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SHORT COMMUNICATION

Cortical depth and differential transport of vegetally localized dorsal and germ line determinants in the zebrafish embryo

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ABSTRACT. In zebrafish embryos, factors involved in both axis induction and primordial germ cell (PGC) development are localized to the vegetal pole of the egg. However, upon egg activation axis induction factors experience an asymmetric off-center shift whereas PGC factors undergo symmetric animally-directed movement. We examined the spatial relationship between the proposed dorsal genes *wnt8a* and *grip2a* and the PGC factor *dazl* at the vegetal cortex. We find that RNAs for these genes localize to different cortical depths, with the RNA for the PGC factor *dazl* at a deeper cortical level than those for axis-inducing factors. In addition, and in contrast to the role of microtubules in the long-range transport of dorsal determinants, we find that germ line determinant transport depends on the actin cytoskeleton. Our results support a model in which vegetal cortex differential RNA transport behavior is facilitated by RNA localization along cortical depth and differential coupling to cortical transport.

KEYWORDS. axis induction, cortical rotation, embryo, germ cell determination, germ plasm, RNA localization, vegetal cortex, zebrafish

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INTRODUCTION

One of the main processes that take place during vertebrate embryogenesis is the establishment of the dorsoventral axis. This process has been extensively studied in amphibians through classic experiments involving embryological manipulations.[1,2] In *Xenopus laevis*, these manipulations indicated that shortly after fertilization, dorsal determinants are transported to the future dorsal side of the embryo coincident with the shift of the outer cortex, the "cortical rotation," relative to the entire cytoplasm.[2,3] Subsequent studies have indicated that this process depends on the microtubule cytoskeleton at the vegetal cortex, and in particular the reorganization of vegetal microtubules as long tracks of aligned, parallel bundles. It has been argued that microtubule alignment coupled to motor function causes the relative shift of the cortex, which feeds back to reinforce further microtubule alignment. In *Xenopus*, these tracks of aligned microtubule bundles extend the relatively long span from the vegetal pole to the prospective dorsal region near the animal pole, and visualization of particles, vesicles and fluorescently labeled factors suggest that these tracks may be acting as a substrate for long range transport. This has led to a model in which cortical rotation and microtubule-dependent transport are interdependent processes that together mediate the transport of dorsal determinants.[2] These determinants, through mechanisms that have not been fully determined, result in the activation of the canonical Wnt/β-catenin signaling pathway,[4,5] leading to dorsal gene expression and the induction of the dorsal organizer.[6]

Similar to *Xenopus*, manipulations of the zebrafish embryo have demonstrated that dorsal determinants are maternally supplied and initially localized at the vegetal pole of the unfertilized egg and the one cell embryo, and subsequently translocate to the prospective dorsal side via a microtubule dependent process.[7-9] Early imaging studies in the zebrafish embryo showed that fluorescent polystyrene beads injected at the vegetal cortex of the embryo move animally along a cortical arc in a microtubule-dependent manner.[7] Moreover, this and subsequent studies showed that zebrafish vegetal cortex microtubules, as in *Xenopus*, become reorganized into parallel bundles.[7,10] Visualization of microtubules in live embryos further showed that the direction of these tracks and cytoplasmic granule movement corresponds to the site of dorsal induction.[10] This led to the idea that, as in *Xenopus*, tracks of aligned cortical microtubules mediate the transport of dorsal determinants from the vegetal pole to the prospective site of dorsal induction in blastomeres at the animal pole. These studies painted a picture of translocation of dorsal axis determinants that was remarkably similar to that in *Xenopus*, with dorsal determinant transport dependent on aligned microtubule tracks and an associated cortical shift.

In spite of similarities between *Xenopus* and zebrafish, previous studies have also highlighted potential differences with regards to the contribution of microtubule alignment to the overall dorsal determinant transport process. In particular, the range of product transport and region covered by aligned microtubules is significantly less in zebrafish, where it is confined only to the vegetal-most 30° arc.[10] Moreover, localized products encoded by genes required for axis induction, such as RNAs coding for the Wnt8a ligand and the intracellular protein Hecate/Grip2a, as well as the protein for the kinesin 1 linker Tokkaebi/Syntabulin, experience a similar restricted shift upon egg activation,[11-13] which roughly coincides with the region of microtubule alignment. *hecate/grip2a* function was shown to be required for the reorganization of vegetal cortex microtubules into aligned bundles, as well as the associated movement of these localized factors.[13] These observations indicated the presence of a short range, off-center shift dependent on *hecate/grip2a* function and vegetal microtubule reorganization, which contrasts with the long-range process based on this system as described in *Xenopus*.

