aura/mid1ip1L regulates the cytoskeleton at the zebrafish egg-to-embryo transition

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Key Words: Mid1, Mid1ip1L, cytoskeleton, cortical granules, membrane exocytosis, cytokinesis, F-actin, zebrafish, Opitz G/BBB syndrome
Summary

Embryos from females homozygous for a recessive maternal-effect mutation in the gene *aura* exhibit defects including reduced cortical integrity, defective cortical granule (CG) release upon egg activation, failure to complete cytokinesis, and abnormal cell wound healing. Subcellular analysis shows that the cytokinesis defects observed in *aura* mutants are associated with aberrant cytoskeletal reorganization during furrow maturation, including abnormal F-actin enrichment and microtubule reorganization. Cortical F-actin prior to furrow formation fails to exhibit a normal transition into F-actin-rich arcs, and drug inhibition is consistent with *aura* function promoting F-actin polymerization and/or stabilization. In mutants, components of exocytic and endocytic vesicles, such as Vamp2, Clathrin and Dynamin, are sequestered in unreleased CGs, indicating a need for CG recycling in the normal redistribution of these factors. However, the exocytic targeting factor Rab11 is recruited to the furrow plane normally at the tip of bundling microtubules, suggesting an alternate anchoring mechanism independent of membrane recycling. A positional cloning approach indicates that the mutation in *aura* is associated with a truncation of Mid1 Interacting Protein 1L (Mid1ip1L), previously identified as an interactor of the X-linked Opitz G/BBB syndrome gene Mid1. A Cas9/CRISPR-induced mutant allele in *mid1ip1L* fails to complement the originally isolated *aura* maternal-effect mutation, confirming gene assignment. Mid1ip1L protein localizes to cortical F-actin aggregates, consistent with a direct role in cytoskeletal regulation. Our studies indicate that maternally provided *aura/mid1ip1L* acts during the reorganization of the cytoskeleton at the egg-to-embryo transition and highlight the importance of cytoskeletal dynamics and membrane recycling during this developmental period.
Introduction

The egg-to-embryo transition in the zebrafish involves multiple cytoskeletal changes, such as the reorganization of the egg cortex, ooplasmic streaming and the preparation of the cytoskeleton for cell division. The zebrafish egg cortex is composed of a meshwork of actin filaments (Hart and Collins, 1991; Becker and Hart, 1996). During oogenesis, membrane-bound cortical granules (CGs) accumulate throughout this egg cortex (Hart et al., 1987; Mei et al., 2009), and activation of the egg through water exposure triggers rapid granule exocytosis (Hart and Collins, 1991; Becker and Hart, 1999). Upon exocytosis, released CG products promote the lifting and hardening of the chorion (Wessel and Wong, 2009).

Inhibition or stabilization of the actin network lead to, respectively, enhanced or reduced CG release, suggesting that the F-actin cytoskeleton acts as a physical barrier that needs to be remodeled and dismantled to allow for CG release. As CGs exocytose, membrane must be retrieved, presumably through a process such as Clathrin-mediated endocytosis (Bement et al., 2000; Tsai et al., 2011), and indeed both Clathrin and Dynamin have been found to localize to at least a subset of CGs (Faire and Bonder, 1993; Kanagaraj et al., 2014). After exocytosis, F-actin undergoes rapid reassembly to enclose edges of endocytosing crypts at sites of previous exocytotic events (Becker and Hart, 1999).

During embryonic cell division the cytoskeleton is involved in the formation and resolution of cell boundaries between blastomeres (Rappaport, 1996). During furrow formation zebrafish blastomeres form an F-actin-based contractile ring (Urven et al., 2006; Li et al., 2008; Webb et al., 2014), as well as pericleavage actin enrichments on both sides of the furrow, that converge to generate adhesive cell walls. The microtubule apparatus also undergoes stereotypic rearrangements during furrow formation (Jesuthasan, 1998; Urven et al., 2006). At the time of furrow initiation, microtubules derived from spindle asters reorganize into an array of bundles parallel to each other and perpendicular to the furrow, the furrow microtubule array (FMA) (Danilchik et al., 1998; Jesuthasan, 1998). As the furrow matures, FMA bundles become enriched at distal ends of the furrow while acquiring a characteristic tilting angle, pointing distally. Subsequently, distally located FMA bundles undergo disassembly.

Vesicular delivery of structural proteins and membrane is necessary to keep the furrow from regressing (Danilchik et al., 1998; Jesuthasan, 1998; Pelegri et al., 1999; Danilchik et al., 2003), and microtubules act as substrates for the trafficking of Rab11-positive vesicles to the membrane (Takahashi et al., 2012). In the early zebrafish embryo,
SNARE-mediated vesicle fusion required for membrane remodeling during cytokinesis relies on microtubules of the FMA and pericleavage F-actin enrichments (Li et al., 2006).

Here, we characterize the role of *aura* in early embryonic development, in processes that include plasma membrane integrity and CG release after egg activation, cell wound healing and cytokinesis. We show that *aura* encodes Mid1ip1L, a maternally-provided zebrafish homologue of Mid1 Interacting protein 1 (Mid1ip1), a protein that interacts with Mid1, the product of the X-linked Opitz G/BBB syndrome causal gene in humans. Our studies indicate a role for maternal *aura/mid1ip1L* in the reorganization of the cytoskeleton at the egg-to-embryo transition, and highlight the importance of cytoskeletal dynamics and membrane recycling for early embryonic development.
Results

Effects of a mutation in aura at the egg-to-embryo transition

Embryos from females homozygous for a mutation in *aura*, for simplicity referred to here as *aura* mutants, exhibit complete embryonic lethality due to a variety of defects (Pelegri et al., 2004; Fig. 1, Fig. S1A,B). In the most severely affected clutches, a significant fraction of embryos or activated eggs undergo lysis immediately after laying (Fig. 1A,B), suggesting a defect in egg membrane integrity. In eggs that do not undergo lysis, *aura* mutants typically exhibit abnormal yolk morphology. In wild-type, the yolk is present as discrete granules in the mature egg (Fig. 1G), which coalesce during egg activation (Fig. 1C, 2-cell). In *aura* mutants, the yolk resembles that in the mature oocyte (Fig. 1D, 2-cell), suggesting a defect in yolk coalescence. In addition, *aura* mutant eggs and embryos often show pockets of ooplasm trapped between membrane and an indented yolk cortex (Fig. 1E, arrow). Blastodisc lifting, a result of ooplasmic streaming during the first several cell cycles (Hisaoka and Firlit, 1960; Leung et al., 2000; Fernández et al., 2006; Fuentes and Fernández, 2010), is mildly reduced in *aura* mutants (Fig. S2A,B). *aura* mutants also exhibit a mild reduction in chorion expansion and integrity during the first cell cycle (Fig. S2C-E, see below). During oogenesis, yolk granule and CG distribution (Fig. S2F-I) as well as enrichment of F-actin and formation of the mitochondrial cloud (Marlow and Mullins, 2008; Gupta et al. 2010) appear normal (Fig. S3A-D).

