

RESEARCH ARTICLE

Modulation of F-actin dynamics by maternal Mid1ip1L controls germ plasm aggregation and furrow recruitment in the zebrafish embryo

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ABSTRACT

During the early embryonic cell cycles, zebrafish germ plasm ribonucleoparticles (RNPs) gradually multimerize and become recruited to the forming furrows. RNPs multimerization occurs prior to and during furrow initiation, as forming aggregates move outward through their association with the tips of growing interphase astral microtubules. Germ plasm RNPs are also associated with short cortical F-actin. We show that, in embryos mutant for the cytoskeletal regulator *mid1ip1l*, germ plasm RNPs fail to become recruited to the furrow, accumulating instead at the periphery of the blastodisc. RNP aggregates are associated with zones of *mid1ip1l*-dependent cyclical local cortical F-actin network enrichments, as well as contractions at both the cortex and the contractile ring. F-actin inhibition in wild-type embryos mimics the RNP peripheral accumulation defect of *mid1ip1l* mutants. Our studies suggest that a common mechanism underlies distinct steps of germ plasm RNP segregation. At the cortex, this process attenuates microtubule-dependent outward RNP movement to retain RNPs in the blastodisc cortex and allow their recruitment to the furrows. F-actin network contraction likely also facilitates higher-order germ plasm RNP multimerization.

KEY WORDS: Mid1ip1L, Germ plasm, Ribonucleoparticles, Microtubules, F-actin, Zebrafish, Embryo

INTRODUCTION

In many animal species, including the zebrafish *Danio rerio*, primordial germ cell specification occurs through the inheritance of the so-called germ plasm, a maternally derived specialized cytoplasm containing specific RNAs and proteins (Wylie, 1999; Strome and Lehmann, 2007; Eno and Pelegri, 2013). In the zebrafish, germ plasm ribonucleoparticles (RNPs) accumulate at the cleavage furrows for first two embryonic cell divisions to generate four large aggregates (Yoon et al., 1997; Pelegri et al., 1999; Knaut et al., 2000). As blastomeres fully cellularize, these aggregates become incorporated into primordial germ cells (Olsen et al., 1997; Yoon et al., 1997; Braat et al., 1999; Knaut et al., 2000). Removal of the cytoplasm in the region harboring the germ plasm results in embryos lacking primordial germ cells (Hashimoto et al., 2004), as do treatments that interfere with germ plasm segregation (Miranda-Rodríguez et al., 2017). Conversely, an increase in germ plasm aggregates results in excess germ cells (Bontems et al., 2009).

Immediately after fertilization, germ plasm RNPs are relatively evenly dispersed through the blastodisc (Theusch et al., 2006), with RNPs appearing under fluorescence microscopy as single spherical units of typically less than a micron in diameter (Eno and Pelegri, 2013; Nair et al., 2013; this study). The assembly of germ plasm masses during the first embryonic cell cycles involves sequential steps in which RNPs, while appearing to remain as distinct units, multimerize to form increasingly larger aggregates (Fig. 1).

An initial step, 'pre-aggregation' occurs in the blastodisc cortex even prior to furrow formation, resulting in multimers, or pre-aggregates, consisting of a small number of RNPs (typically fewer than 20; Nair et al., 2013). During pre-aggregation, RNPs are cleared from the center of the blastodisc, while a wave-front of RNP multimers forms at the inner edge of the surrounding RNP-containing band (Theusch et al., 2006; Nair et al., 2013). Previous analysis suggests that the outwardly RNP clearing and associated RNP multimerization wave-front is driven by sperm aster and mitotic spindle astral microtubules (Theusch et al., 2006; Nair et al., 2013), which undergo radially outward growth (Wühr et al., 2010) and whose tips are associated with germ plasm RNPs (Nair et al., 2013). Function of Birc5b (also known as Motley), a maternal-specific duplicate product for the chromosomal passenger complex (CPC) Survivin, has been shown to be a necessary link between astral microtubule ends and the germ plasm aggregate (Nair et al., 2013).

A second step, 'furrow recruitment', occurs as early blastomeres undergo cytokinesis. It involves the local gathering of RNPs to the forming furrow through the action of astral microtubules originating from opposite sides of the mitotic spindle (Wühr et al., 2010; Nair et al., 2013) and through maternal kinesin-1 motor protein Kif5Ba function (Campbell et al., 2015). This generates elongated, rod-like arrangements of aggregates along the base of the furrow, altogether involving a large number of RNPs (~500-1000 for furrows corresponding to the first two cell cycles; Eno and Pelegri, 2013). A third step, 'distal compaction', occurs as the furrow matures and involves the transition of the set of aggregates distributed along the furrow base into a single compact mass at each of the furrow distal ends (Pelegri et al., 1999; Eno and Pelegri, 2013).

The steps of RNP pre-aggregation, furrow recruitment and distal compaction are reiterated through the early embryonic cycles, allowing the systematic gathering of germ plasm RNPs into multiple germ plasm masses (Eno and Pelegri, 2013). The largest four masses, those that form at the furrows for the first and second cell cycles, subsequently become incorporated into primordial germ cells. This work focuses on F-actin-dependent mechanisms underlying multimerization and movement during these three stages of germ plasm segregation. The accompanying article (Eno et al., 2018) addresses cues mediating the directional movement of

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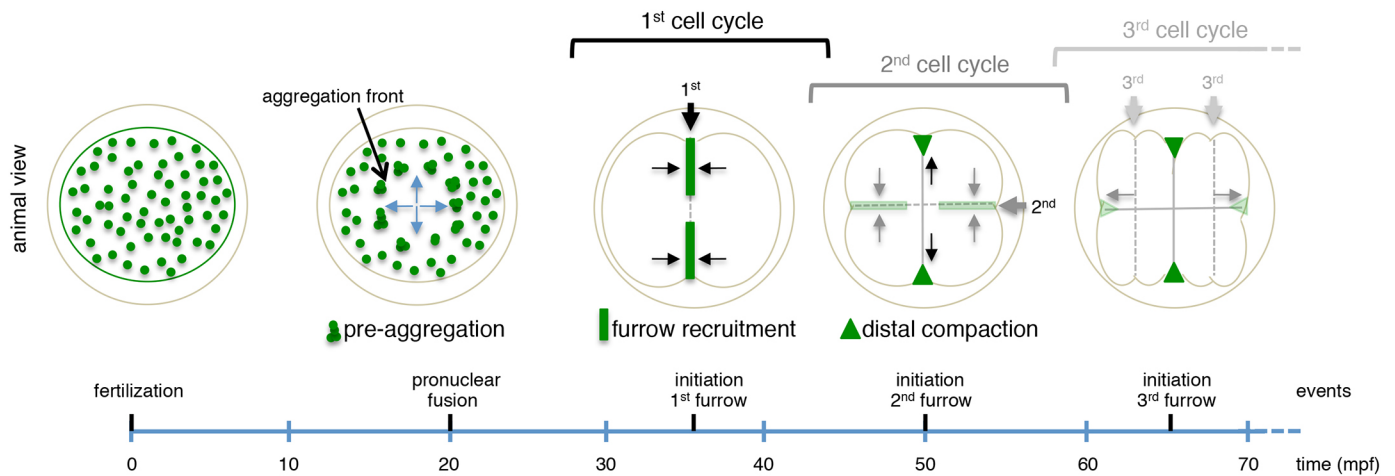


Fig. 1. Stages of RNP segregation leading to germ plasma aggregates at the furrow. Radial astral microtubule growth results in the outward movement of RNPs, which begin a process of pre-aggregation even prior to furrow formation. During cell division, the same process, but now driven by microtubules from a bipolar spindle (not shown), results in the RNP recruitment at the furrow. Medial-to-distal movement along the furrow generates compact masses at the furrow distal ends. The process is systematically repeated through the early cell divisions, although only the largest four germ plasma masses, corresponding to those at the first and second cleavage furrows, are stable (Eno and Pelegri, 2013, 2016).

the forming germ plasma mass towards furrow distal ends during the distal compaction stage.

In addition to their attachment to microtubule ends, during their multimerization and movement germ plasma RNPs are associated with short F-actin fragments (Theusch et al., 2006; Nair et al., 2013). Similar to germ plasma RNPs, cortical F-actin undergoes a gradual microtubule-dependent movement towards the blastodisc periphery, where it accumulates forming circumferential arcs composed of short F-actin (Theusch et al., 2006; Nair et al., 2013). The close association of F-actin fragments to RNPs during their multimerization and outward movement suggests that F-actin is involved in one or both of these processes.

We recently reported that the maternal-effect gene *aura* encodes the cytoskeletal regulator Mid1p1L (MID1 interacting protein 1 like) and is required for the reorganization of cortical F-actin in the early embryo, including the formation of circumferential arcs (Eno et al., 2016). Here, we show that, in *aura* mutant embryos, germ plasma RNP aggregates fail to become recruited to the forming furrow, instead accumulating in peripheral regions of the blastodisc. This phenotype is associated with an accelerated rate of outward RNP movement in mutants. Inhibition of the F-actin network mimics, and stabilization of F-actin partially rescues, the *aura* mutant germ plasma segregation phenotype, suggesting that dynamics events within the F-actin cortex regulate germ plasma RNP movement. *aura/mid1p1l* function is required for the generation of cyclical patterns of dynamic cortical F-actin enrichment as well as cortical trench-like contractions corresponding to circumferential F-actin arcs. *aura/mid1p1l*-dependent F-actin local contractions are also observed at the contractile band during furrow formation, where they are also associated with RNP aggregate fusion. Our studies indicate that regulated *aura/mid1p1l*-dependent cortical F-actin dynamics mediate germ plasma segregation prior to and during furrow formation.