Previous studies had shown extensive movement of ooplasm in the zebrafish one-cell embryo, including in cortical mediolateral regions;[14,15] could it be possible that zebrafish embryos rely on a general transport mechanism and a second phase of animally-oriented

transport? This was directly tested in zebrafish embryos using the bead injection assay. Remarkably, beads injected into the vegetal pole of *hecate/grip2a* mutant embryos could still travel animally along mediolateral cortical tracks even though these mutants lack an early short-range asymmetry.[13] Even more surprising, beads injected near the vegetal pole, but at opposite sides of the embryo, were equally able to undergo animally-oriented transport.[13] These observations clearly indicated a transport mechanism at the mediolateral cortex of the early zebrafish embryo that is independent of *hecate/grip2a* function and vegetal microtubule reorganization, and indicated that the entire cortex, and not just that along the prospective dorsal region, is competent for this transport.

These studies have highlighted similarities and differences between mechanisms of dorsal determinant transport in *Xenopus* and zebrafish. In both cases there appears to be a cortical rotation-like process that is associated with microtubule alignment. However, in *Xenopus* this process appears to be an integral part of the mechanism that conveys dorsal determinants to their final location in animal blastomeres, while in zebrafish the end-point transport appears to depend on 2 sequential processes. In zebrafish, an initial short-range phase of transport involves a cortical rotation-like mechanism, dependent on vegetal cortex microtubule alignment, itself dependent on *hecate/grip2a* function.[13] Subsequently, a second, long-range transport phase involves animally-directed movement along the mediolateral region by a mechanism that is neither spatially restricted nor dependent on *hecate/grip2a* function or vegetal microtubule reorganization.[7,13] Thus, the zebrafish embryo constitutes a remarkable example in which a small, random change, such as the orientation of microtubule alignment, leads to an off-center shift that creates an early embryonic asymmetry. This subtle difference is subsequently amplified by a less specific mechanism that generates a larger embryo-wide response.

In this 2-step pathway, the off-center shift of localized factors involved in dorsal axis induction is a key determinative event. However, the zebrafish embryo contains other vegetally localized factors, in particular RNAs that eventually become associated with the zebrafish germ plasm, a cytoplasmic structure containing ribonucleoparticles (RNPs) that determines the germ cell fate. Two germ plasm-associated RNAs, for the genes *deleted in azoospermia-like* (*dazl*) and *Bruno-like*, are originally localized to the vegetal pole of the mature egg.[16,17] Upon egg activation, RNPs containing these vegetally localized RNAs are transported along cortical paths toward the animal pole, where they become incorporated into germ plasm masses.[18,19] These masses are associated with the cellular furrows corresponding to the first and second cell cycles[20] and, due to the alternate pattern of furrow orientation in the zebrafish,[21] become distributed in 4 quadrants of the embryo. Observations indicate that both animally-directed transport of vegetal germ plasm RNPs along the mediolateral cortex and recruitment of these RNPs at the furrows occurs evenly throughout the embryo, without a dorsoventral bias.[19,20,22] This suggests that, in contrast to the asymmetric movement of products involved in dorsal axis induction, animally-oriented translocation of vegetal germ plasm RNPs occurs symmetrically along the mediolateral cortex.

Here, we study the underlying basis of the differential behavior of these 2 types of determinants, namely asymmetric movement of factors involved in axis induction (*wnt8a* and *grip2a* RNAs) and the symmetric movement of vegetal germ plasm RNPs (*dazl* RNA). We use fluorescent in situ hybridization (FISH) to detect these RNAs at the subcellular level and find that they are present in non-overlapping sets of RNPs. Asymmetrically-transported RNPs containing factors involved in axis induction are preferentially found in the outermost cortical region, whereas symmetrically-transported vegetal germ plasm RNPs are enriched in more internal regions of the embryo. In contrast to the proposed requirement for microtubules in the long-range transport of dorsal determinants,[7] we find that animally-directed movement of the germ plasm factor *dazl* is dependent on F-actin function. Thus, a pre-pattern of RNP localization at different cortical depths, coupled to microtubule-based cortical rotation and subsequently implemented by different cytoskeletal systems, underlies the

differential migratory behavior of axis induction and vegetal germ plasm factors.