Fertilized *aura* mutant embryos exhibit defective cell division. In wild-type, the region between the two central blastomeres (corresponding to the furrow for the first cell cycle) exhibits by the 8-cell stage a clearly visible membrane septum (Fig. 1C, 8-cell), which contributes to cell-cell adhesion. In *aura* mutants, furrows ingress normally, generating a normal cleavage pattern (Fig. 1D, 4-cell, Fig. S3E,F). However, at a time corresponding to the 8-cell stage, when the furrow for the first cell cycle should have undergone completion, *aura* mutants display no clearly defined septum and instead exhibit either the initial rounded morphology corresponding to the ingressing blastomeres or regress (Fig. 1D, 8-cell; S3G,H).

At a time when wild-type embryos are forming a cellularized blastula, *aura* mutants contain irregularly-sized blastomeres and rounded cells at the surface (Fig. 1, 64-cell, Fig. S3G,H). When wild-type embryos display a mass of cells on top of the yolk at the 512-cell and 1000-cell stages (Fig. 1C), *aura* mutants are either fully syncytial or display pockets of cells aggregated atop a syncytial region (Fig. 1D, 256- and 1000-cell), resembling other cell division mutants (Pelegri et al., 1999; Dosch et al., 2004; Yabe et al., 2009). At a time coincident with the initiation of epiboly in wild-type, the syncytial mass in *aura* mutants
expands downward over the yolk (Fig. 1F and Fig. S3I-L), and mutants typically undergo lysis by 4-10 hours post fertilization (hpf).

**Maternal aura function is essential for furrow maturation during late cytokinesis**

During development of the 1st furrow, F-actin is recruited to form of the contractile ring (Fig. 2A,A’, white arrow). In addition, wild-type zebrafish embryos show accumulation of pericleavage F-actin along the furrow (Fig. 2A,A’, arrowheads). As the furrow matures, pericleavage F-actin forms lamella-like structures that converge at the furrow center to form an adhesive cell wall (Fig. 2C,C’). This process is repeated in subsequent cell cycles (Fig. 2E,E’). In aura mutants during furrow initiation (Fig. 2B,B’), F-actin accumulations occur in the contractile ring (arrow) and pericleavage (arrowheads) regions, although the level of F-actin intensity in pericleavage regions appears reduced (Fig. 2B,B’). During furrow maturation pericleavage F-actin in mutants does not converge or form an adhesive wall (Fig. 2D,D’), and lack of blastomere coherence becomes apparent by the third cellular cycle (Fig. 2F,F’; Fig. S3H).

To better visualize cell adhesion junctions, we labeled wild-type and aura mutant embryos to detect β-catenin (Fig. 3). The same embryos were additionally labeled to detect microtubules, which have a role in the exocytosis of vesicles containing cell-adhesion junction components (Jesuthasan, 1998). In wild-type embryos, β-catenin aggregates along the cleavage plane in mature furrows (Fig. 3C-E, arrows). In contrast, β-catenin accumulation is severely reduced in aura mutant embryos (Fig. 3H-J, arrows). Surprisingly, in aura mutant embryos β-catenin localizes to ectopic vesicles present throughout the blastocyst (Fig. 3 F’-J’, asterisks; see below).

The labeled embryos also allowed us to detect potential defects in microtubule reorganization during cytokinesis. During furrow initiation, wild-type embryos organize the FMA as arrays of microtubules parallel to each other and perpendicular to the furrow (Danilchik et al., 1998; Jesuthasan, 1998; Pelegri et al., 1999; Urven et al., 2006; Fig. 3A-D, bracket in C, C’; Fig. S4A, double arrows). As furrows mature, microtubules in these arrays progressively accumulate at the furrows distal ends and tilt their orientation, to form V-shape structures pointing distally (Fig. 3E,E’, bracket; Fig. S4A, arrows). Subsequently, FMA tubules disassemble (Fig. S4A, arrowheads). In aura mutants, FMA microtubules appear to align relatively normally as arrays perpendicular to the furrow (Fig. 3F-I, brackets in H, H’; Fig. S4B, double arrows). However, during furrow maturation, FMA tubules in aura mutants
maintain their original conformation, perpendicular to the furrow and without any apparent bulk distal enrichment (Fig. 3J,J’, brackets; Fig. S4B, double arrows) until they eventually become undetectable (Fig. S4B, dashed double arrows). Although microtubule alignment can appear distorted in furrow regions that contain ectopic vesicles (Fig. 3F-I), defects in FMA reorganization can be detected regardless of the presence of ectopic vesicles (Fig. S4C,D). These observations suggest that *aura* is required for FMA reorganization.

**aura** mutant embryos show defects in cortical granule exocytosis

The size and the large number of vesicles observed in *aura* mutants suggested that they are unreleased CGs (Becker and Hart, 1999), which we confirmed using the glycoconjugate-binding dyes Maclura Pumifera Agglutinin (MPA) and Wheat Germ Agglutinin (WGA) (Becker and Hart, 1999; El-Mestrah and Kan, 2001; Bembenek et al. 2007). Quantification of CG densities in mature, extruded eggs from wild-type and *aura* mutant females indicate that initial CG densities are not significantly different (paired t-test, p-value=0.56). At 2 minutes post fertilization (mpf) (Becker and Hart, 1999), wild-type and *aura* mutants still exhibit a similar density of CGs (Fig. 4A,B). In wild-type, exocytosis of CGs leads to the complete or near complete absence of CGs by 10 mpf (Fig. 4C, 2.0% unreleased, see Methods). In contrast, at 10 mpf *aura* mutant embryos exhibit a significant number of unreleased CGs (Fig. 4D, 53% unreleased), which are observed in the most cortical 12 µm and with highest densities in the region immediately below the cortex (Fig. S5A-D and data not shown). These observations suggest that CGs accumulate normally in developing *aura* mutant oocytes, but experience defective release upon egg activation. A reduction in CG exocytosis can also explain observed defects in chorion expansion and integrity (Fig. S2C-E; Wessel and Wong, 2009).