RESULTS

Mutations in the maternal gene *aura/mid1p1l* lead to failed germ plasma RNP furrow recruitment

As previously reported (Yoon et al., 1997; Pelegri et al., 1999; Theusch et al., 2006; Eno and Pelegri, 2013), germ plasma RNPs

present in the blastocyst cortex of wild-type embryos, such as those containing *vasa* RNA, form a characteristic rod-like structure along the forming furrows for the first two mitotic cycles (35 mpf; Fig. 2A,A'; 96%, $n=45$). Previous studies have shown that maternal homozygosity for mutations in *aura/mid1p1l* leads to defects in the early embryo, including cortical granule release and the completion of cytokinesis, defects that invariably result in embryonic lethality (Pelegri et al., 2004; Eno et al., 2016). However, using molecular markers, we find that, in embryos from females homozygous for mutations in *aura/mid1p1l*, animal germ plasma RNPs fail to localize to the forming furrow (Fig. 2B,B', Fig. S1), accumulating

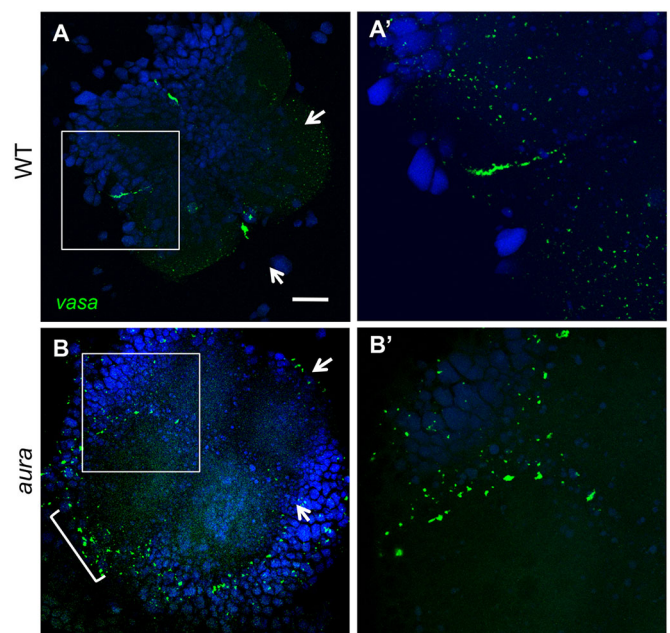


Fig. 2. *aura* mutant embryos fail to recruit germ plasma to the furrows. (A–B') *vasa* RNA localizes to the furrow in wild type (A,A') but not in *aura* mutants (B,B'), where it instead localizes to the blastodisc periphery (B, bracket). Arrows indicate cleavage planes for the first and second cell cycles. Boxed regions in A,B are shown in A',B'. Scale bar: 100 μ m in A,B; 50 μ m in A',B'.

instead as aberrantly large aggregates at the periphery of the blastodisc (Fig. 2B, bracket; 11% with furrow localization, $n=45$). This phenotype is observed in embryos from females homozygous for either the mutant allele *aur*⁴⁹⁷⁹² allele (Fig. 2, Fig. S1), which deletes a conserved C-terminal domain in the Midlip1L protein (Eno et al., 2016), or the CRISPR/Cas9-induced allele *aur*^{mw39} (Fig. S2), which deletes most of the Midlip1L protein and is therefore presumed to be a null allele (Eno et al., 2016). For simplicity, we refer to embryos from *aura/midlip1l* homozygous mutant females as *aura/midlip1l* mutant embryos.

aura/midlip1l mutant embryos exhibit defects in furrow formation, but these defects occur at late stages of cytokinesis (45–55 mpf). However, furrow initiation in *aura/midlip1l* mutants at earlier stages (30–40 mpf), a time coincident with the observed defect in animal germ plasma RNP furrow recruitment, is largely normal (Eno et al., 2016). Thus, the RNP recruitment phenotype observed in *aura/midlip1l* mutants is likely caused by cellular events that occur prior to and during furrow initiation, independent of the late cytokinesis defects.

Germ plasma RNP aggregation is associated with cortical F-actin surface trenches

Because *aura/midlip1l* mutants exhibit defects in cortical F-actin dynamics even prior to furrow formation (Eno et al., 2016), and as animal germ plasma RNPs are associated with cortical F-actin (Theusch et al., 2006; Nair et al., 2013), we explored whether the RNP furrow recruitment phenotype in *aura/midlip1l* mutants is related to cortical F-actin defects. For these experiments, and in order to increase sensitivity and preserve subcellular morphology, we took advantage of the ability of anti-P-myosin monoclonal antibody to label most germ plasma RNAs in the one- to four-cell stage embryo (Nair et al., 2013). Our previous studies have shown that wild-type embryos form cortical F-actin arcs, composed of short F-actin fragments, that run along the periphery of the blastodisc (Theusch et al., 2006; Nair et al., 2013; Eno et al., 2016) (Figs 3A and 4A–D). *aura/midlip1l* mutants, on the other hand, exhibit reduced formation of circumferential F-actin arcs (Eno et al., 2016) (Figs 3B and 4E–H). Orthogonal views of these projections reveal that the F-actin arcs found in wild type correspond to trench-like indentations at the cortex (Fig. 3A', A''). Such cortical trenches are often associated with RNP aggregates in their cytoplasmic side (Fig. 3A'). In *aura/midlip1l* mutants, although RNPs appear associated with cortical F-actin, the F-actin cortex appears as a flat surface that lacks cortical trenches (Fig. 3B', B'').

aura/midlip1l mutants show an increased outward germ plasma RNP movement along the blastodisc cortex

In a time course analysis of fixed samples, cortical F-actin and associated germ plasma RNPs are initially distributed throughout most of the blastodisc (Fig. 4A, A'), and show the expected outward movement during sperm aster formation immediately prior to and during the first few cell divisions (Theusch et al., 2006; Nair et al., 2013; Fig. 4B–D, white double-headed arrows denote RNP domain). In *aura* mutant embryos, the initial distribution and number of RNPs appears normal compared with wild type (Fig. 4E; Fig. S3). However, concomitant with the cortical F-actin reorganization defect, RNP pre-aggregates accumulate to the distal edge of the blastodisc at a rate that appears to be faster than wild type (Fig. 4F–H). We quantified the rate of outward movement of cortical RNPs by determining the ratio of the RNP-free domain, from the center of the blastodisc to the RNP aggregation front [at the inner rim of the RNP peripheral band (red double-headed arrows)]

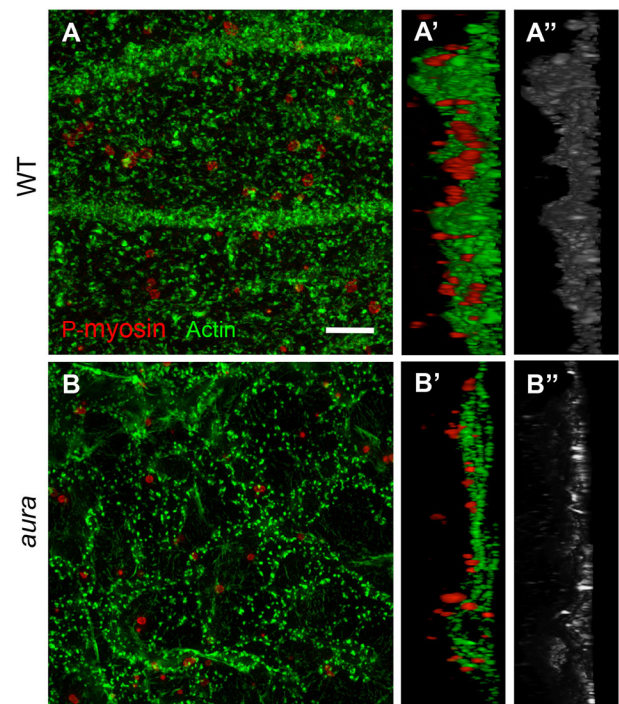


Fig. 3. Germ plasma RNP aggregates associate with Midlip1L-dependent cortical F-actin contractions. (A–B'') SIM images of cortical F-actin and germ plasma RNPs (anti-P-myosin) in wild type (A–A'') and *aura* mutants (B–B''), with orthogonal views (A', B', F-actin/RNPs; A'', B'', F-actin alone). Scale bar: 5 μ m.

relative to the radius of the blastodisc. This analysis shows a significantly increased outward movement of the germ plasma RNP aggregation front in mutants (Fig. 4I).

F-actin dynamics modulates microtubule-dependent outward movement of germ plasma RNPs

Given the role of *aura/midlip1l* in cortical F-actin dynamics (Eno et al., 2016), we tested the effect of F-actin inhibitors and stabilizers on germ plasma RNP furrow recruitment in embryos co-labeled for the germ plasma marker P-myosin and F-actin (Fig. 5), assaying at a time when, in control wild-type embryos, RNPs have become recruited to the furrows for the first two cycles (60 mpf, 84% of furrows with large aggregates, $n=50$) and a fraction of RNPs remains associated with F-actin arcs at the blastodisc margin (Eno and Pelegri, 2013; Nair et al., 2013; Fig. 5A). Treatment of wild-type embryos with the F-actin inhibitor cytochalasin D results in a reduced fraction of furrows with germ plasma RNP furrow recruitment (30%, $n=20$), defects that are similar to those observed in control *aura/midlip1l* mutants (25%, $n=20$). In cytochalasin D-treated wild-type embryos, cortical F-actin arcs fail to form and RNP aggregates are found mostly as large aggregates at the outer periphery of the blastodisc (Fig. 5B), again reminiscent of the *aura/midlip1l* mutant phenotype (Fig. 5E). Cytochalasin D treatment of *aura/midlip1l* mutants results in an apparent enhancement of the F-actin and RNP segregation phenotype (0% furrow recruitment, $n=8$), with RNPs accumulating at the margin (Fig. 5F). Treatment of wild-type embryos with the F-actin stabilizer phalloidin does not have a significant effect on RNP furrow localization (90%, $n=20$) and does not affect F-actin arc formation or lead to ectopic marginal RNP aggregates (Fig. 5C). However, phalloidin treatment restores RNP furrow recruitment in *aura/midlip1l* mutants (95%, $n=20$), allowing