RESULTS

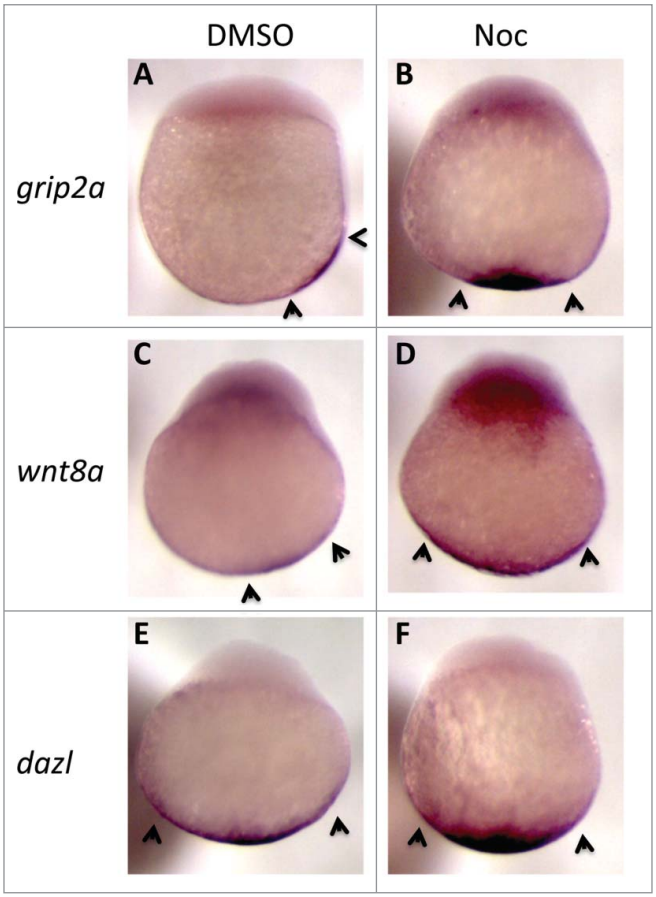
In zebrafish, parallel microtubules become aligned at the vegetal cortex beginning at approximately 14 mpf and start to dissociate at ~26 mpf.[10] Formation of an array of aligned vegetal microtubules is essential for the off-center asymmetric shift experienced by several factors involved in axis induction, such as *wnt8a* RNA,[12] *hecate/grip2a* RNA[13] and Syntabulin protein.[11] In wild type embryos at 10 and 30 mpf, RNA localization domains for *hecate/grip2a* and *wnt8a*, respectively, show an off-center movement, a shift not observed in embryos where the microtubule network is inhibited due to exposure to nocodazole. In contrast, the RNA of the vegetally localized germ plasm gene *dazl* is at this time point also localized at the base of the vegetal cortex but does not experience an asymmetric movement (**Fig. 1**). Instead, vegetal pole localization of *dazl* RNA remains symmetric even as it begins animally-directed transport, and this symmetry is similar regardless of the integrity of the microtubule network.

In order to understand the basis for this differential transport behavior, we examined at the subcellular level the spatial relationship between these RNAs during the first cell cycle (20 mpf, when the microtubule array is maximally aligned and RNP movement is likely occurring). We carried out double fluorescence in situ hybridization to detect pairs of RNAs for axis induction factors (*wnt8a*, *grip2a*) and vegetal germ plasm components (*dazl*) at the vegetal-most embryonic cortex (**Fig. 2**). Two-dimensional projections indicate the presence of transcripts as discreet punctae in the case of *wnt8a* and *dazl* RNAs and a more diffuse pattern albeit with noticeable enrichments in the case of *grip2a* RNA. Overlay of fluorescence channels shows virtually no signal overlap between the 3 RNA pair combinations: *wnt8a/dazl, grip2a/dazl* and *wnt8a/grip2a*. This indicates that these 3 vegetally localized RNAs, in spite of being present in the same general embryonic region, are present in separate RNPs. The observation that *wnt8a* and *grip2a* RNAs are present in different particles indicates that multiple types of RNPs have a role in the process of axis induction.

To better understand the localization of these RNPs at the vegetal cortex, we generated 3-D renderings of these pair-wise comparisons of doubly-labeled fluorescence in situ hybridizations (**Fig. 3**). Co-detection of *wnt8a* and *dazl* RNAs show that *wnt8a* RNA-containing particles tend to be present in the cortical most region, whereas *dazl* RNA-containing particles occur in more internal regions (**Fig. 3A**). Dual labeling of *grip2a* and *dazl* RNAs exhibit a similar pattern, with *grip2a* -containing particles in the cortical most region and *dazl* RNA-containing particles enriched in deeper regions (**Fig. 3B**). In contrast, co-detection of *wnt8a* and *grip2a* RNAs do not show any apparent differential distribution between these RNAs with respect to cortical depth (**Fig. 3C**). We quantified these distributions by binning particles in 3 sub-cortical regions of equal thickness, corresponding to the cortical-most (outer), intermediate, and most internal regions within a region 9 microns below the membrane surface (**Figs. 3D-F**; see Methods). This analysis confirms a statistically significant difference in particle distribution for both the *wnt8a/dazl* and *grip2a/dazl* comparisons, in which *wnt8a* and *grip2a* RNAs are enriched at the outer cortical region and *dazl* RNA is enriched in the inner cortical region. Together, these data indicate that the particles containing dorsal factor RNAs, which experience an asymmetric shift and act during axis induction, are preferentially localized in the outer most region of the cortex, whereas particles containing *dazl* RNA, which do not experience an asymmetric shift, are located in a more internal regions of the cortex.