Previous studies have shown that during oogenesis CGs are embedded in an F-actin network and that CG release is dependent on F-actin dynamics (Becker and Hart, 1999). At 2 mpf, CGs appear similarly embedded in an F-actin network in both wild-type and *aura* mutants (Fig. 4A,B, Fig. S5A,C). At 10 mpf, F-actin in wild-type has undergone a dramatic rearrangement (Fig. 4C’,C’’, Fig. S5B) and appears enriched in cortical punctae that likely correspond to remnants of exocytic events ((Becker and Hart, 1999); Fig. 4C’,C’’). In contrast, unreleased CGs in *aura* mutants at 10 mpf appear encased in an F-actin network similar to that at earlier time points (Fig. 4D,D’,D’’, Fig. S5D). Our observations suggest that *aura* function is required for dynamic F-actin reorganization required for CG release.
**aura** mutant embryos display defects in wound repair

The high frequency of lysed eggs found in severely affected **aura** mutants suggested that **aura** mutants may have a defect in wound repair. To test this, wild-type and **aura** mutant embryos were wounded by pricking the cell membrane with an injection needle at 10-15 mpf. At 30 mpf, nearly all pricked wild-type embryos had fully re-sealed their cell membranes (2% with wounds, n=150) (Fig. 5A,B), whereas nearly all pricked **aura** mutant embryos continued to display signs of wounding (94%, n=243) (Fig. 5D,E).

Previous studies have shown that wound healing of the embryo cell membrane is mediated by the recruitment and contraction of F-actin to the edge of the wound site (Bement et al., 1999; Mandato and Bement, 2001). We therefore fixed embryos 1 minute after wounding and labeled them to detect F-actin. At this time point, wild-type embryos showed small-diameter rings with high levels of F-actin at the wound edge (Fig. 5C). In contrast, similarly treated **aura** mutant embryos exhibit large-diameter cortical openings with reduced levels of F-actin (Fig. 5F). These observations indicate that **aura** function is required for F-actin enrichment at sites of wound repair.

**Distribution of exocytic and endocytic markers is affected in **aura** mutants**

Dynamic membrane release and retrieval, two processes affected in **aura** mutant embryos, are known to be important in CG release (Bement et al., 2000), furrow maturation (Albertson et al., 2005; Li et al., 2006; Albertson et al., 2008; Li et al., 2008; Takahashi et al., 2012) and wound closure (McNeil, 2002; Togo and Steinhardt, 2004; Idone et al., 2008). This led us to characterize the distribution of exocytic and endocytic markers in **aura** mutants. Rab11, a GTPase involved in the docking and fusion of vesicles during exocytosis (Takahashi et al., 2012) required for cytokinesis completion (Pelissier et al., 2003; Giansanti et al., 2007) accumulates at the tips of bundling FMA tubules in wild type embryos (Fig. 6A,A’), and this accumulation is normal in **aura** mutants (Fig. 6B,B’). On the other hand, localization of Vamp2, a marker for docking and/or fusion of membrane vesicles (Conner et al., 1997; Li et al., 2006) is severely affected in **aura** mutants. In wild-type embryos, Vamp2 accumulates along the plane of the mature furrow (Li et al., 2006, Fig. 6C,C’; Fig. S5E,E’). In **aura** mutants, Vamp2 instead localizes to the surface of ectopic vesicles corresponding to unreleased CGs (Fig. 6D,D’; Fig. S5F,F’). Thus, some but not all steps in vesicle exocytosis are dependent on **aura** function.

We also analyzed the localization of two endocytic factors, Clathrin, which forms the lattice structure of endocytic coated-vesicles (Pearse, 1976; Royle, 2006) and Dynamin, a
large GTPase that mediates their scission (Hinshaw, 2000). In wild-type, these factors appear diffusely distributed through the cortex (Fig. 6E,G). On the other hand, in aura mutants Clathrin and Dynamin, like Vamp2, exhibit localization to ectopic CGs throughout the embryo (Fig. 6F,H). In the case of Clathrin, the labeling is relatively diffuse throughout the surface of the CG. Dynamin also exhibits diffuse localization (Fig. S5M,N) but can also be observed to form ring-like structures (Fig. 6H,H’), reminiscent of Dynamin-based rings described in other systems (Hinshaw, 2000). These data are consistent with previous studies that identify Clathrin and Dynamin as components of CGs in oocytes (Faire and Bonder, 1993; Tsai et al., 2011; Fig. S5G,H).

Our results suggest that the defect in CG exocytosis during egg activation, which occurs in aura mutants, results in the sequestering of exocytic and endocytic factors.

**F-actin reorganization defects in aura mutants**

To gain a better understanding of aura function we focused on cortical F-actin reorganization during the first cell cycle (Fig. 7), as this process is amenable to observation and drug treatment and occurs prior to furrow formation, facilitating the interpretation of the phenotype. High magnification views of F-actin in wild-type embryos during the period 5-35 mpf show that cortical F-actin transitions from an initial field of punctate structures at 5 mpf (Fig. 7A,A’), through transient association as aggregates at 10-20 mpf (Fig. 7B,B’,C,C’), to develop band-like structures consisting of F-actin aggregates (27-35 mpf; Fig. 7D,E,E’, arrows). These F-actin bands correspond to previously described circumferential F-actin arcs parallel to the outer rim of the blastodisc (Theusch et al., 2006; Nair et al., 2013b). At later stages (42-63 mpf), which coincide with the period of cytokinesis for the first cell cycle, circumferential F-actin bands continue to be present and a contractile band develops at the site of furrow formation (Fig. 2 and data not shown). In aura mutants, the early cortical F-actin field appears punctate as in wild-type (Fig. 7F,F’). However, instead of undergoing the reorganization observed in wild-type, in mutants the cortical F-actin develops into fine meshwork (Fig. 7G,G’H,H’). This meshwork gradually hollows, forming an increasing area of F-actin-free patches, while F-actin coalesces into lines of single aggregates (Fig. 7J’, arrowheads). Although F-actin bands can also be observed in aura mutants, these exhibit a less ordered orientation with respect to the blastodisc periphery and appear to be less continuous and composed of a smaller number of aggregates than in wild-type (Fig. 7I, arrows). These trends continue at later stages (data not shown).
We also observed cortical F-actin in live wild-type and mutants using the LifeAct transgene (Behrndt et al., 2012). This analysis reveals a dynamic wave-like pattern of cortical F-actin in wild-type, which temporarily coalesces into F-actin bands (Movie S1). *aura* mutants fail to show this dynamic pattern, instead exhibiting the gradual aggregation detected in fixed embryos (Movie S2).