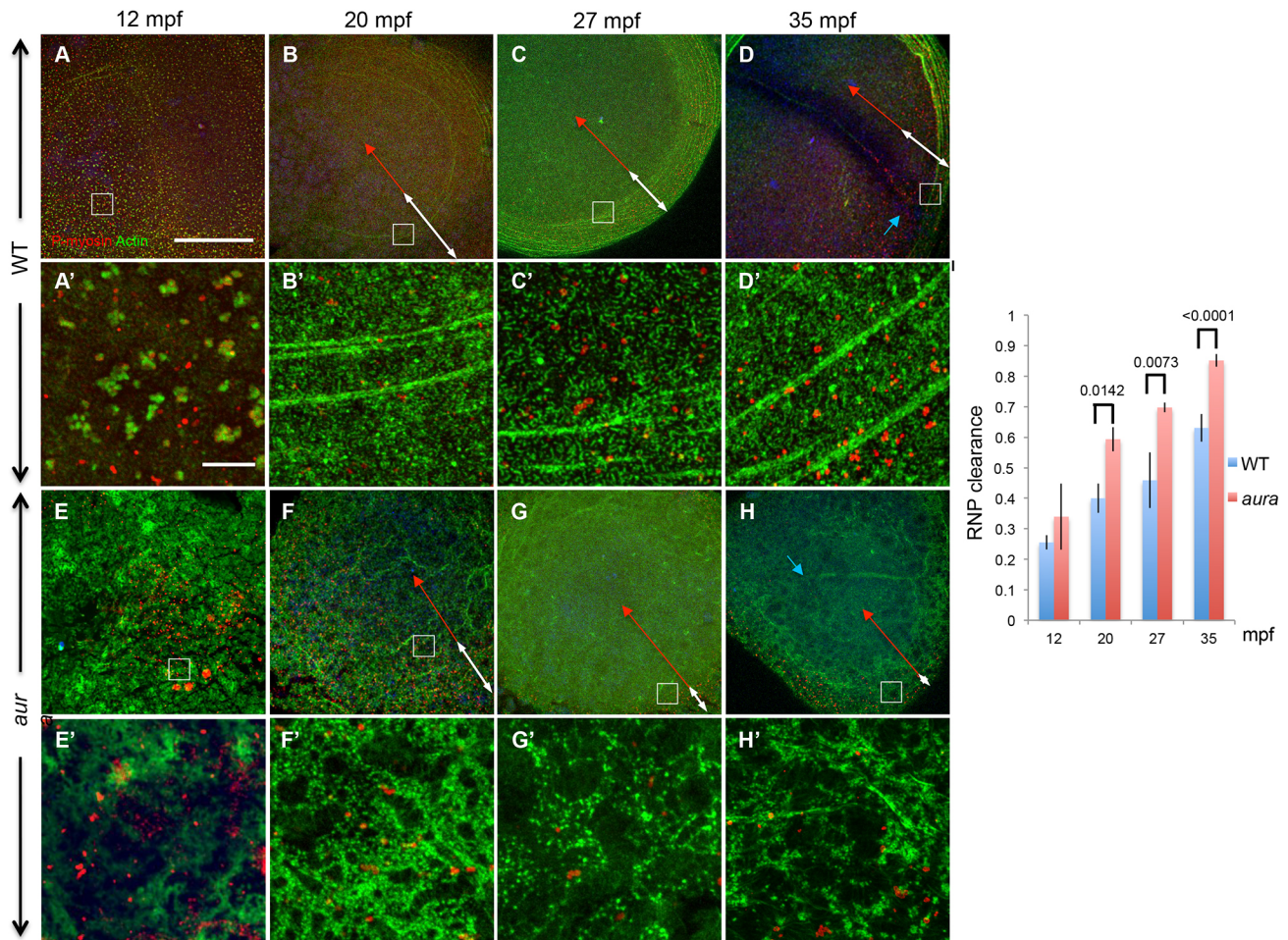


Fig. 4. Increased outward movement of germ plasm RNPs in *auramid1ip1* mutants. (A-D) In wild-type embryos, RNPs, which are initially distributed throughout the blastodisc, progressively accumulate as a peripheral band coincident with F-actin arc formation and peripheral movement. (E-H) In *aura* mutants, F-actin arcs fail to form and the field of RNPs narrows at a faster rate. White double-headed arrows highlight the RNP field; red double-headed arrows indicate the distance from the center of the embryo to the inner rim of aggregates at the RNP field. Blue arrows (D,H) indicate furrow F-actin. A'-H' are magnified regions of A-H at the location indicated by insets. Scale bars: in A, 100 μ m for A-H; in A', 10 μ m for A'-H'. (I) Quantitation of outward RNP movement, with the location of the RNP aggregating front determined upon magnification of the image. Significance was determined using an unpaired *t*-test. Data are mean \pm s.e.m.

F-actin arc formation and reducing ectopic marginal RNP aggregates (Fig. 5G). Similar effects on germ plasm RNP segregation were obtained when visualizing germ plasm with a probe to detect *dead end* RNA (Weidinger et al., 2003) using *in situ* hybridization (Fig. S4). Quantification of the position of the RNP aggregation front at 60 mpf shows that inhibition of the F-actin network results in an increase in the extent of outward RNP movement (95.4%, $n=9$; Fig. 5I) compared with control wild type (70.3%, $n=13$), similar to the increase observed in *aura/mid1ip1* mutant embryos (93.0%, $n=7$, see also Fig. 4). These results suggest that Mid1ip1L-dependent cortical F-actin dynamics function in the modulation of outward clearing of germ plasm RNP segregation, leading to furrow recruitment.

As expected (Theusch et al., 2006; Nair et al., 2013), treatment of wild-type embryos with the microtubule depolymerizing agent nocodazole results in germ plasm RNPs being distributed in more central regions of the cortex due to a reduced degree of outward movement of RNP aggregates (29.1% RNP clearance from blastodisc center, $n=13$, compared with 80.9% in control wild-type, $n=4$; Fig. 5D,J). In *aura/mid1ip1* mutants, nocodazole treatment also results in decreased outward RNP clearance (53.3%, $n=10$, compared with 91.3% in control mutants, $n=4$;

Fig. 5J), and reverses the ectopic marginal accumulation of RNP aggregates (Fig. 5H). Thus, the formation of peripheral ectopic aggregates in *aura/mid1ip1* mutants, as in wild-type (Theusch et al., 2006; Nair et al., 2013), is dependent on microtubule-dependent outward RNP movement. Altogether, these data suggest that Mid1ip1L function and the F-actin network are required to attenuate microtubule-dependent outward movement of cortical germ plasm RNPs, and that this modulation is necessary for their recruitment to the furrows.

Cortical F-actin exhibits *auramid1ip1*-dependent cyclical enrichment

To investigate further the dynamic nature of cortical F-actin and its dependence on Mid1ip1L, we carried out live imaging of actin in wild-type and mutant embryos using a LifeAct transgene (Behrndt et al., 2012). In wild-type early embryos before first furrow formation (between 15-30 mpf), cortical F-actin exhibits repeated cycles of F-actin enrichments (Fig. 6A, white and red circles; Movie 1). These F-actin enrichments appear to reappear in the same location with a periodicity of about 80 s, in a manner reminiscent of previously described cyclical cortical F-actin polymerization patterns in *Xenopus* and starfish oocytes, and in early embryonic

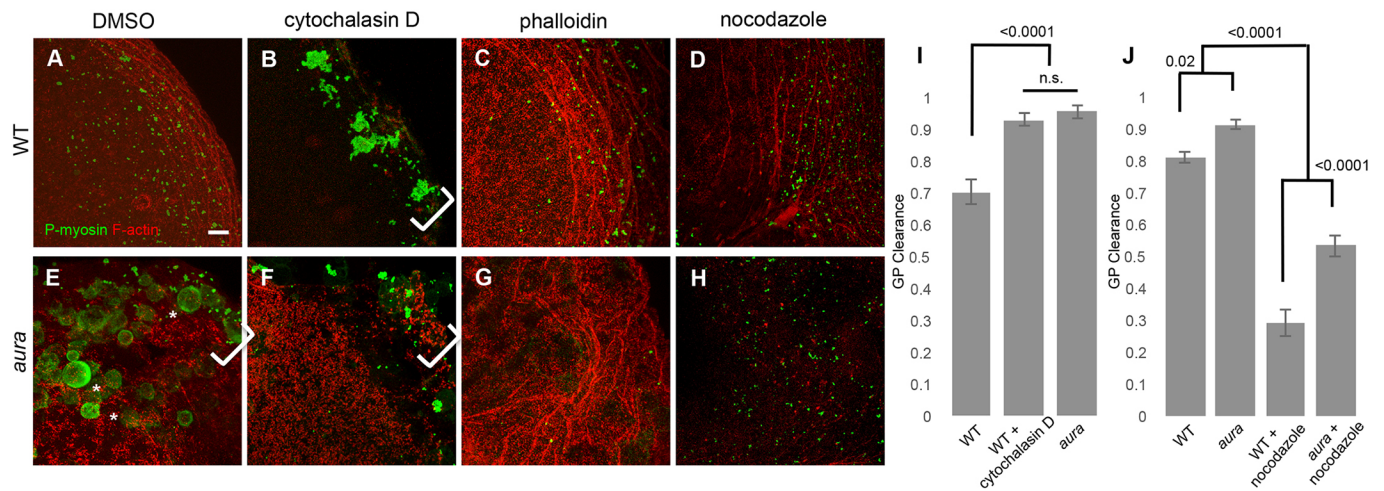


Fig. 5. Modulation of germ plasm RNP outward movement depends on cortical F-actin. (A-H) RNPs (green) and cortical F-actin (red) in control- (DMSO), cytochalasin D-, phalloidin- and nocodazole-treated wild-type (A-D) and *aura* mutant (E-H) embryos. F-actin inhibition with cytochalasin D in wild-type embryos leads to large RNP aggregates at the distal edge of the blastodisc (B), mimicking the *aura* mutant phenotype (E; large spherical structures are retained cortical granules; Eno et al., 2016). F-actin stabilization with phalloidin (C,G) does not have a significant effect in wild type (C) but reduces ectopic RNP aggregate accumulation in *aura* mutants (G). Inhibition of microtubules with nocodazole (D,H) results in reduced outward RNP movement in both wild type (D; Theusch et al., 2006) and *aura* mutants (H). Brackets indicate ectopic marginal RNP aggregate accumulation. (I,J) Quantitation of RNP outward clearing. Significance was determined using an unpaired *t*-test. Data are mean \pm s.e.m. Scale bar: 10 μ m.

cells (Bement et al., 2015). *aura* mutant embryos do not show these cyclical patterns, exhibiting instead a relatively static network that gradually reorganizes into punctate circular patterns similar to those observed in fixed samples (Fig. 6B,D,D'; Movie 1; see also Eno et al., 2016). F-actin ring-like structures, though smaller and not as punctate or frequently observed as in *aura/mid1ip1* mutants, can also be discerned amid cortical F-actin enrichments in wild-type embryos (Fig. 6C,C'). Kymograph analysis from live imaging data shows that in wild-type cortical F-actin exhibits a characteristic

undulating pattern over time, involving slight lateral shifts of F-actin enrichment (Fig. 6C''). In contrast, in *aura* mutants localized cortical F-actin enrichments do not undergo lateral shifts, remaining instead in a static pattern that dissipates over time (Fig. 6D''). As expected from the analysis of fixed samples, wild-type embryos treated with cytochalasin D and phalloidin exhibit reduced and dynamic F-actin networks, respectively (Movies 2 and 3), and phalloidin treatment partially rescues F-actin dynamics in live *aura/mid1ip1* mutants (Movie 4).

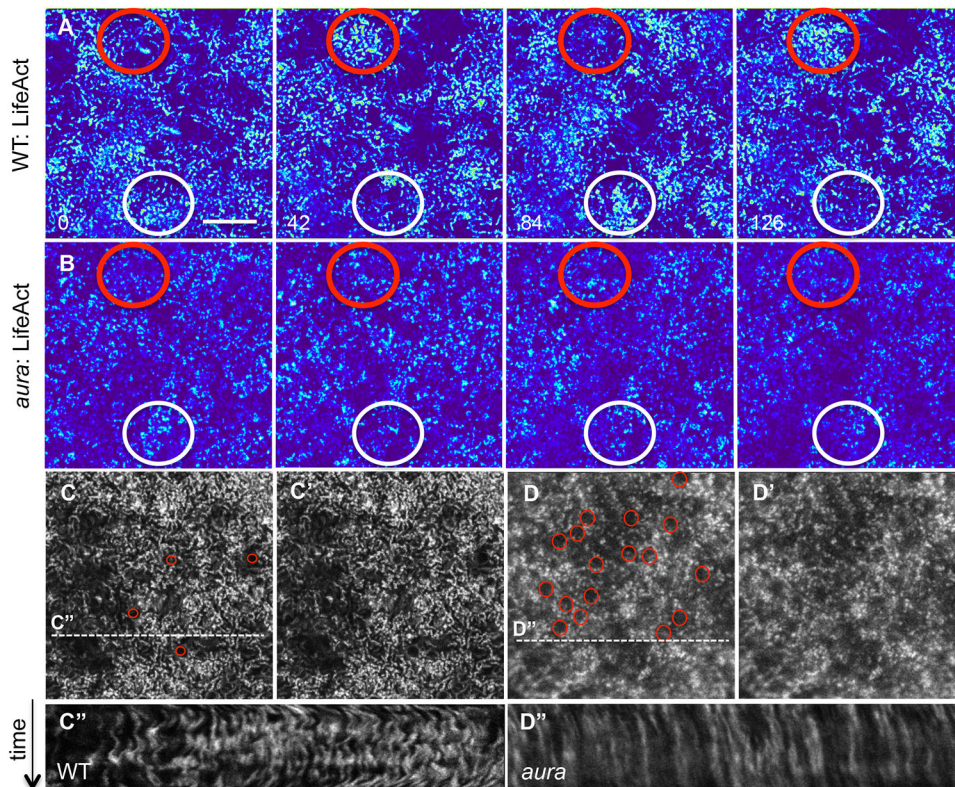


Fig. 6. Dynamic waves of cortical F-actin depend on *auramid1ip1* function. (A,B) In live wild-type embryos, cortical F-actin shows cyclical patterns of apparent enrichment, whereas this network appears static in *aura* mutants (B) (in each case, red and white circles highlight two different cortical locations). Still images of LifeAct transgenic embryos spaced 42 s apart (Movie 1). (C-D') The F-actin network towards the end of the movie sequence highlights that F-actin forms a punctate pattern as well as numerous round F-actin structures (red circles) in mutants (D,D') that are present in lower numbers in wild type (D,D'). (C'',D'') Kymographs of cortical F-actin (data from dashed lines in C',D', representing 70 time points in a 140 s period) show lateral undulations in wild type and a static network in *aura* mutants. Scale bar: 10 μ m.