Animally-directed movement of dorsal determinants toward the animal pole is thought to depend on an intact microtubule network.[7] Given the different initial cortical localization and transport behavior of the germ plasm component *dazl* RNA, we tested the cytoskeletal requirement for its animally-directed movement. Exposure of early embryos to the microtubule inhibitor nocodazole, whose effects were confirmed by a complete inhibition of

FIGURE 1. Differential behavior of localized determinants at the vegetal pole of the embryo.(**A-D**) Asymmetric movement of mRNAs for dorsal factors. Whole mount in situ hybridization shows that *grip2a* and *wnt8a* mRNA are localized at the base of the vegetal cortex and experience an off-center shift in control (DMSO-treated) embryos ((**A**) *grip2a:* 88%, n = 27 ; (**C**) *wnt8a:* 85%, n = 13). Treatment with nocodazole inhibits asymmetric movement ((**B**) *grip2a:* 84%, n = 19, (**D**) *wnt8a*: 85%, n = 13). (**E-F**) Symmetric localization of the germ line *dazl* mRNA. *dazl* mRNA is also localized to the base of the vegetal cortex in control embryos ((**E**) 90%, n = 11) and this distribution appears unaffected by nocodazole treatment ((**F**) 100%, n = 12). Embryos fixed at 40 mpf. Animal pole up, dorsal (when known) right. Arrowheads demark RNA localization domains. (Color figure available online.)

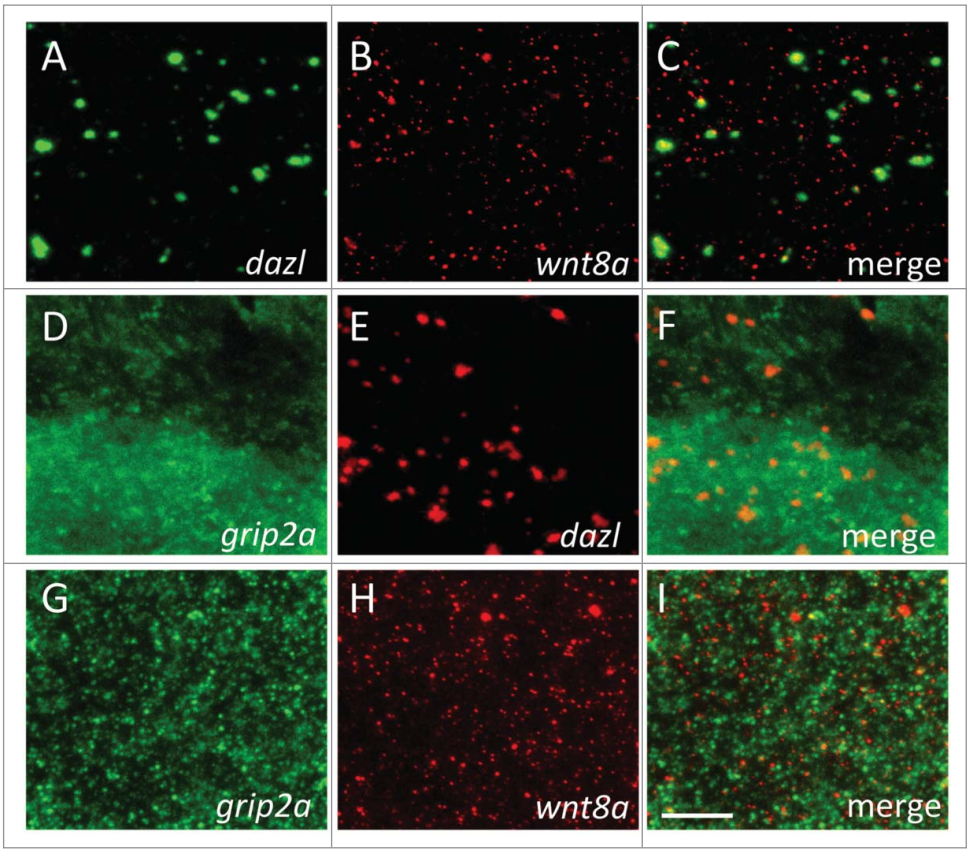


blastomere furrowing, did not have an apparent effect on *dazl* RNA animally-directed movement as judged by the presence of cortical *dazl* RNPs in mediolateral and animal cortex regions (**Fig. 4A,A′ and B,B′** and data not shown). However, *dazl* RNA fails to migrate animally toward the furrows when actin assembly is inhibited using latrunculin A (**Fig. 4C, C′**). These results suggest that the actin cytoskeleton is important for the animally-directed transport of vegetally-localized germ plasm RNPs prior to their recruitment at the distal ends of cleavage furrows.