We additionally tested the effect of cytoskeletal dynamics inhibitors on the F-actin network of wild-type and mutant embryos (Fig. 8), in particular whether these drugs modify the phenotype normally observed in an untreated mutant (Fig. 8F,F’). Exposure to the F-actin polymerization inhibitor cytochalasin D dramatically enhances the *aura* cortical F-actin phenotype (Fig. 8G,G’). In contrast, exposure to the F-actin stabilizer phalloidin appears to ameliorate the mutant F-actin phenotype (Fig. 8H,H’, arrow). Exposure to microtubule inhibiting- (nocodazole) and stabilizing- (taxol) drugs do not have major effects on the mutant F-actin phenotype (Fig. 8I,I’,J,J’).

Together, our observations are consistent with a role for *aura* function in the dynamic rearrangement of cortical F-actin, possibly by promoting enhanced actin polymerization or stabilization.

**aura encodes Mid1 Interacting Protein 1L**

We undertook a positional cloning approach to identify *aura* through bulk segregation analysis, using markers polymorphic between the AB/Tü strain that carries the *aur*<sup>9792</sup> mutation and the WIK strain, followed by fine mapping (see Methods). The analysis of key recombination events placed the mutation within a critical region containing 9 predicted genes and identified a polymorphic marker fully linked to the mutation in 700 meioses (Fig. S6A). Sequencing of amplified genomic and cDNA products for genes closest to this marker identified an A to T transversion in the gene *mid1 interacting protein 1L* (*mid1ip1L*). This sequence change results in the conversion of a codon encoding lysine to a stop codon, causing the truncation of the 24 C-terminal amino acids of Mid1ip1L (Fig. 9A,B). The C-terminal region truncated in the mutant *aura* allele deletes over half of a 45-amino acid block that is highly conserved in Mid1ip1 homologues found in various organisms, including human, mouse, frog and chicken (Fig. 9C). Protein prediction analysis (PredictProtein) suggests this mutation occurs within the last helical structure of Mid1ip1L, and mutation-effect predictions (SuSPect) suggest its truncation leads to severe effects on protein function (Fig. S6B).
Attempts to prove gene identity through functional manipulation of in vitro maturing oocytes (Nair et al., 2013a) or early embryos failed to yield conclusive results (data not shown). We therefore induced mutations in mid1ip1L through CRISPR-mediated gene targeting and tested a newly induced allele for complementation against the existing maternal-effect aura (aur<sup>9792</sup>) mutation. A CRISPR-generated mid1ip1L mutant was made by targeting mid1ip1L in an N-terminal region of the Mid1Ip1L protein (see Methods). The resulting CRISPR-generated allele, mid1ip1L<sub>uw39</sub>, has a two base pair insertion after 116 bp of the coding region, which results in a normal protein sequence until amino acid position 39, followed by out-of-frame sequence until amino acid position 90 and translational termination (Fig. 9A; Fig. S6C). Females transheterozygous for this mid1ip1L<sub>uw39</sub> allele and the maternal-effect mutation aur<sup>9792</sup> were generated through natural crosses. The resulting transheterozygous females were, like aura homozygotes, viable and capable of producing embryos. These embryos exhibited a 100% penetrant maternal-effect phenotype essentially identical to that of aura mutant embryos (Fig. 9D-G), indicating that these mutations are allelic. Similar maternal-effect phenotypes were observed in embryos from females homozygous for the mid1ip1L<sub>uw39</sub> allele (Fig. S1 and data not shown). We do not observe any zygotic phenotype associated with the mid1ip1L<sub>uw39</sub> mutation. Altogether, our experiments demonstrate that aura codes for Mid1ip1L.

**Expression of aura/mid1ip1L indicates a predominant role in the early embryo**

RT-PCR analysis suggests aura/mid1ip1L RNA is present at high levels as maternal RNA early in development (up to 3.7 hpf) with increasingly reduced expression at later developmental stages (Fig. 10A,N). Whole mount in situ hybridization to detect mid1ip1L in wild-type embryos corroborated RT-PCR results, with uniformly distributed aura/mid1ip1L RNA present at high levels during early development (2-256 cell stage; Fig. 10B,E,F) and experiencing a severe reduction (Fig. 10G,N and data not shown). Levels and distribution of maternal RNA during the early stages appear similar in aura mutant embryos (Fig. 10D). Previous studies have shown that zygotic mid1ip1L transcription becomes initiated in the pharyngula (24-hr) stage embryo, with specific expression in hatching gland precursor (polster) cells (Thisse et al., 2001).

To detect Mid1ip1L protein expression and localization, we generated antibodies against Mid1ip1L protein regions (see Methods). Western analysis with antibodies against Mid1ip1L protein shows they recognize a single band of the expected size in wild-type but not mutant embryos (Fig. S6D). Immunolabeling of fixed wild-type embryos shows
Mid1ip1L protein is distributed within the cortical F-actin network (Fig. 10H,K), at the developing furrows (Fig 10L) and at induced wounds (Fig. 10M). As expected, labeling is reduced and undetectable, respectively, in mutants for the *aur*<sup>9792</sup> and *mid1ip1L<sup>inv39</sup>* alleles (Fig. 10I,J). High magnification imaging of cortical F-actin reveals punctate Mid1ip1L within F-actin aggregates (Fig. 10K).

In addition to *aura/mid1ip1L*, there are two other *mid1ip1L*-related genes in zebrafish, *mid1ip1a* and *mid1ip1b*. RT-PCR analysis shows that these genes produce maternal transcripts at levels lower than *mid1ip1L* (Fig. 10N). Both *mid1ip1a* and *mid1ip1b* become zygotically active during embryonic development: *mid1ip1a* exhibits a burst of activity at the initiation of epiboly (4.5 hpf; Fig. 10N; Kassahn et al., 2009), and *mid1ip1b* begins expression at the bud stage (10 hpf; Fig. 10N and data not shown). Bioinformatic searches of available databases indicate that vertebrates including humans, mouse, *Xenopus tropicalis* and chicken contain a single *mid1ip1* gene, whereas teleost fish contain three *mid1ip1*-like copies: *mid1ip1(a)*, *mid1ip1b* and *mid1ip1L*. Phylogenetic analysis is consistent with the Mid1ip1B form in fish lineages retaining closer relatedness to tetrapod mid1ip, with functional diversification of the L and A forms in fish lineages (Fig. 10O, Fig. S6E).
Discussion

Here, we show that the gene aura, previously identified as a maternal-effect lethal mutation, corresponds to a mid1 interacting protein 1-family zebrafish gene, mid1ip1L. Our analysis shows that aura mutant embryos exhibit a variety of defects at the egg-to-embryo transition, including CG release, cortical integrity, cytokinesis completion and wound repair, and suggests defective cytoskeletal reorganization as a likely underlying cause for these defects.