***aura/mid1ip1l* is required for F-actin cortical waves associated with germ plasm RNP aggregates**

3-D rendered orthogonal views of the cortical F-actin cytoskeleton show key differences between wild-type and mutant embryos, as well as embryos treated with cytoskeletal inhibitors (Fig. 7). Wild-type embryos show cortical trenches (Fig. 7A, corresponding to Fig. 3A'), whereas these structures are entirely lacking in *aura/mid1ip1l* mutants (Fig. 7G, corresponding to Fig. 3B') or cytochalasin D-treated wild-type embryos (Fig. 7C). Although phalloidin-treated wild-type embryos did not exhibit a statistically significant increase in cortical trenches (Fig. 7E), phalloidin treatment caused a significant increase in cortical F-actin trench formation in *aura/mid1ip1l* mutants compared with control-treated mutant embryos (Fig. 7I, quantification in K), consistent with a rescuing effect through F-actin stabilization of the phenotypes associated with *aura/mid1ip1l* mutations (Eno et al., 2016). Thus, Mid1ip1L function is required for the formation of cortical F-actin trench-like structures associated with germ plasm RNP pre-aggregates.

Shared *aura/mid1ip1l*-dependent features at the cortex and furrows associated with germ plasm RNP aggregation

Remarkably, a pattern of repeated wave-like structures was also observed in optical cross-sections of F-actin at the furrow, with wild-type furrows showing repeated indentations (Fig. 7B). Similar to the case for the F-actin cortex, these indentations are not present in *aura* mutants (Fig. 7H). Also similar to cortical F-actin trench formation (Fig. 7C), cytochalasin treatment of wild-type embryos results in furrow F-actin without indentations (Fig. 7D), mimicking the *aura* mutant phenotype. Moreover, and also reminiscent of the effect on cortical trenches (Fig. 7I), phalloidin treatment restores the ability of furrow F-actin to form indentations in *aura/mid1ip1l* mutant embryos (Fig. 7J, quantification in L). In addition, F-actin

accumulations at the furrow, as seen in a face on (animal) view, corresponding to furrow indentations, appear continuous with F-actin enrichments at the abutting cortex, corresponding to cortical trenches (Fig. S5). Thus, these two types of F-actin structures, cortical trenches and furrow indentations, are induced by a functionally related Mid1ip1L-dependent mechanism and are structurally linked.

As with the case of surface contraction trenches at the cortex (Fig. 8A-C, see also Fig. 3A'), F-actin indentations at the furrow are associated with germ plasm RNP aggregates (Fig. 8D-F), which appear to be in close association with, and typically in between, furrow indentations (Fig. 8G,G'). In cases in which the RNP aggregate at the furrow is a relatively elongated structure, it is often observed to be associated via one of its ends to the furrow indentation, with most of the aggregate mass protruding into the cytoplasm (Fig. 8G').

Notably, cortical trenches and furrow indentations exhibit a directional accumulation over time. Cortical trenches span a progressively reduced and more distal location of the blastodisc in a radial direction (Fig. 8A-C', quantified in H) mirroring the outward movement of the germ plasm RNP aggregation front. Furrow indentations also occur in a progressively more distally confined region, in this case in a medial-to-distal direction along the furrow (Fig. 8D-F', quantified in I), mirroring the compaction of the RNP aggregate.

The directional tightening of F-actin cortical trenches and furrow indentations is consistent with a role in their gradual encroachment of RNP aggregates to facilitate their fusion into increasingly larger aggregates, which eventually accumulate at the furrow distal ends. Indeed, RNPs can be observed as streams of single particles in between adjacent aggregates at the furrow (Fig. 8G,G'), a behavior consistent with inter-aggregate fusion. Thus, wild-type embryos

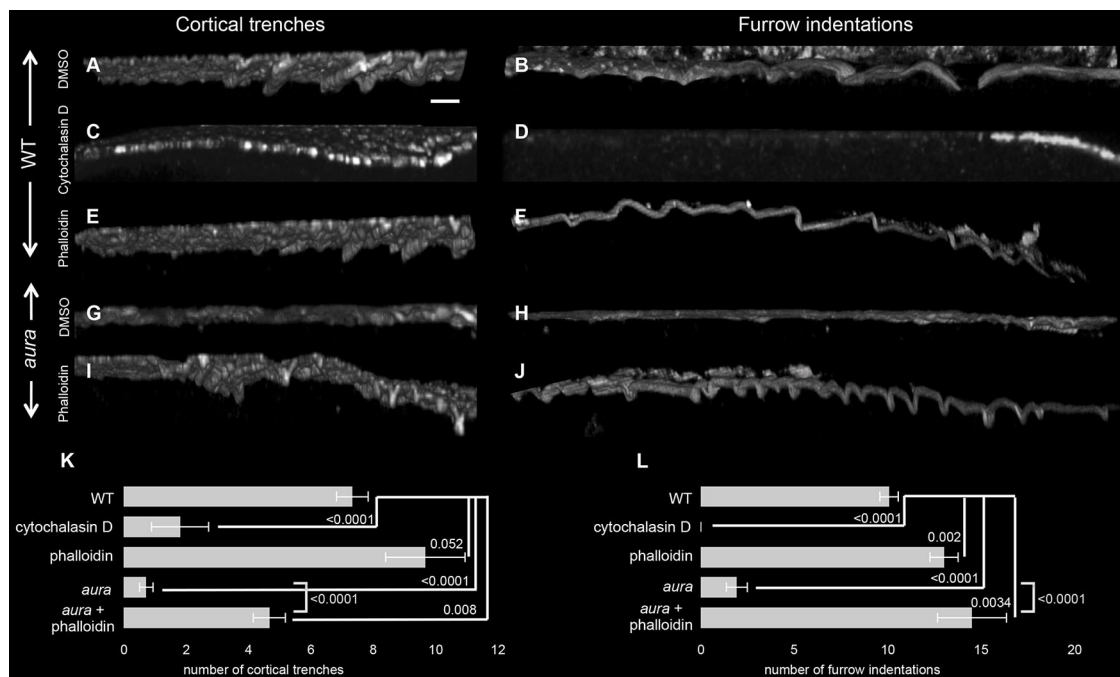


Fig. 7. F-actin cortical trenches and furrow indentations depend on *aurami1ip1L* function. (A–J) Orthogonal views of cortical and furrow F-actin in wild-type and *aura* mutant embryos treated with drugs targeting F-actin. Wild-type embryos exhibit cortical trenches (A) and furrow indentations (B), which are missing in *aura* mutants (G,H). In wild type, actin inhibition with cytochalasin D abolishes the formation of both cortical trenches (C) and furrow indentations (D). Treatment with the F-actin stabilizer phalloidin allows cortex trenches (E) and furrow indentations (F) in wild type and rescues the formation of these structures in *aura* mutants (I,J). (K,L) Quantitation of the number of cortical trenches and furrow indentations under the various conditions, using data from at least seven images per condition. Significance was determined using an unpaired *t*-test. Data are mean \pm s.e.m. Scale bar: 10 μ m.