DISCUSSION

Recent advances have provided new insights on mechanisms of axis induction in amphibians and teleosts, including the identities of primary inducing molecules.[2] In addition, studies have shown that a dramatic reorganization of the microtubule apparatus is required for the

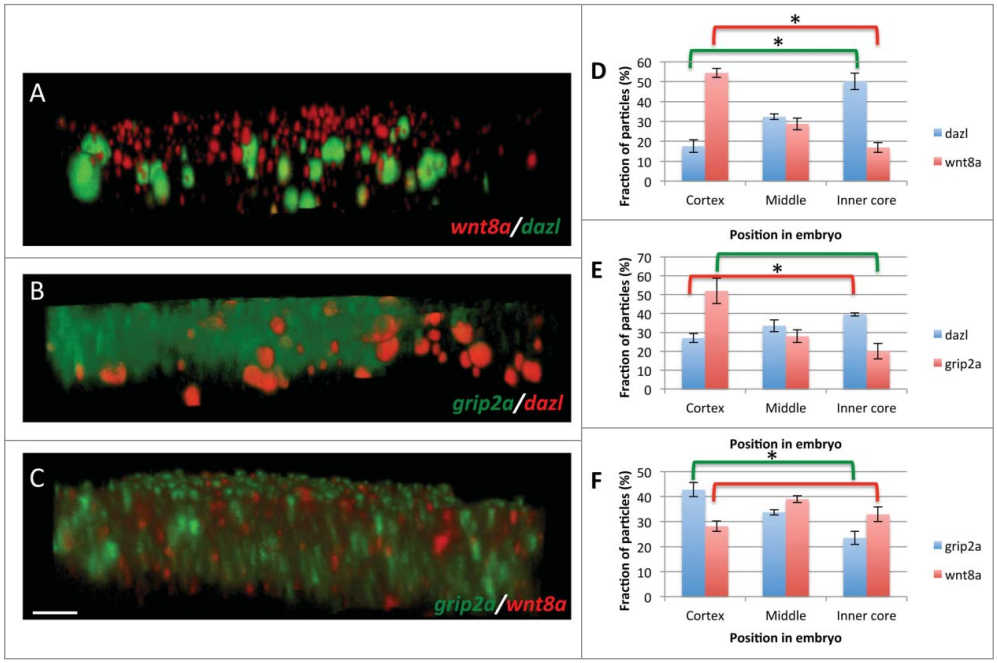
FIGURE 2. Dual label FISH of pairwise comparisons of *wnt8a*, *grip2a* and *dazl* mRNA localization at the vegetal cortex. (**A-C**) *dazl* (green) and *wnt8a* (red). (**D-F**) *grip2a* (green) and *dazl* (red). (**G-I**) *grip2a* (green) and *wnt8a* (red). In all cases mRNAs localize to discrete units, which do not overlap between different RNAs. Embryos are wild type fixed at 20mpf. Panels are 2-D projections of imaged vegetal cortex samples. Scale bar in (**I**) represents 5μm for all images. (Color figure available online.)



movement of these axis-induction determinants from their initial localization at the vegetal pole to the site of dorsal induction in blastomeres at the animal pole.[2] In amphibians, a long history of studies have described a so-called cortical rotation, the movement of the cortex in relation to the inner core of the early embryo, which facilitates the movement of these determinants.[3,27] The cortical rotation was originally identified by the appearance of new cytoplasmic crescent after rotation, reflective of the resulting misalignment of the pigmented animal cortex and the underlying fertilized egg inner core. Later, this cortical rotation was associated with the function of an array of parallel microtubule bundles at the vegetal cortex, which in amphibians spans the length of the arc between the vegetal pole and the prospective dorsal site.[28-30] The spatial extension of this microtubule array, coupled with the observation of movement of vegetal localizing factors to the animal pole region, has led to the postulation that this array is also involved in the long-range transport of dorsal determinants.