Identification of aura as encoding Mid1ip1L and use of Cas9/CRISPR gene editing to confirm gene identification

A positional cloning approach reveals that the maternal-effect mutation in aura is associated with a 24-amino acid C-terminal truncation in the gene mid1 interacting protein1L (mid1ip1L), which in its wild-type form codes for a 164-amino acid protein. The C-terminal region deleted in the maternal-effect aura mutation (auro19792) is highly conserved across mid1ip1 genes in other species, suggesting that the original mutation interferes with protein function.

Corroboration of gene identity is a challenge for maternal-effect genes, as their products typically act immediately or shortly after egg activation (Lindeman and Pelegri, 2012; Nair et al., 2013a,b). Indeed, our attempts to manipulate Aura function during oogenesis led to variable results. In order to fully prove that aura and mid1ip1L are the same gene, we employed the CRISPR/Cas-9 system to generate an additional allele in mid1ip1L. This CRISPR-generated mutation proved to be allelic to aura in a genetic complementation assay. Thus, CRISPR-mediated gene knock-out allowed bypassing underlying difficulties in gene identity confirmation.

Aura/Mid1ip1 is essential for cytoskeletal dynamics at the egg-to-embryo transition

The mid1ip1 gene, also known as MIG12, was originally identified as coding for a protein interactor of Mid1 (Berti et al., 2004). Mid1 encodes a TRIM/RBCC protein implicated in X-linked Opitz G/BBB syndrome in humans, a genetic syndrome characterized by a variety of midline abnormalities (Quaderi et al. 1997; Gaudenz et al., 1998; Cox et al., 2000; De Falco et al., 2003; Winter et al., 2003). Mid1ip1 family proteins are known to associate with the microtubule cytoskeleton and are thought to aid Mid1 in the regulation of microtubule dynamics (Berti et al., 2004), and have also been shown to play a role in the regulation of lipogenesis (Inoue et al., 2011).
Our studies show that maternally provided Aura/Mid1ip1L product is essential for a variety of processes at the egg-to-embryo, including CG release and the completion of cytokinesis (Fig. 10), as well as membrane integrity and repair. One of the processes affected in aura mutants is the restructuring of the FMA during furrow maturation, in agreement with the previously proposed role for Mid1ip1 in microtubule dynamics (Berti et al., 2004). Other microtubule-based processes, such as spindle formation and function, appear unaffected.

Our analysis also suggests a role for Aura/Mid1ip1L in F-actin regulation. Previous studies have shown that CG release depends on the disassembly of the F-actin cortex during egg activation (Becker and Hart, 1999), and the CG release defect in aura/mid1ip1L mutants suggests a role for Aura/Mid1ip1L in this cytoskeletal restructuring. Similarly, aura/mid1ip1L mutants show a defect in the dynamic reorganization of F-actin at the cortex prior to furrow formation and its accumulation at the cleavage plane of during furrow formation, as well as during wound repair. Accordingly, Mid1ip1L protein localizes to F-actin. Together with the effects of inhibitors of actin dynamics in these mutants, our results suggest that Mid1ip1L promotes F-actin reorganization by facilitating F-actin polymerization and/or stabilization. As in the case of the microtubule cytoskeleton, not all F-actin based processes are strongly affected in aura/mid1ip1L mutants: blastodisc cell lifting, known to depend on F-actin (Leung et al., 2000), is only mildly affected, and the initial contraction of the furrow, presumably dependent on the contractile ring, appears unaffected in these mutants.

Our findings are consistent with a previously proposed role for Mid1ip1 in the regulation of cytoskeletal dynamics and expand its targets to include the actin cytoskeleton. We cannot rule out that some of the observed effects are secondary consequences of undetected earlier developmental defects. However, imaging and inhibitor studies, including Mid1ip1L protein localization, argue for a direct role for this factor in F-actin dynamics in the early embryo. Further research will be required to better understand the precise role of Aura/Mid1ip1L in cytoskeletal regulation, as well as its potential connection to Mid1 and Opitz G/BBB syndrome.

**Sequestration of a membrane subcompartment in aura/mid1ip1L mutants**

Embryonic cell division involves a marked increase in surface area, which depends on the exocytosis of internal membrane during cytokinesis. The early defect in aura/mid1ip1L mutants generates the unusual situation in which embryonic processes proceed in spite of the
absence of early CG exocytosis. Instead, the membrane subcompartment corresponding to CGs is arrested in the early embryo (Fig. 11).

Previous studies have shown that in normal embryos, CG release is tightly coupled to membrane endocytosis in order to regulate membrane surface area and reconstitute internal stores essential for membrane addition during embryonic cell division. Exocytic markers such as Vamp2 do not accumulate at the furrow in *aura* mutants, exhibiting instead localization to unreleased CGs. This is consistent with the idea that CGs constitute an early embryonic membrane compartment that is at a later stage reused for membrane addition during cell division. In contrast, the exocytic targeting factor Rab11 exhibits normal localization at the furrow, coincident with the tip of FMA tubules. This finding suggests that Rab11 localization to the furrow occurs through transport along microtubules, consistent with previous studies (Takahashi et al., 2012), and suggests that this transport is independent of membrane compartment association.

Surprisingly, unreleased CGs in *aura* mutants also accumulate membrane proteins involved in membrane endocytosis, such as Dynamin and Clathrin (Hinshaw, 2000; Royle, 2006). In addition to its role in endocytosis, Dynamin has been shown to have a role in the regulation of fusion pore widening after exocytosis (Anantharam et al., 2011). Thus, Dynamin rings observed on unreleased CGs may represent intermediates with stalled exocytosis. Alternatively, the localization of these endocytic proteins to unreleased CGs may reflect that anchors for these factors pre-exist in the CG membrane compartment, in anticipation of immediate endocytosis after CG release (Hart and Collins, 1991; Bement et al., 2000). Indeed, Dynamin has been shown to act in reinternalization mechanism involving direct membrane retrieval after exocytosis (Holroyd et al., 2002; Ryan, 2003).