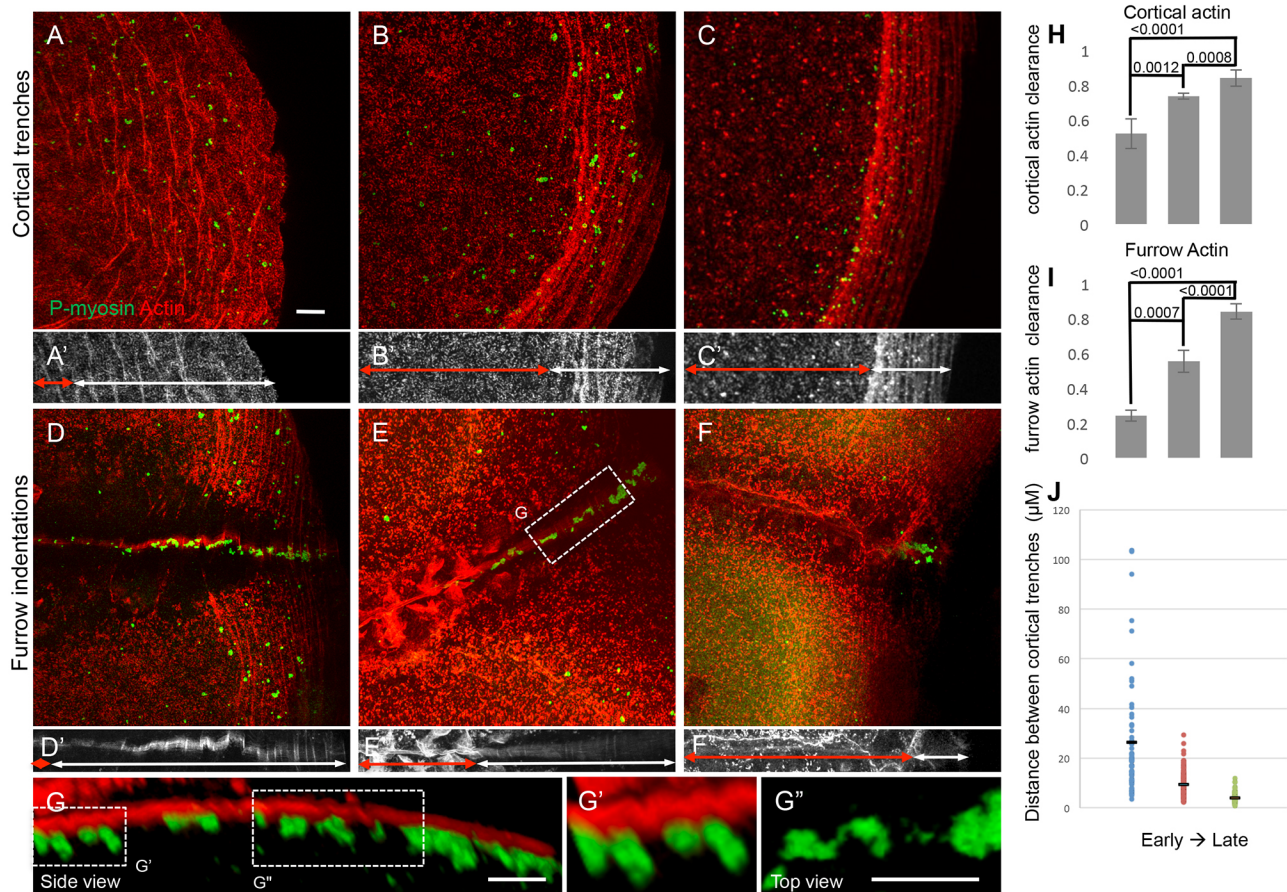


Fig. 8. F-actin cortical trenches and furrow indentations exhibit a directional progression and are associated with germ plasm RNPs. (A-C) Wild-type embryos fixed prior to furrow formation (A, 35 mpf; B, 50 mpf; C, 65 mpf; animal with blastodisc center to the left and blastodisc periphery to the right). F-actin arcs, associated with RNP aggregates, move outwards as they form a more-dense network. A'-C' highlight the region of F-actin arcs (white double-headed arrows) and the distance from the center of the blastodisc to the inner rim of the F-actin arc region (red double-headed arrows). (D-F) Wild-type embryos fixed during furrow formation (D, 35 mpf; E, 50 mpf; F, 65 mpf; animal view with furrow medial region to the left and furrow distal end to the right). The region containing RNPs, which progressively becomes more distally restricted, corresponds to that exhibiting F-actin furrow indentations, as revealed by orthogonal views (D'-F'). In D'-F', white double-headed arrows encompass the region with F-actin indentations and red double-headed arrows the more medial region with F-actin of an even and wider span, and with a punctate appearance, which likely correspond to the forming cell adhesive wall (Jesuthasan, 1998; Urven et al., 2006). (G) Orthogonal view of the furrow showing association of RNP aggregates with F-actin furrow indentations. (G') RNP aggregates wedged within cytoplasmic spaces between indentations. (G'') An apparent stream of individual RNPs between larger aggregates (rotated as a top view for better visualization). In all images, RNPs (P-myosin) are in green and F-actin is in red, except the RNP and F-actin channels have been omitted for clarity in A'-F' and G'', respectively. (H) Quantification of cortical actin clearance as the ratio of the distance from the center of the blastodisc to the region of cortical F-actin trenches (red double-headed arrows in A'-C') over the blastodisc radius. (I) Quantification of furrow actin clearance as the distance from the center of the furrow to the region of furrow F-actin indentations (red double-headed arrows in D'-F') over the half-length of the furrow. (J) Quantification of the distance between individual F-actin arcs (distance between their peak intensity) within the blastodisc arc region (double-headed arrows in A'-C'), showing increasing density of F-actin network during its peripheral restriction. Significance was determined using an unpaired *t*-test. Data are mean \pm s.e.m. Scale bars: 10 μ m.

exhibit *aura/midlip1l*-dependent wave-like contractions at the cortex and furrows, which in both cases are associated with germ plasm RNP aggregates and appear to regulate their movement and aggregation.

Midlip1L associates with dynamic cortical F-actin

We used structured illumination microscopy (SIM) to visualize Midlip1L protein in relation to cortical F-actin with increased resolution compared with previous studies (Eno et al., 2016). In control wild-type embryos, Midlip1L protein largely colocalizes with cortical F-actin (Fig. 9A), as well as in F-actin-enriched regions at the furrow (Fig. 9B). Interestingly, Midlip1L protein and F-actin do not fully colocalize, revealing regions within or immediately adjacent to F-actin enrichments that contain higher levels of Midlip1L protein, and vice versa. In mutants for the *aura*⁹⁷⁹² allele, which lacks the majority of the protein except for a conserved

C-terminal domain, Midlip1L protein is present, but the localization to actin is drastically reduced (Eno et al., 2016; data not shown).

Interfering with F-actin polymerization with cytochalasin D treatment results in the expected reduction in cortical F-actin. Under this condition, Midlip1L protein appears highly punctate, showing reduced overlap with any remaining cortical F-actin (Fig. S6B). Treatment of wild-type embryos with phalloidin also results in an apparently reduced degree of localization between Midlip1L protein and F-actin (Fig. S6C). In contrast to treatments that affect F-actin dynamics, embryos treated with the microtubule inhibitor nocodazole do not exhibit apparent differences in the pattern of colocalization of Midlip1L protein and F-actin observed in control wild-type embryos (Fig. S6D). Thus, Midlip1L protein binds specific regions of short cortical F-actin, and this binding depends on F-actin dynamics (see Discussion).

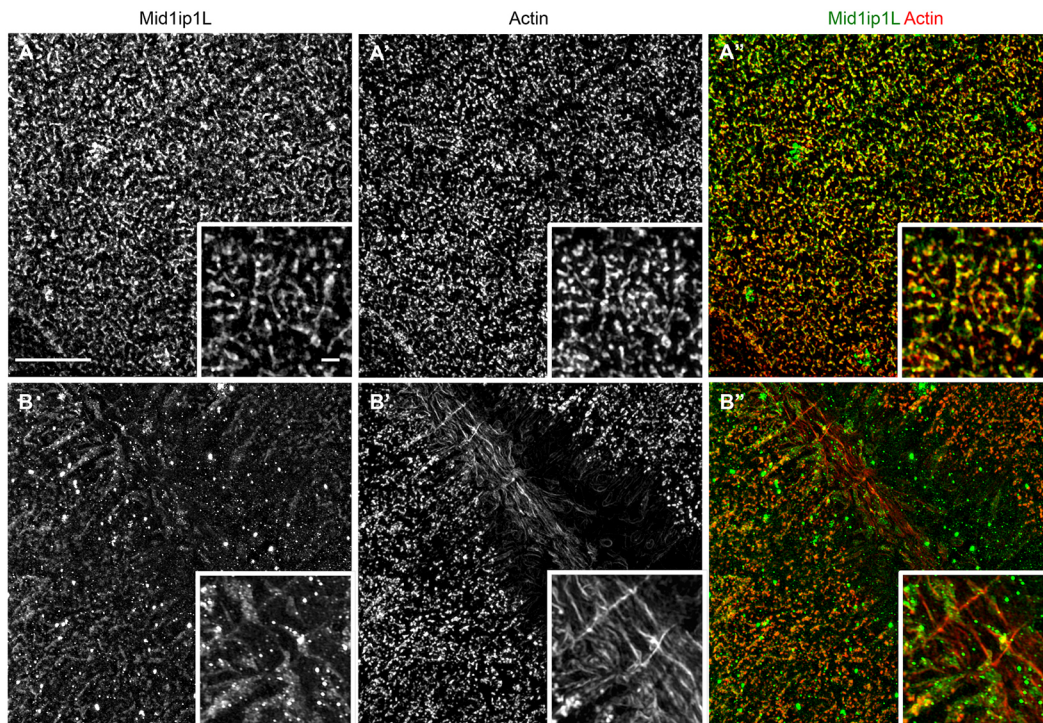


Fig. 9. Mid1ip1L protein localizes with cortex and furrow actin. (A-B'') SIM images of single and merged channels showing partial colocalization of Mid1ip1L protein with F-actin in wild-type embryos. (A-A'') At the cortex, Mid1ip1L protein partially colocalizes with the cortical F-actin. (B-B'') At the furrow, Mid1ip1L protein also partially colocalizes with transverse F-actin at furrow distal ends, which corresponds to furrow F-actin indentations (see also Fig. S5). Insets show a magnified view within the field. Scale bar: 10 μ m in A-B''; 1 μ m in insets.

DISCUSSION

Previous studies have shown that zebrafish germ plasm RNPs are associated with cortical F-actin, and that microtubule growth mediates the movement of RNPs associated with the actin network, which results in RNP multimerization and localization to the furrows (Theusch et al., 2006; Nair et al., 2013). Here, we show that dynamic reorganization of the cortex, mediated by the function of maternal Mid1ip1L, is required to retain RNPs to the cortex during the early cleavage cycles in order to allow their recruitment to the forming furrows. Surprisingly, *mid1ip1l*-dependent F-actin dynamic mechanisms appear conserved at both the early embryo cortex and furrows, showing shared mechanisms associated with germ plasm RNP movement and multimerization prior to and during furrow formation.

Mid1ip1L-dependent germ plasm RNP cortical retention is essential for furrow recruitment

Zebrafish germ plasm is inherited in the egg as RNPs associated with the animal cortex of the early zygote (Theusch et al., 2006; Nair et al., 2013; Eno and Pelegri, 2016). Association of these animal germ plasm RNPs to the plus ends of astral microtubules during early embryonic development results in their outward movement, with aggregates forming along what appear to be outwardly moving aggregation waves at the inner rim of an RNP cortical band. Soon after fertilization, germ plasm RNPs appear associated with short F-actin seeds, with the CPC protein Birc5b acting as necessary link between the ends of growing astral microtubules and a complex involving RNPs and F-actin (Nair et al., 2013). RNP recruitment during cell division depends on their gathering at the furrow from adjacent cortical regions, mediated by the action of interphase astral microtubules (Eno and Pelegri, 2013; Nair et al., 2013).

Aided by a process of internal priming (Ishihara et al., 2014), interphase astral microtubules appear to reach the outermost regions of the blastodisc and forming blastomeres (Wühr et al., 2009, 2010), an ability that allows for furrow induction in large dividing blastomeres (Yabe et al., 2009). We find that ectopic localization of RNP aggregates to the blastodisc periphery in *aura/mid1ip1l* mutant embryos correlates with an increased rate of outward movement of the RNP aggregation front in the embryo, and that inhibition of cortical actin results in similar effects. These observations suggest a modulatory function for Mid1ip1L and cortical F-actin to reduce the rate of outward RNP movement. In wild type, the attenuation of germ plasm RNP peripheral movement by Mid1ip1L function and cortical F-actin allows cortical RNPs to be partially retained in the blastodisc cortex, where astral microtubules for the following cell cycle can promote further RNP furrow recruitment (Fig. 10). In the absence of this attenuation, as in *aura/mid1ip1l* mutants, outward forces bring RNPs prematurely to the blastodisc periphery, rather than to the furrow (Fig. 10, *aura*). Thus, our studies suggest that the rate of germ plasm RNP aggregate outward movement is finely regulated in order to balance two opposing forces: microtubule-dependent outward RNP movement, which promotes their collection at the furrow plane by bipolar spindles during cell division; and Mid1ip1L-dependent RNP retention at the cortex, which allows for the gradual collection of RNPs in furrow planes during consecutive cell cycles.