Movement of cortical granules toward the prospective dorsal site has also been observed in the early zebrafish embryo, along with the observation of parallel-arrayed microtubule bundles oriented along this movement.[7,10] However, in the zebrafish embryo these bundles span a much less extensive domain

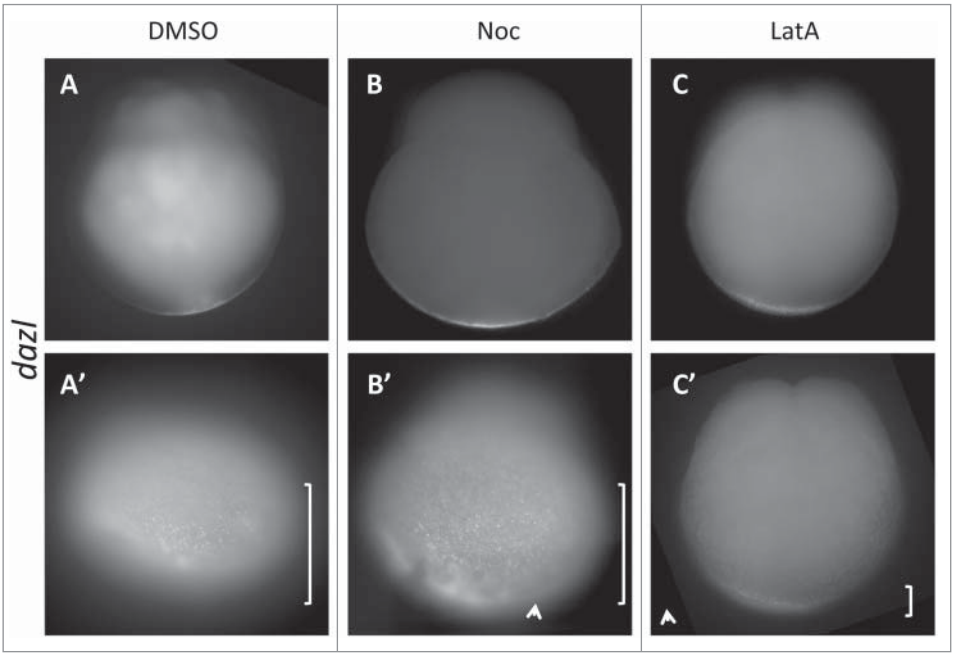
FIGURE 3. Colocalization analysis of *wnt8a*, *grip2a* and *dazl* mRNA localization. (**A-C**) 3-D rendering of dual labeled FISH for pairwise comparisons: (**A**) *wnt8a* (red) and *dazl* (green); (**B**) *grip2a* (green) and *dazl* (red); and (**C**) *grip2a* (green) and *wnt8a* (red). (**D-F**) Quantification of particles according to cortical depth within a 9 μm section of the vegetal half of the embryo divided into outer (0 – 2.997 μm), intermediate (3.087 – 5.994 μm) and most internal (6.084 – 9 μm) (see Methods). Particles containing *wnt8a* and *grip2a* mRNAs are enriched in the outer section whereas particles containing *dazl* mRNA are enriched in the inner section. Brackets indicate comparisons evaluated for statistical significance, with green and red bracket colors corresponding to FISH label. Asterisks indicate statistical significance according to a Comparing Means T-test (StatPlus), with p-values as follows: (**D**) p = 0 .02 and 0.006 for *dazl* and *wnt8a*, respectively; (**E**) p = 0 .001 and 0.07 for *dazl* and *grip2a*, respectively; (**F**) p = 0 .01 and 0.5 for *grip2a* and *wnt8a*, respectively. Scale bar in (**C**) represents 5μm for panels (**A-C**). (Color figure available online.)



encompassing only about a 30-degree arc from the vegetal pole. The remainder of the distance to dorsal blastomeres at the animal pole appears to be dependent on a separate process independent of microtubule reorganization in the vegetal region. This is suggested by the observation that embryos mutant for the *hecate/grip2a* gene, which are defective in vegetal microtubule reorganization, appear unaffected in the animally-directed transport of injected beads along the mediolateral cortex.[13]

In spite of these differences, both amphibian and teleost embryos exhibit an initial symmetry-breaking event dependent on the reorganization of vegetal cortex microtubules and associated cortical movement, which results in the asymmetric localization of dorsal factors such as *wnt8a* and *grip2a* RNAs. This asymmetric transport contrasts with the segregation pattern of a second type of cell determinant also localized to the vegetal pole region, namely germ plasm components. In the case of *Xenopus*, germ plasm RNPs are present in the vegetal pole and segregate into 4 large germ cell-determining masses that remain in the vegetal region of the embryo.[31] In the case of zebrafish, germ plasm components appear to be split into 2 types of particles.[19] One set of zebrafish germ plasm RNPs, containing RNAs for genes such as *vasa*, *nanos1* and *dead end*, is present at the animal pole.[19,20,32,33] A second set of zebrafish germ plasm RNPs, containing

