein in the clearing of f-actin short fragments away from the furrow (Guha et al., 2005; Murthy and Wadsworth, 2005). Our data are consistent with a similar role for myosin activity in cleaving zebrafish cells.

In summary, we show that myosin function is required for furrow maturation during zebrafish cytokinesis, specifically the recruitment of cortical components at the forming adhesive membrane septum, the remodeling of the FMA microtubules and the disassembly of the contractile ring apparatus. Because these effects occur in the absence of apparent defects in furrow ingress, our observations suggest additional function(s) for myosin during furrow maturation and thus add to the growing list of roles of myosin function during cytokinesis.

Materials and Methods

Embryo manipulations and drug treatments

Zebrafish of the AB strain were grown and embryos obtained under standard conditions (Brand et al., 2002). Embryos were collected approximately 5 minutes after they were laid, manually dechorionated following a brief exposure to pronase and exposed to the drug diluted in embryonic medium (E3) (Pelegri and Schulte-Mmerker, 1999) within 20 minutes of fertilization. Blebbistatin (Sigma) was prepared as a 10 mM stock in DMSO and used at 100 μM (concentrations tested up to 400 μM led to similar results). ML7 (Sigma) was prepared as a 75 mM stock in DMSO and used at 300 μM (concentrations higher than 300 μM resulted in the rapid lysis of the embryos). Control embryos were treated in the same manner and exposed to a similar concentration of carrier solvent (DMSO). For drug injections, about 1 nl of solutions of blebbistatin (0.2 mM) and ML7 (1.5 mM) was injected into one-cell stage embryos.

Detection using fluorescent probes

Labeling of DNA, f-actin and microtubules was carried out as previously described (Theusch et al., 2006). Labeling of β-catenin was as previously described (Pelegri et al., 1999), except that embryos were dechorionated before fixation. Fixation was carried out using microtubule fix buffer (Theusch et al., 2006) using anti-β-catenin polyclonal antibodies (Sigma, made in rabbit) at a 1:1000 dilution. Labeling of non-muscle myosin was performed as described in Becker and Hart (Becker and Hart, 1999), with the exception that dechorionated embryos were fixed using a
glutaraldehyde-based fixative (Theusch et al., 2006) and the methanol step was omitted.

In situ hybridization
Whole mount in situ hybridization to detect the vasa mRNA (Yoon et al., 1997) was carried out as in Theusch et al. (Theusch et al., 2006) using digoxigenin as a hapten and NBT/BCIP as a color substrate.

Image acquisition
Fluorescent images were acquired using either a Zeiss Axioplan 2 fluorescence microscope and Open Lab imaging software or a Bio-Rad confocal microscope. Confocal images are flat projections of z-stack optical sections spanning the width of the imaged embryo or subcellular structure. For images in animal views, embryos labeled with fluorescent probes were deyolked prior to labeling and the blastodiscs mounted flat on a microslide. Images of live embryos were obtained using DIC optics and a Zeiss Axioplan 2 microscope.

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