The end result of this process is the formation of four large germ plasm masses, one at each furrow end for the first and second cell cycles (Eno and Pelegri, 2013). Cell cleavage orientation in the zebrafish embryo is specified independently of the axis of dorsal induction (Abdelilah and Driever, 1997). We speculate that the location of these germ plasm masses, which generate primordial germ cells in all four quadrants of the embryo, maximizes the

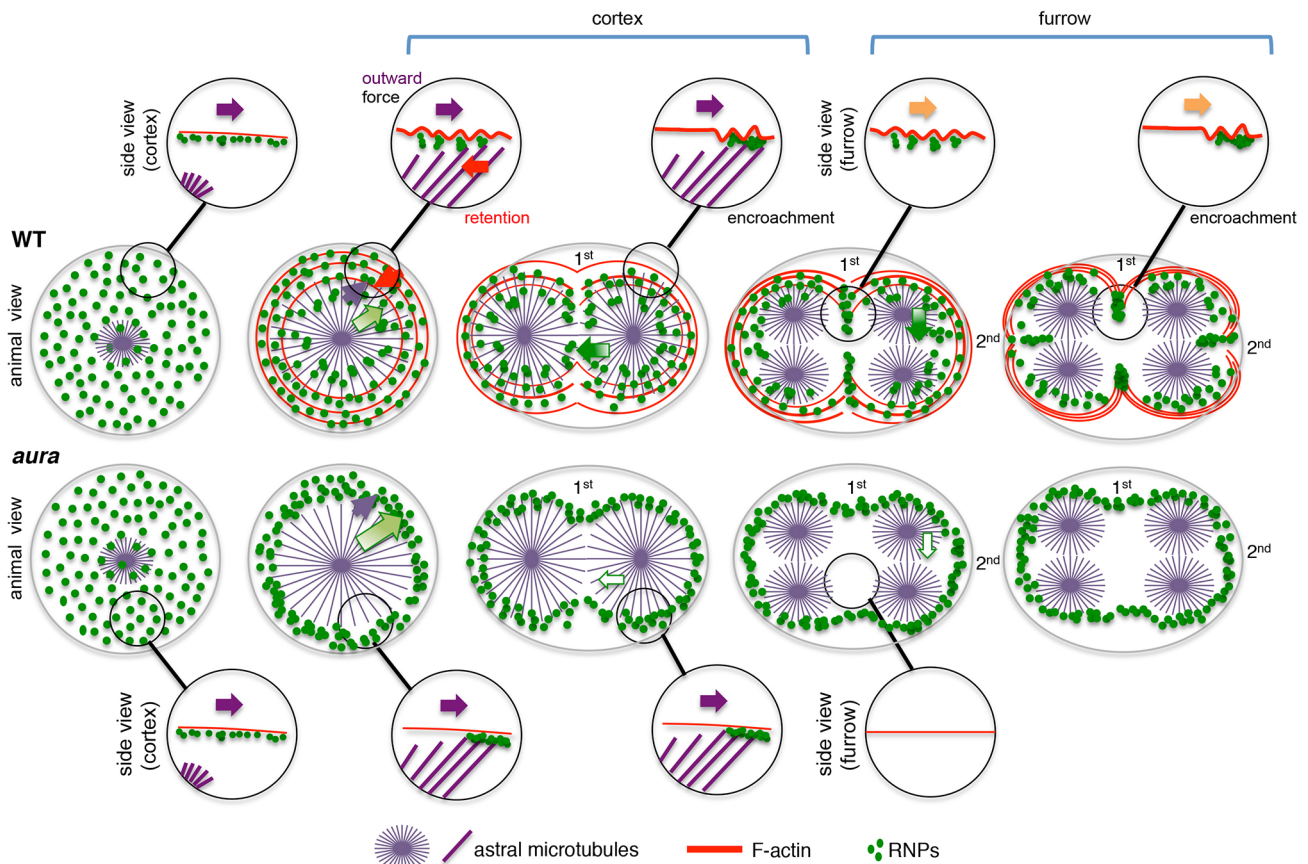


Fig. 10. Model for the Mid1ip1L- and F-actin-dependent modulation of germ plasm segregation. In wild type (WT, animal view) during the first few cell cycles, Mid1ip1L-dependent F-actin dynamics attenuate (red arrow) a microtubule-dependent outward movement (purple arrows), resulting in the regulated outward movement of RNPs (graded light-green arrows). Regulated outward RNP movement allows the partial persistence of RNPs at the cortex, making them available for recruitment to the furrows for the next cell cycles (graded dark-green arrows). In *aura* mutants (animal view), Mid1ip1L-dependent modulation is affected, causing unregulated outward RNP movement. This results in low persistence of RNPs at the cortex, leading to reduced RNP furrow recruitment (white arrows) and increased peripheral accumulation. Insets as side views depict shared aspects of Mid1ip1L-dependent surface contractions at the cortex and along the furrow, the gradual increase in density of which facilitates RNP aggregate fusion through encroachment. Growing astral microtubules likely provide a directional outward radial cue to influence cortical contractions. A second signal (yellow arrows) provides medial-to-distal directionality along the furrow [see accompanying work (Eno et al., 2018); furrow-associated microtubules are not shown].

potential to populate the gonad through mechanisms of chemokine-guided cell migration (Paksa and Raz, 2015).

Mid1ip1L-dependent F-actin dynamics and surface wave formation

We find that wild-type embryos exhibit cycles of Mid1ip1L-dependent cortical F-actin polymerization and depolymerization. We additionally find the presence of F-actin indentations at the cortex, which also depend on Mid1ip1L function, corresponds to previously described circumferential F-actin bands (Theusch et al., 2006; Nair et al., 2013). We do not yet know the precise interconnection between F-actin polymerization cycling, which is observed in cortices of live embryos, and the formation of cortical F-actin bands and trenches observed in fixed samples. However, both of these events are dependent on Mid1ip1L function and, in live embryo cortices, we observe F-actin alignment, albeit only partial, that is reminiscent of F-actin bands in fixed embryos (Fig. 6A). It is also possible that fixation arrests cycles of cortical contraction at a specific point in the F-actin cycle, highlighting the elongated circumferential trenches that can be observed in fixed samples. In addition to a potential connection to cortical contraction formation (see below), Mid1ip1L-dependent lateral cortical

movement may help accelerate RNP-RNP encounters and therefore multimerization of single RNPs into the observed pre-aggregates.

Some of the processes we observe in zebrafish may be conserved, as *Xenopus* germ plasm aggregation has been found to depend on surface contraction waves at the vegetal pole of the embryos (Savage and Danilchik, 1993). Moreover, cortical F-actin in *Xenopus* and starfish oocytes and early embryonic cells exhibits Rho-dependent cycles of polymerization and depolymerization that initiate at anaphase onset, leading to cytokinesis (Bement et al., 2015). The latter are similar to those we observe in the zebrafish embryo, although in *Xenopus* and starfish these cycles occur independently of the segregation of germ cell determinants, which are restricted to the vegetal pole in these species (Whittington and Dixon, 1975; Ransom and Dixon, 1988; Savage and Danilchik, 1993; Juliano et al., 2010). It is possible that the early zebrafish embryo has co-opted an already existing cortical process to precisely regulate germ plasm segregation.

Super-resolution images show that Mid1ip1L localizes to the ends of short F-actin, a localization that is reduced by both inhibition of actin polymerization with cytochalasin D and F-actin stabilization with phalloidin. Similar to the effect on germ plasm

RNP segregation, defects in cortical contractions in *aura/mid1p1l* mutants are mimicked by F-actin depolymerization and partially rescued by F-actin stabilization (see also Eno and Pelegri, 2016). Considering these findings, we envision that Mid1p1L protein either promotes F-actin polymerization at its plus (barbed) end (Fig. S7, top) or negatively regulates F-actin depolymerization at its minus (pointed) end (Fig. S7, bottom). Stabilization of F-actin with phalloidin may allow for proper filament length in *aura* mutant embryos, and the formation of surface contractions (Fig. S7), although additional defects in *aura* mutants such as in internal membrane recycling (Eno et al., 2016) and furrow maturation (Eno et al., 2016; Eno et al., 2018) likely preclude the ability of phalloidin to fully rescue the *aura* cytokinesis defect (Eno et al., 2016; C.E. and F.P., unpublished).

Several possible mechanisms may allow Mid1p1L-dependent F-actin dynamics to produce cortical trenches, including F-actin ruffling (Ballestrem et al., 1998), polymerization coupled to membrane attachment (Nawaz et al., 2015; Zuchero et al., 2015), F-actin flow (Comrie et al., 2015) or actomyosin contraction (Schaefer et al., 2002; Burnette et al., 2011). Mid1p1L protein, initially identified as an interacting factor of the X-linked causal gene for Opitz G/BBB syndrome in humans (Quaderi et al., 1997; Gaudenz et al., 1998; Cox et al., 2000; De Falco et al., 2003; Winter et al., 2003), does not contain obvious functional motifs to explain these interactions, although the protein has been shown to regulate both microtubules (Berti et al., 2004) and F-actin (Eno et al., 2016).

The periodic nature of the observed trenches is intriguing. In motile cells, while treadmilling is constitutive during F-actin ruffling, ruffles only appear at certain points (Ballestrem et al., 1998). Similarly, F-actin hotspots accumulate periodically where F-actin undergoes continuous assembly and disassembly (Ganguly et al., 2015). In the latter system, F-actin accumulation occurs at 3–4 μm apart, a distance similar to that between F-actin arcs in the early zebrafish embryo. It will be interesting to determine how apparently constitutive mechanisms determine the observed periodic patterns of cortical F-actin.

Further work will also be required to understand how Mid1p1L-dependent F-actin dynamics and cortical indentations attenuate the outward movement of germ plasm RNPs. Previous studies suggest dynamic actin and actomyosin contractility can actively influence cell-surface molecules to form transient clusters (Goswami et al., 2008; Gowrishankar et al., 2012) and can alter diffusion of GPI-anchored cortical proteins (Saha et al., 2015). Competitive binding of germ plasm RNPs between microtubule tips and F-actin (Alberico et al., 2016; Mohan and John, 2016; Cabrales-Fontela et al., 2017), as RNPs move outwardly through the action of astral microtubule growth, may also explain the modulation of RNP movement by the actin network.

During the early cell cycles in the zebrafish embryo, intracellular components undergo active redistribution through ooplasmic streaming, a process that depends on F-actin function (Roosen-Runge, 1938; Kane and Kimmel, 1993; Leung et al., 2000; Fernández et al., 2006; Fuentes and Fernández, 2010; Li-Villarreal et al., 2015). We cannot rule out scenarios in which Mid1p1L- and F-actin-mediated RNP retention occurs indirectly through the modulation of cytoplasmic flow. However, the bulk of ooplasmic streaming into the blastodisc occurs through internal yolk cell channels, likely generating a central-to-peripheral flow in the blastodisc (e.g. Li-Villarreal et al., 2015). Such a flow would be unlikely to contribute to RNP retention, which we infer occurs in the opposite direction. In addition, we observe RNP aggregates only in cortical locations (Theusch et al., 2006; Eno and Pelegri, 2013; Nair

et al., 2013; this study), and not in internal regions if these were to cycle through cytoplasmic streams.