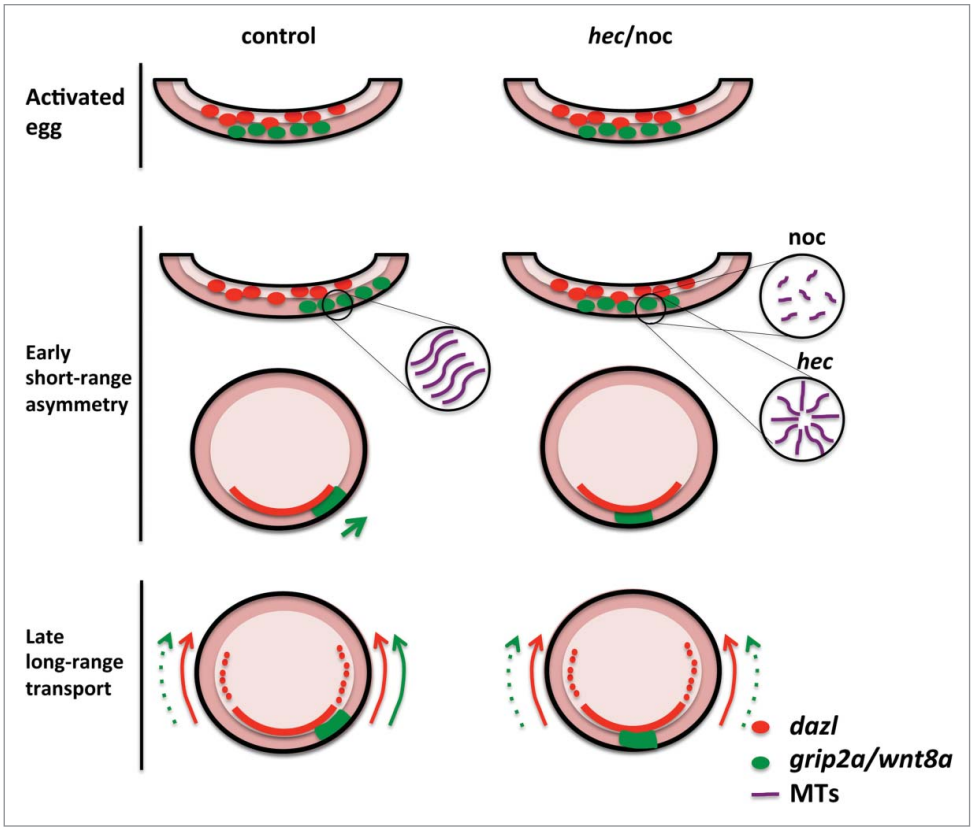
FIGURE 4. Fluorescent in situ hybridization to detect animally-directed movement of *dazl* RNA. (**A-A'**) In DMSO control embryos, *dazl* RNPs are transported toward the animal pole (79% with transport, n = 19). (**B-B'**) Movement toward the animal pole appears unaffected by treatment with nocodazole (86% with transport, n = 21). (**C-C'**) Injection of latrunculin A inhibits the transport of *dazl* RNPs toward the animal pole region (31% with transport, n = 13 ; the effects of injected latrunculin A are expected to be non-uniform due to inhibition of ooplasmic movement and resulting uneven drug distribution, as has been previously observed).[19] Top and bottom panels present different focal planes of the same embryos, focusing on side edge and frontal cortex, respectively. Movement of *dazl* RNA along the cortex is best visualized in the frontal cortex (extent of RNA movement is highlighted by brackets). Nocodazole exposure was initiated at 5 mpf and latrunculin A injection was carried out at 7–12 mpf. All embryos were fixed at 60 mpf. Injection of carrier solvent to control for latrunculin A injection did not affect *dazl* RNP transport (85% with transport, n = 7).



RNAs for genes such as *dazl* and *bruno-like*, is, as in amphibians, initially localized to the vegetal region.[17,26] Upon egg activation, vegetally-localized zebrafish germ plasm RNPs migrate animally to join the animal germ plasm RNPs, to generate germ plasm masses in forming blastomere furrows.[18,19] In spite of species-specific differences, in both systems dorsal and germ plasm factors are initially localized to the vegetal cortex and dorsal determinants undergo an asymmetric movement. Moreover, no dorsoventral asymmetry can be detected in zebrafish *dazl* RNA localization, nor in the size of germ plasm or the number of induced PGCs in neither amphibians nor teleosts.[20,22,31] This implies that, in spite of a similar localization pattern at the vegetal pole, dorsal and PGC determinants undergo different transport mechanisms.