The CG release defect in *aura/mid1ip1L* mutants results in the sequestration of a membrane subcompartment within the embryo, making it unavailable for subsequent use during cell cleavage. This sequestration likely contributes to the defect in late cytokinesis, which requires membrane addition (Skop et al., 2001; Strickland and Burgess, 2004; Barr and Gruneberg, 2007). The association of the cell-adhesion junction component β-catenin to ectopic CGs in *aura/mid1ip1L* mutants further suggests that CGs may be pre-loaded with factors used at later stages of development, such as anchors for cell-adhesion junction components that may become active during cytokinesis. Wound healing is also known to depend on membrane exocytosis (McNeil, 2002) and, although CGs do not appear to participate in this process (McNeil et al., 2000), aberrant membrane recycling may also contribute to the wound healing defect observed in *aura* mutants.
Diversification of mid1ip1 genes in the zebrafish

The zebrafish genome contains three mid1ip1-like genes. This is somewhat surprising considering expected duplicate copies from a whole genome duplication event that occurred early in the teleost lineage. *mid1ip1b*, whose sequence is most closely related to that of other vertebrate *mid1ip1* genes, is widely expressed in the zebrafish embryo. In contrast, both *mid1ip1a* and *aura/mid1ip1L* have specific patterns of expression: *mid1ip1a* as a temporal spike at the beginning of gastrulation and *mid1ip1L* largely as a maternal transcript also zygotically expressed in hatching gland precursor cells. These expression patterns indicate functional *mid1ip1* gene diversification within the teleost lineage.

In summary, we have determined that a maternal-effect mutation *aura* corresponds to a mutation in *mid1ip1L*, a gene previously known to associate with the Opitz G/BBB syndrome factor Mid1 and thought to contribute to microtubule dynamics. We show that zebrafish *aura/mid1ip1L* function is required for cytoskeletal reorganization at the egg-to-embryo transition, including not only microtubules but also F-actin. In addition, cortical granule sequestration in *aura/mid1ip1L* mutants suggests multiple pathways for exocytic factors and underscores the requirement for membrane recycling in the early embryo.
Materials and Methods

See Table S1 for a list of used primers.

Genetic Methods

Fish were maintained under standard conditions at 28.5°C (Brand et al., 2002). *aur*<sup>9792</sup> was generated in an AB/Tübingen hybrid background (Pelegri et al., 2004). Wild-type stocks were WIK for the mapping crosses and AB for functional analysis. Wild-type embryos are tested as a mixed pool and mutant embryos are tested as separate clutches, in both cases derived from 2 – 4 females. Transgenic lines were EMTB (Tg(EMTB-3GFP)) (Wühr et al., 2010) and Life Act (Tg(actb2:LIFEACT-GFP)) (Behrndt et al., 2012). All animal experiments were conducted according to University of Wisconsin – Madison and Institutional Animal Care and Use Committee (IACUC) guidelines (University of Wisconsin - Madison Assurance number A3368-01)

Positional cloning was carried as described (Pelegri and Mullins, 2011). First-pass mapping with 214 SSLP markers distributed 10 cM apart showed linkage of *aura* to LG 14. A second phase of mapping from genotyped parents narrowed the genomic region to a 1.2 cM interval and a polymorphism from the left end of BAC CR388071.10 to 0.23 Mb (Fig. S6).

Fluorescence Imaging

Embryos were fixed with paraformaldehyde-glutaraldehyde after dechorionation (Urven et al., 2006) and oocyte labeling was carried out as previously described (Gupta et al. 2010). Primary antibodies: Mouse anti-α-Tubulin (1:2500, Sigma), Rabbit anti-β-catenin (1:1000, Sigma), Rabbit anti-Vamp2 (1:200, Abcam), Rabbit anti-Rab11b (1:200, Genetex), Rabbit anti-Dynamin2 (1:100, Genetex), Rabbit anti-Clathrin (1:200, Abcam), Rabbit anti-Vamp4 (1:400, SySy). Mouse anti-Mid1ip1L (1:400, Antibodies-Online) was derived from 3 separate peptides in the middle of Mid1ip1L, thus able to detect *aur*<sup>9792</sup> but not *mid1ip1L<sup>w39</sup>.

For CG analysis, embryos were fixed with paraformaldehyde, dechorionated, washed in PBS/1% Triton X-100 and labeled with Alexa-488 conjugated phalloidin 1hr at room temperature and Alexa-555 conjugated WGA (LifeTechnologies, 1:100) for 30m at 37°C. When co-labeling with anti-Clathrin and anti-Dynamin2, WGA was used first as WGA binds antibodies. Embryos were semi-flat mounted. For F-actin, paraformaldehyde fix contained 0.2U/mL Rhodamine-phalloidin for preservation and imaging. Immunofluorescence data is from at least duplicate trials. Quantification of the CG release phenotype was carried out by comparing the total number of CGs in 60x magnification cortical fields at 0 and 10 minutes.
post activation from the same females (aura: 6 fields/time point; wild-type: 4 fields/time point). Epifluorescence images were carried out using a Zeiss Axioplan 2. Confocal microscopy images were carried out using a Zeiss LSM 510 (fixed images, live movies: low magnification) or Zeiss LSM 780 (live movies: high magnification) and processed with FIJI.

**Drug Treatment**

Wild-type and aur^{9792} embryos were dechorionated, and exposed to drug treatment at 15 mpf. Concentration of inhibitors in E3 medium were: cytochalasin D 20μg/mL, phalloidin 10μg/mL, nocodazole 2μg/mL, and taxol 10μg/mL.

**Histology and in situ Hybridization**

Dissected wild-type and aur^{9792} ovaries were fixed overnight in paraformaldehyde, washed in PBS and dehydrated in EtOH. Ovaries were embedded in paraffin, sectioned, stained, and mounted as described previously (Gupta et al. 2010). mid1ip1L was amplified using wild-type 2-cell embryo cDNA with a forward and reverse primers, the latter including a T7 promoter. RNA was prepared with mMessage Machine (Ambion), and carried out as described previously (Pelegri and Maischein, 1998). Images of labeled embryos and sectioned ovaries were acquired using a Leica-FLIII and Spot Insight. Data is from at least duplicate trials.