Parallel Mid1p1L-dependent mechanisms in the blastodisc cortex and the furrow

Surprisingly, we found indentations in furrow F-actin that share similarities to trenches found in the blastodisc cortex. In both cases, these indentations are Mid1p1L dependent and are affected by inhibitors of F-actin dynamics. Trenches and indentations exhibit an apparent movement towards more peripheral regions of the blastodisc (outwardly for cortical trenches and medial-to-distal for furrow indentations). These similarities strongly suggest that similar underlying mechanisms drive F-actin deformations at both the cortex and the furrow.

The directionality of inferred movement suggests the involvement of traveling F-actin waves (t-waves; Allard and Mogilner, 2013), which may be responding to different signals at the cortex compared with the furrow. At the cortex, outward directionality may be caused by the growth of interphase astral microtubules, whereas at the furrow, medial-to-distal directionality may be conferred by furrow-associated directional cues (Eno et al., 2018).

An encroachment mechanism for germ plasm RNP aggregation

Germ plasm has been proposed to generate a fluid phase that is different from that of the cytoplasm in various systems (Brangwynne et al., 2009; Updike et al., 2011; Wang et al., 2014; Nott et al., 2015), including the zebrafish (Riemer et al., 2015), which by virtue of differential affinity may promote particle-particle binding. An increase in particle density is expected to facilitate particle encounters whose different properties may cause particle-particle adhesion. Outward movement of germ plasm RNPs linked to growing astral microtubules result in an increase in RNP relative density that likely facilitates inter-particle encounters.

We find an association of germ plasm RNP aggregates with cortical F-actin trenches at the blastodisc cortex, as well as F-actin indentations at the furrow, with RNP aggregates connected to the cytoplasmic side of the trench/indentation. Considering this association, the gradual convergence of trenches and indentations (into peripheral blastodisc cortex and distal furrow regions, respectively) likely acts to gradually encroach RNP aggregates, increasing RNP density and promoting multimerization. Indeed, we observe instances in which adjacent RNP aggregates appear to have particles streaming from one aggregate to another, consistent with the fluid phase character of the aggregates, and which may constitute intermediate stages in the aggregate fusion process.

Our work shows that Mid1p1L-dependent cortical F-actin dynamics and deformations regulate the movement of germ plasm RNPs, allowing their recruitment to the forming furrows and likely facilitating RNP multimerization. These events occur through an intrinsic Mid1p1L-dependent mechanism that is modified by successive extrinsic polarity signals, first radially outward on the cortex, and subsequently medial-to-distally along the furrow. These processes constitute sequential steps that facilitate the movement and aggregation of germ plasm RNPs in the early embryo.

MATERIALS AND METHODS

Fish maintenance and genetic methods

Fish stocks were raised and maintained under standard conditions at 28.5°C (Brand et al., 2002). All animals were handled in strict accordance with good animal practice, as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate

committee (University of Wisconsin-Madison assurance number A3368-01). Wild-type stocks were AB and *mid1ip1l* mutants included both *aura*¹⁹⁷⁹² (Pegri et al., 2004) and *mid1ip1*^{tw39} (Eno et al., 2016). Fish were identified either through their phenotype or genotypic typing using custom-designed LNA probes (IDT) to distinguish mutant from wild type via end-point genotyping on a Roche LC480 qPCR system (Table S1). For embryo collection and synchronization, fish were mated and embryos collected within a 5 min interval during mating.

Immunofluorescence and *in situ* hybridization

For immunofluorescence detection, embryos were dechorionated using pronase and fixed in 4% paraformaldehyde, 0.25% glutaraldehyde, 5 mM EGTa, 0.2% Triton X-100 and 0.2 U/ml rhodamine (or Alexa-488)-conjugated phalloidin for 3–5 h at room temperature. Embryos were treated with 0.5 mg/ml NaBH₄, washed with PBS-T (Triton-X 0.1%), blocked with PBST+10% BSA for 1 h at room temperature (Theusch et al., 2006), and incubated ON with primary antibodies: p-NMII (1:50; CST, 3710) and Mid1ip1L (1:200; Antibodies Online). Embryos were incubated with secondary antibodies against mouse or rabbit conjugated to Alexa 488 (1:200; Thermo Fisher) or Cy3 (1:200; Jackson Immuno Labs) and rhodamine-phalloidin (1:200; Cytoskeleton) or Alexa-488-phalloidin (1:200; Thermo Fisher).

For fluorescent *in situ* hybridization, embryos were fixed with 4% PFA while still in their chorions. Fixed embryos were labeled using a fluorescein-labeled antisense RNA probe for the gene *vasa* (Yoon et al., 1997) and detected using the Fluorescein system (Perkin Elmer) following 'Triple fluorescent *in situ*' protocol (modified Talbot protocol, wiki.zfin.org/display/prot/Triple+Fluorescent+In+Situ). For all immunofluorescent and *in situ* experiments, the embryos were de-yolked after labeling, stained with DAPI (Theusch et al., 2006) and the blastodiscs were mounted flat on slides for imaging. Percentage recruitment presented is based on the number of furrows, typically two to four per embryo imaged. Embryos were imaged using a Zeiss LSM510 for confocal or 780 for structured illumination microscopy and analyzed using FIJI, including 3D Viewer.

Live imaging

Both wild-type and *aura* (Eno et al., 2016) embryos carrying the Life Act [Tg(actb2:LIFEACTION-GFP)] (Behrmdt et al., 2012) transgene were dechorionated and placed in glass bottomed microscopy slides (Ibidi) in E3. Embryos laying animal pole side down were imaged on an inverted Zeiss LSM780. Images were taken every 2 s at 100× magnification with 2× digital zoom. 3D rendering, projections, movies, montages and kymographs were generated using FIJI.

Drug exposure

Wild-type and *aura*¹⁹⁷⁹² embryos were dechorionated using pronase (Pegri and Schulte-Merker, 1999) and exposed to the drug in E3 medium by 15 mpf. Concentrations of inhibitors in E3 medium were: cytochalasin D (Thermo Fisher), 20 µg/ml; phalloidin (P3457), 10 µg/ml; nocodazole (Sigma), 2 µg/ml; and taxol (Sigma), 10 µg/ml. Controls for drug treatment were solutions carrying the same concentration of carrier solvent (DMSO).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.E., F.P.; Methodology: C.E.; Validation: C.E.; Formal analysis: C.E.; Investigation: C.E.; Data curation: C.E.; Writing - original draft: C.E.; Writing - review & editing: C.E., F.P.; Visualization: C.E.; Supervision: F.P.; Project administration: F.P.; Funding acquisition: F.P.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.156596.supplemental>

References

- Abdelilah, S. and Driever, W. (1997). Pattern formation in *janus*-mutant zebrafish embryos. *Dev. Biol.* **184**, 70–84.
- Alberico, E. O., Zhu, Z. C., Wu, Y.-F. O., Gardner, M. K., Kovar, D. R. and Goodson, H. V. (2016). Interactions between the microtubule binding protein EB1 and F-actin. *J. Mol. Biol.* **428**, 1304–1314.
- Allard, J. and Mogilner, A. (2013). Traveling waves in actin dynamics and cell motility. *Curr. Opin. Cell Biol.* **25**, 107–115.
- Ballestrem, C., Wehrle-Haller, B. and Imhof, B. A. (1998). Actin dynamics in living mammalian cells. *J. Cell Sci.* **111**, 1649–1658.
- Behrmdt, M., Salbreux, G., Campinho, P., Hauschild, R., Oswald, F., Roensch, J., Grill, S. W. and Heisenberg, C.-P. (2012). Forces driving epithelial spreading in zebrafish gastrulation. *Science* **338**, 257–260.
- Bement, W. M., Leda, M., Moe, A. M., Kita, A. M., Larson, M. E., Golding, A. E., Pfeuti, C., Su, K.-C., Miller, A. L., Goryachev, A. B. et al. (2015). Activator-inhibitor coupling between Rho signalling and actin assembly makes the cell cortex an excitable medium. *Nat. Cell Biol.* **17**, 1471–1483.
- Berti, C., Fontanella, B., Ferrentino, R. and Meroni, G. (2004). Mig12, a novel Opitz syndrome gene product partner, is expressed in the embryonic ventral midline and co-operates with Mid1 to bundle and stabilize microtubules. *BMC Cell Biol.* **5**, 9.
- Bontems, F., Stein, A., Marlow, F., Lyautey, J., Gupta, T., Mullins, M. C. and Dosch, R. (2009). Bucky ball organizes germ plasm assembly in zebrafish. *Curr. Biol.* **19**, 414–422.
- Braat, A. K., Zandbergen, T., van de Water, S., Goos, H. J. T. and Zivkovic, D. (1999). Characterization of zebrafish primordial germ cells: morphology and early distribution of vasa RNA. *Dev. Dyn.* **216**, 153–167.
- Brand, M., Granato, M. and Nüsslein-Volhard, C. (2002). Keeping and raising zebrafish. In *Zebrafish - A Practical Approach*, Vol. 261 (ed. C. Nüsslein-Volhard and R. Dahm), pp. 7–37. Oxford: Oxford University Press.
- Brangwynne, C. P., Eckmann, C. R., Courson, D. S., Rybarska, A., Hoege, C., Gharakhani, J., Jülicher, F. and Hyman, A. A. (2009). Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* **324**, 1729–1732.
- Burnette, D. T., Manley, S., Sengupta, P., Sougrat, R., Davidson, M. W., Kachar, B. and Lippincott-Schwartz, J. (2011). A role for actin arcs in the leading-edge advance of migrating cells. *Nat. Cell Biol.* **13**, 371–381.
- Cabral-Fontela, Y., Kadavath, H., Biernat, J., Riedel, D., Mandelkow, E. and Zweckstetter, M. (2017). Multivalent cross-linking of actin filaments and microtubules through the microtubule-associated protein Tau. *Nat. Commun.* **8**, 1981.
- Campbell, P. D., Heim, A. E., Smith, M. Z. and Marlow, F. L. (2015). Kinesin-1 interacts with Bucky ball to form germ cells and is required to pattern the zebrafish body axis. *Development* **142**, 2996–3008.
- Comrie, W. A., Babich, A. and Burkhardt, J. K. (2015). F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *J. Cell Biol.* **208**, 475–491.
- Cox, T. C., Allen, L. R., Cox, L. L., Hopwood, B., Goodwin, B., Haan, E. and Suthers, G. K. (2000). New mutations in MID1 provide support for loss of function as the cause of X-linked Opitz syndrome. *Hum. Mol. Genet.* **9**, 2553–2562.
- De Falco, F., Cainarca, S., Andolfi, G., Ferrentino, R., Berti, C., Rodríguez Criado, G., Rittinger, O., Dennis, N., Odent, S., Rastogi, A. et al. (2003). X-linked Opitz syndrome: novel mutations in the *MID1* gene and redefinition of the clinical spectrum. *Am. J. Hum. Genet.* **120A**, 222–228.
- Eno, C. and Pegri, F. (2013). Gradual recruitment and selective clearing generate germ plasm aggregates in the zebrafish embryo. *Bioarchitecture* **3**, 125–132.
- Eno, C. and Pegri, F. (2016). Germ cell determinant transmission, segregation and function in the zebrafish embryo. In *Insights from Animal Reproduction* (ed. R. P. Carreira), pp. 115–142. Rijeka, Croatia: InTech.
- Eno, C., Solanki, B. and Pegri, F. (2016). *aura* (*mid1ip1l*) regulates the cytoskeleton at the zebrafish egg-to-embryo transition. *Development* **143**, 1585–1599.
- Eno, C., Gomez, T., Slusarski, D. C. and Pegri, F. (2018). Slow calcium waves mediate furrow microtubule reorganization and germ plasm compaction in the early zebrafish embryo. *Development* **145**, dev156604.
- Fernández, J., Valladares, M., Fuentes, R. and Ubilla, A. (2006). Reorganization of cytoplasm in the zebrafish oocyte and egg during early steps of ooplasmic segregation. *Dev. Dyn.* **235**, 656–671.
- Fuentes, R. and Fernández, J. (2010). Ooplasmic segregation in the zebrafish zygote and early embryo: pattern of ooplasmic movements and transport pathways. *Dev. Dyn.* **239**, 2172–2189.
- Ganguly, A., Tang, Y. A., Wang, L., Ladit, K., Loi, J., Dargent, B., Leterrier, C. and Roy, S. H. (2015). A dynamic formin-dependent deep F-actin network in axons. *J. Cell Biol.* **210**, 401–417.

- Gaudenz, K., Roessler, E., Quaderi, N. A., Franco, B., Feldman, G., Gasser, D. L., Wittwer, B., Montini, E., Opitz, J. M., Ballabio, A. et al. (1998). Opitz G/BBB syndrome in Xp22: mutations in the MID1 gene cluster in the carboxy-terminal domain. *Am. J. Hum. Genet.* **63**, 703-710.
- Goswami, D., Gowrishankar, K., Bilgrami, S., Ghosh, S., Raghupathy, R., Chadda, R., Vishwakarma, R., Rao, M. and Mayor, S. (2008). Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity. *Cell* **135**, 1085-1097.
- Gowrishankar, K., Ghosh, S., Saha, S., C., R., Mayor, S. and Rao, M. (2012). Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. *Cell* **149**, 1353-1367.
- Hashimoto, Y., Maegawa, S., Nagai, T., Yamaha, E., Suzuki, H., Yasuda, K. and Inoue, K. (2004). Localized maternal factors are required for zebrafish germ cell formation. *Dev. Biol.* **268**, 152-161.
- Ishihara, K., Nguyen, P. A., Groen, A. C., Field, C. M. and Mitchison, T. J. (2014). Microtubule nucleation remote from centrosomes may explain how asters span large cells. *Proc. Natl. Acad. Sci. USA* **111**, 17715-17722.
- Jesuthasan, S. (1998). Furrow-associated microtubule arrays are required for the cohesion of zebrafish blastomeres following cytokinesis. *J. Cell Sci.* **111**, 3695-3703.
- Juliano, C. E., Swartz, S. Z. and Wessel, G. M. (2010). A conserved germline multipotency program. *Development* **137**, 4113-4126.
- Kane, D. A. and Kimmel, C. B. (1993). The zebrafish midblastula transition. *Development* **119**, 447-456.
- Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H. and Nüsslein-Volhard, C. (2000). Zebrafish *vasa* RNA but not its protein is a component of the germ plasm and segregates asymmetrically before Germline specification. *J. Cell Biol.* **149**, 875-888.
- Leung, C. F., Webb, S. E. and Miller, A. L. (2000). On the mechanism of ooplasmic segregation in single-cell zebrafish embryos. *Dev. Growth Differ.* **42**, 29-40.
- Li-Villarreal, N., Forbes, M. M., Loza, A. J., Chen, J., Ma, T., Helde, K., Moens, C. B., Shin, J., Sawada, A., Hindes, A. E. et al. (2015). Dachsous1b cadherin regulates actin and microtubule cytoskeleton during early zebrafish embryogenesis. *Development* **142**, 2704-2718.
- Miranda-Rodríguez, J. R., Salas-Vidal, E., Lomeli, H., Zurita, M. and Schnabel, D. (2017). RhoA/ROCK pathway activity is essential for the correct localization of the germ plasm mRNAs in zebrafish embryos. *Dev. Biol.* **421**, 27-42.
- Mohan, R. and John, A. (2016). Microtubule-associated proteins as direct crosslinkers of actin filaments and microtubules. *Int. Union Biochem. Mol. Biol.* **67**, 395-403.
- Nair, S., Marlow, F., Abrams, E., Kapp, L., Mullins, M. C. and Pelegri, F. (2013). The chromosomal passenger protein Birc5b organizes microfilaments and germ plasm in the zebrafish embryo. *PLoS Genet.* **9**, e1003448.
- Nawaz, S., Sánchez, P., Schmitt, S., Snaidero, N., Mitkovski, M., Velte, C., Brückner, B. R., Alexopoulos, I., Czopka, T., Jung, S. Y. et al. (2015). Actin filament turnover drives leading edge growth during myelin sheath formation in the central nervous system. *Dev. Cell* **34**, 139-151.
- Nott, T. J., Petsalaki, E., Farber, P., Jarvis, D., Fussner, E., Plochowitz, A., Craggs, T. D., Bazett-Jones, D. P., Pawson, T., Forman-Kay, J. D. et al. (2015). Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol. Cell* **57**, 936-947.
- Olsen, L. C., Aasland, R. and Fjose, A. (1997). A *vasa*-like gene in zebrafish identifies putative primordial germ cells. *Mech. Dev.* **66**, 95-105.
- Paksa, A. and Raz, E. (2015). Zebrafish germ cells: motility and guided migration. *Curr. Opin. Cell Biol.* **36**, 80-85.
- Pelegri, F. and Schulte-Merker, S. (1999). A gynogenesis-based screen for maternal-effect genes in the zebrafish, *Danio rerio*. In *The Zebrafish: Genetics and Genomics*, Vol. 60 (ed. W. Detrich, L. I. Zon and M. Westerfield), pp. 1-20. San Diego: Academic Press.
- Pelegri, F., Knaut, H., Maischein, H.-M., Schulte-Merker, S. and Nüsslein-Volhard, C. (1999). A mutation in the zebrafish maternal-effect gene *nebel* affects furrow formation and *vasa* RNA localization. *Curr. Biol.* **9**, 1431-1440.
- Pelegri, F., Dekens, M. P. S., Schulte-Merker, S., Maischein, H.-M., Weiler, C. and Nüsslein-Volhard, C. (2004). Identification of recessive maternal-effect mutations in the zebrafish using a gynogenesis-based method. *Dev. Dyn.* **231**, 324-335.
- Quaderi, N. A., Schweiger, S., Gaudenz, K., Franco, B., Rugarli, E. I., Berger, W., Feldman, G., Volta, M., Andolfi, G., Gilgenkrantz, S. et al. (1997). Opitz G/BBB syndrome, a defect of midline development, is due to mutations in a new RING finger gene on Xp22. *Nat. Genet.* **17**, 285-291.
- Ressom, R. E. and Dixon, K. E. (1988). Relocation and reorganization of germ plasm in *Xenopus* embryos after fertilization. *Development* **103**, 507-518.
- Riemer, S., Bontems, F., Krishnakumar, P., Gömann, J. and Dosch, R. (2015). A functional Bucky ball-GFP transgene visualizes germ plasm in living zebrafish. *Gene Exp. Patterns* **18**, 44-52.
- Roosen-Runge, E. C. (1938). On the early development-bipolar differentiation and cleavage of the zebra fish, *Brachydanio rerio*. *Biol. Bull.* **75**, 119-133.
- Saha, S., Lee, I.-H., Polley, A., Groves, J. T., Rao, M. and Mayor, S. (2015). Diffusion of GPI-anchored proteins is influenced by the activity of dynamic cortical actin. *Mol. Biol. Cell* **26**, 4033-4045.
- Savage, R. M. and Danilchik, M. V. (1993). Dynamics of germ plasm localization and its inhibition by ultraviolet irradiation in early cleavage *Xenopus* embryos. *Dev. Biol.* **157**, 371-382.
- Schaefer, A. W., Kabir, N. and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J. Cell Biol.* **158**, 139-152.
- Strome, S. and Lehmann, R. (2007). Germ versus soma decisions: lessons from flies and worms. *Science* **316**, 392-393.
- Theusch, E. V., Brown, K. J. and Pelegri, F. (2006). Separate pathways of RNA recruitment lead to the compartmentalization of the zebrafish germ plasm. *Dev. Biol.* **292**, 129-141.
- Updike, D. L., Hachey, S. J., Kreher, J. and Strome, S. (2011). P granules extend the nuclear pore complex environment in the *C. elegans* germ line. *J. Cell Biol.* **192**, 939-948.
- Urven, L. E., Yabe, T. and Pelegri, F. (2006). A role for non-muscle myosin II function in furrow maturation in the early zebrafish embryo. *J. Cell Sci.* **119**, 4342-4352.
- Wang, J. T., Smith, J., Chen, B.-C., Schmidt, H., Rasoloson, D., Paix, A., Lambrus, B. G., Calidas, D., Betzig, E. and Seydoux, G. (2014). Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in *C. elegans*. *eLife* **3**, e04591.
- Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., Thisse, C., Thisse, B. and Raz, E. (2003). *dead end*, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. *Curr. Biol.* **13**, 1429-1434.
- Whittington, P. M. and Dixon, K. E. (1975). Quantitative studies of germ plasm and germ cells during early embryogenesis of *Xenopus laevis*. *J. Embryol. Exp. Morph.* **33**, 57-74.
- Winter, J., Lehmann, T., Suckow, V., Kijas, Z., Kulozik, A., Karlsheuer, V., Hamel, B., Devriendt, K., Opitz, J. M., Lenzner, S. et al. (2003). Duplication of the *MID1* first exon in a patient with Opitz G/BBB syndrome. *Hum. Genet.* **112**, 249-254.
- Wühr, M., Dumont, S., Groen, A. C., Needleman, D. J. and Mitchison, T. J. (2009). How does a millimeter-sized cell find its center? *Cell Cycle* **8**, 1115-1121.
- Wühr, M., Tan, E. S., Parker, S. K., Detrich, H. W. and Mitchinson, T. J. (2010). A model for cleavage plane determination in early amphibian and fish embryos. *Curr. Biol.* **20**, 2040-2045.
- Wylie, C. (1999). Germ cells. *Cell* **96**, 165-174.
- Yabe, T., Ge, X., Lindeman, R., Nair, S., Runke, G., Mullins, M. C. and Pelegri, F. (2009). The maternal-effect gene *cellular island* encodes Aurora B kinase and is essential for furrow formation in the early zebrafish embryo. *PLoS Genet.* **5**, e1000518.
- Yoon, C., Kawakami, K. and Hopkins, N. (1997). Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* **124**, 3157-3165.
- Zuchero, J. B., Fu, M.-M., Sloan, S. A., Ibrahim, A., Olson, A., Zaremba, A., Dugas, J. C., Wienbar, S., Capriariello, A. V., Kantor, C. et al. (2015). CNS myelin wrapping is driven by actin disassembly. *Dev. Cell* **34**, 152-167.