**Functional Manipulation and Molecular Methods**

RNA was generated from cDNA product (above) using T7-RNA ULTRA mMessage Machine Kit (Ambion) and poly-A tailed. RNA was mixed with 0.2M KCl injected into oocytes (Nair et al., 2013). The MO sequence (GeneTools) targeting the Mid1ip1L translational start site is 5’ CGTTGCTGAGCTGCATCATGGCAAA 3’. Embryo RNA was extracted with Trizol, with RT-PCR using gene-specific mid1ip1L/a/b and β-actin1 primers.

**Phylogenetic Analysis**

Phylogenetic trees, sequence alignment and phenotypic prediction were carried out using Phylogeny.fr (Dereeper et al., 2008; Dereeper et al., 2010), PRALINE (CIBVU) and SuSPect (Yates et al., 2014), respectively.
CRISPR mutant methods

sgRNA was designed using http://crispr.mit.edu, PCR-amplified with universal primer (Bassett et al., 2013) and in vitro transcribed using T7 Flash kit (Epicentre). Plasmid #46757 pT3S::nCas9n (Jao et al., 2013; Moreno-Mateos et al., 2015) was digested with XbaI, purified and transcribed using T3 mMessage Machine Kit (Ambion). Cas9 RNA and sgRNA were injected at 100 ng/μl and 30 ng/μl, respectively. Injected fish were raised and mated to wild-type fish. The next generation was raised and sequenced (Eno and Pelegri, unpublished). Sequencing in transheterozygotes was carried out through a nested PCR approach.

Acknowledgements

We thank Drs. Martin Wühr and Tim Mitchison (Harvard U.) and Carl-Philippe Heisenberg (IST, Austria) for transgenic lines, and Drs. Moreno-Mateos and Giraldez for their unpublished CRISPR/Cas9 protocol. This work was supported by NIH grant GM065303 to F.P. and NIH F31 grant GM108449 to C.E.

Author Contributions

C.E. performed phenotypic, cytoskeletal, CG release, membrane factor, expression, and wounding analysis, confirmed gene identification, and prepared the manuscript. B.S. performed linkage, cytoskeletal and CG release analysis. F.P. prepared the manuscript.
Figure 1: Developmental time course of *aura* mutant embryos. (A,B) Embryo clutches from wild-type (A) and *aura* (B) mothers at 10 mpf showing egg lysis in mutants. (C,D) Wild-type (C) and *aura* (D) mutant time course. *aura* embryos do not display normal yolk coalescence (red arrow) instead resembling inactive wild-type mature oocytes (G). At the 8-cell a septum is apparent in wild-type (black arrow), whereas *aura* mutants lack this septum (blue arrow). *aura* mutants subsequently exhibit rounded, non-adhesive cells (purple arrow). In the cleavage stages, *aura* embryos are partially or fully syncytial (orange arrows). Other phenotypes include discontinuities between the egg plasma membrane and the yolk along any animal-vegetal position (E, burnt red arrow). Syncytia in *aura* mutants undergo an epiboly-like movement (F, green arrows indicate migrating edge).
Figure 2: *aura* embryos exhibit reduced accumulation of pericleavage and adhesive junction F-actin. (A,A’) In wild-type (25/25), F-actin becomes recruited to the contractile ring (arrows) and pericleavage F-actin regions (arrowheads). (B,B’) In *aura* mutants the contractile ring pericleavage F-actin is reduced (30/30). (C,C’) At the 4-cell stage, the furrow corresponding to the first cell cycle has formed adhesion junctions containing F-actin (white arrow). (D,D’). At the same stage, *aura* embryos do not exhibit adhesion junction F-actin cables (white arrow). (E,E’,F,F’) Defects continue to be observed in subsequent furrows. White and blue arrows indicate furrows for the first and second cell cycles, respectively. (A’-F’) are higher magnification views of (A-F). Scale bars: A,B: 20 μm; C,D,E,F: 100 μm; A’-F’: 10 μm.
**Figure 3:** *aura* embryos do not recruit cell adhesion components to furrows and exhibit aberrant furrow microtubule dynamics. (A-E) In wild-type (12/12), the FMA forms as tubules become arranged in a parallel fashion (A,A’, B,B’). As the furrow matures, FMA tubules become tilted and accumulate at the furrow distal end (E,E’). During furrow maturation, cell adhesion components like β-catenin accumulate at the furrow (C-E). (F-J) In *aura* mutants, FMA tubules form (F-H, brackets in H,H’) but remain in a parallel array arrangement throughout the length of the furrow (J,J’, brackets), failing to aggregate at the furrow distal ends (assayed at 49 – 63 mpf (15/15)). All *aura* mutant embryos examined also fail to recruit β-catenin to the furrow (H-J, arrows). Instead, β-catenin localizes to ectopic CGs (F’-J’, asterisks). (A’-J’) are higher magnification views of (A-J). Scale bars: A-J: 100 μm; A’-J’: 10 μm
**Figure 4: aura mutants retain cortical granules.** (A,B) During egg activation (2 mpf) CGs appear embedded in an F-actin network in both wild-type (A) and aura mutants (B). (C,D) By 10 mpf wild-type eggs have extruded nearly all CGs (C), whereas a large fraction of CGs are retained in aura mutants (D) (chi-square, p-value<0.0001). By 10 mpf, the cortical F-actin cytoskeleton appears as a network of short F-actin in aura mutants (D’’), rather than largely de-assembled in wild-type (C’’). (A’’-D’’) and (A’-D’) are sequentially higher magnification views of (A-D). Scale bars: A-D: 100 μm; A’-D’ and A’’-D’’: 10 μm.
Figure 5: Wound healing defect in *aura* mutant embryos. (A,B,D,E) Embryos from both wild-type (A,B) and *aura* mutant (D,E) mothers were pricked with a glass injection needle at 10-15 mpf and allowed to recover until 30 mpf. After recovery, most wild-type embryos (A,B) have resealed their membrane, whereas a majority of *aura* mutant embryos (D,E) show continued leakage of yolk and ooplasm (asterisks). (C,F) Wild-type (C) and *aura* (F) embryos were fixed 1 minute after wounding and labeled with phalloidin. Wild-type embryos recruit F-actin to the closing wound edge (C, arrows, 7/7). All *aura* embryos examined show reduced F-actin enrichment at the wound edge and a larger wound diameter (F, arrows, 13/13). Scale bar: C,F: 100 μm.
Figure 6: Distribution of exocytic and endocytic factors in *aura* mutants.

(A-D) Exocytic factors Rab11 and Vamp2. (A,B) Rab11 properly localizes to the tips of microtubules at the furrow in wild-type (5/5) and *aura* mutant embryos (6/6). (C,D) Vamp2 localizes to mature furrows in wild-type (5/5) but not in mutants (7/7). (E-H) Endocytic factors Clathrin and Dynamin2 become enriched in ectopic CGs in *aura* mutants (F, F’,H,H’), whereas they exhibit a disperse cortical distribution in wild-type (E, E’,G,G’). Clathrin appears localized to the CG surface in ectopic CGs in *aura* mutants (5/5 embryos, compared to 10/10 wild-type embryos with diffuse cortical labeling). Dynamin2 also localizes to ectopic CGs (15/15 embryos, compared to 13/13 wild-type embryos with diffuse cortical labeling), which can appear as ring-like structures (3/15 embryos as shown in H’) or throughout the granule surface (12/15 embryos, Fig. S5M,M’). Ectopic localization to CGs was confirmed by co-labeling with WGA (not shown). (A’-H’) are magnifications of (A-H). Scale bars: A-H: 100 μm, A’-H’: 10 μm
Figure 7: *aura* embryos do not undergo dynamic actin rearrangement. (A-E) Wild-type embryos undergo dramatic actin rearrangement at the cortex leading to organized actin arcs (arrows); whereas *aura* embryos fail cortical actin rearrangement resulting in a punctate F-actin aggregation (F-J, arrowheads). (A’-J’) are magnifications of (A-J), with ≥ 3 embryos imaged per time point. Scale bars: 10 μm
Figure 8: *aura* phenotype is enhanced by actin polymerization inhibitor and ameliorated by actin stabilizer. In wild-type, cytochalasin D leads to a lack of F-actin arcs (B,B’) (17/17) and phalloidin does not have an effect (C,C’) (23/23). In *aura* embryos, cytochalasin D leads to strongly punctate F-actin (G,G’) (17/17), and phalloidin reverses the phenotype, stabilizing F-actin arcs (arrows, 11/11) (H,H’). Microtubule inhibiting- (nocodazole, D,D’,I,I’) and stabilizing- (taxol, E,E’,J,J’) drugs do not change the dynamic F-actin reorganization in wild-type (16/16 and 15/15, respectively) or *aura* embryos (15/15 and 10/10, respectively). (A’-J’) are magnifications of (A-J). Embryos at 35 mpf. Scale bars: 10 μm
Figure 9: *aura* codes for *Mid1ip1L*. (A) Mid1ip1L protein in wild-type and *aura* mutant alleles. Burn red boxes indicate predicted alpha helices. The *aur*<sup>9792</sup> mutation generates a premature stop that truncates the last conserved helix. CRISPR/Cas9-generated *mid1ip1L*<sup>inv39</sup> allele results in a frame shift translated region (red box) followed by an early stop (A). (B) DNA sequencing trace of the *aur*<sup>9792</sup> allele, creating a stop codon in amino acid 142. (C) Amino acid sequence comparison between various *mid1ip1* homologs in the *aur*<sup>9792</sup> mutation site region. The mutation in *aur*<sup>9792</sup> occurs at highly conserved lysine. Red asterisks in (A) and (C) indicate amino acid affected by the mutations. (D-G) The CRISPR/Cas9-generated *mid1ip1L*<sup>inv39</sup> allele does not complement *aur*<sup>9792</sup>. (D,E) All embryos from *aur*<sup>9792</sup>/*mid1ip1L*<sup>inv39</sup> transheterozygous females (E) exhibit reduced membrane integrity (asterisk), reduced yolk coalescence (arrowhead) and regressed furrows (arrow; see also Fig. S1A,B), whereas control embryos from heterozygous siblings (D) are wild-type. (F,G) 8-cell embryos from transheterozygous females exhibit reduced β-catenin accumulation in mature furrows (27/27, G,G’, arrowheads), ectopic CGs throughout the cortex (G) and apparently stabilized FMA (G’, arrow), compared to control siblings (0/25, F,F’). Scale bars: F,G: 100 μm; F’,G’: 10 μm.
Figure 10: Expression of mid1ip1L and gene phylogeny. (A) Developmental RT-PCR analysis time of mid1ip1L RNA, co-amplifying β-actin RNA as an internal control. mid1ip1L RNA is maternally expressed, with subsiding levels at later stages. (B-G) In situ hybridization analysis: mid1ip1L anti-sense probes label early cleavage stage embryos (B, 25/25,E,F) with a reduction by dome stage (20/20, G). Levels or distribution of mid1ip1L RNA is similar in aura mutants (from aur^9792/aur^9792 females (D)). Control sense RNA (C). (H-J) Mid1ip1L antibodies label cortical F-actin in wild-type (26/26, H). Reduced labeling is observed in aura^9792 embryos (20/20, I) and no localization in mid1ip1Luv39 embryos (21/21, J). (K) Higher resolution imaging of wild-type (from H) shows Mid1ip1L protein localizing as punctae within cortical F-actin aggregates (8/8). Insert in (K) corresponds to the shown field, amplified 2-fold. In wild-type, Mid1ip1L protein becomes enriched to the forming furrow (4/4, L) and induced wounds (4/4, M). Embryos in (H-L) and (M) are at 45 and 15 mpf, respectively. (N) RT-PCR analysis of mid1ip1a, mid1ip1b, and mid1ip1L. RT-PCR data in (A,N) is representative of two trials. (O) Phylogenetic tree for Mid1ip1 protein shows
zebrafish mid1ip1b as most closely related to other vertebrate mid1ip1 genes, and mid1ip1a and mid1ip1L as products of gene duplication. I. scapularis (deer tick) is an outgroup. Numbers in red indicate branch support values, number in black indicates branch scale bar. Scale bars: H-J,L,M: 10 μm; K: 5 μm
Figure 11: Summary of the effects on cytoskeletal and membrane dynamics observed in *aura/mid1ip1L* early mutant embryos. Key processes in early wild-type embryos (left column) and defects in *aura/mid1ip1L* mutants (right column), focusing on cytoskeletal dynamics and exocytosis of internal membrane. In mutants during egg activation, CGs fail to be released, possibly due to the inability to restructure cortical actin. During furrow formation, the microtubule-based FMA fails to reorganize and pericleavage F-actin-rich regions are reduced. Failure of pericleavage F-actin enrichment and sequestration of CG internal membrane in mutants results in the inability to form inter-blastomere adhesive membrane. Exocytic and endocytic components, such as Vamp2, Clathrin and Dynamin2, become localized to unreleased CGs in mutants and unavailable. The exocytic factor Rab11 exhibits normal furrow localization.
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