We examine the basis of this differential transport by visualizing at high resolution the vegetal pole localization of RNAs corresponding to dorsal and PGC factors. We find that RNAs for these 3 factors are found in different particles at the vegetal cortex. Moreover, the RNAs for dorsal factors, *wnt8a* and *grip2a*, are enriched in the outermost layer of the cortex, whereas the RNA for the PGC factor *dazl* is present in more internal regions. This observation supports the idea that a cortical rotation-like process is, as in amphibians, involved in generating the asymmetry of dorsal

FIGURE 5. Cortical depth and cytoskeletal-based movements involved in axis induction and germ cell determination in the zebrafish. Top two rows present a magnified view of the vegetal cortex, whereas the bottom 2 rows present embryo overviews. In the activated egg, RNAs for both dorsal-inducing factors and germ line determinants are localized to the vegetal pole at different cortical depths. Upon fertilization, microtubules become aligned into parallel bundles and mediate the off-center movement of outer cortex components with respect to the central core (green short arrow). The differential cortical depth of dorsal (*wnt8a*, *grip2a*, in the outer cortex) and germ line (*dazl*, in a more internal layer) RNAs is coupled to this cortical movement and together these processes contribute to a differential transport: dorsal factor RNAs are transported to generate an early, short-range asymmetry, whereas germ line RNAs remain in a symmetric pattern. Three situations concerning the vegetal cortex microtubule (MT) array reorganization at 20 mpf are presented: normal array formation in the left column, and abnormal reorganization (as in *hecate/grip2a* mutants) or microtubule inhibition (nocodazole-treated embryos) in the right column. Long arrows in the bottom row represent the subsequent paths of long-range factor movement by general transport mechanisms: F-actin-dependent transport of germ cell factors in more internal layers (red arrows) and microtubule-dependent transport of dorsal factors in the outermost cortex (green long arrows).[7,13] Stippled arrows represent long-range transport system that are competent but nevertheless do not contribute to actual factor transport due to the spatial factor distribution resulting from upstream events in the pathway. (Color figure available online.)



determinants in the early zebrafish. As also proposed in amphibians, in the zebrafish embryo the higher density of the yolk cell would be expected to maintain the core of the egg in a stable conformation after a cortical rotation event, so that the movement of the vegetal cortex in relation to the core would result in a shift of cortical components away from the

vegetal-most pole of the embryo, as is observed for dorsal factors. Thus, a location of dorsal determinants in the outer most region of the cortex would facilitate the development of an off-center asymmetry through cortical rotation. On the other hand, an enrichment of germ plasm RNPs, such as those containing *dazl* RNA, in a more internal region would facilitate their being excluded from the shifting cortex and instead allow their symmetric distribution.

A more internal location for vegetal germ plasm RNPs may also facilitate their movement toward the animal pole relying on general mechanisms for the movement of ooplasm components in the early embryo. These mechanisms are currently unknown, although they may be related to those driving animally-directed ooplasmic streaming.[14,15,34,35] Consistent with this idea, our results indicate that mediolateral transport of *dazl* RNPs toward the animal pole is affected by inhibition of the actin cytoskeleton, itself known to be required for ooplasmic streaming.[35] Our results do not rule out alternative possibilities, for example that cortical actin anchors *dazl* RNPs as they are transported toward the animal pole. Our observation that latrunculin A, known to act by making actin monomers unavailable for polymerization, inhibits animally-directed movement of *dazl* RNPs, suggests that dynamic F-actin changes, including filament growth, are required for this process.

In contrast to F-actin inhibition, we also find that inhibition of microtubule function, which interferes with transport of injected beads as markers for dorsal determinants,[7] does not affect animally-directed cortical transport of *dazl* RNPs. Thus, the available data show a correlation for cortical depth and long-range transport cytoskeletal requirement, with dorsal factors using microtubule-based system in the outer cortex, and PGC factors a more internal F-actin-dependent process. However, current studies lack an adequate number of endogenous factors as markers for long-range transport, which would be required to validate these conclusions.

Interestingly, even within the cortical most region we find that *wnt8a* RNA- and *grip2a* RNA-containing particles are distinct, suggesting the presence of multiple type of particles and potentially different mechanisms for asymmetric transport even within the outer cortex. The shift of the entire cortical region through a cortical rotation-like mechanism would insure that all factors of the cortex move coordinately, even if present in different particles. Differences in the cellular anatomies of zebrafish and *Xenopus* eggs and early embryos suggest, however, that a cortical rotation-like process in these 2 species may not be entirely analogous. In particular, the specialized cortex at the animal pole of the meroblastically-cleaving teleost zygote, from which the blastodisc forms,[36] may impose restrictions on global cortical displacement, restrictions that may not be present in the holoblastically-cleaving amphibian zygote. It is possible that a cortical rotation movement in zebrafish occurs locally, solely in the vegetal region. If this is the case, it is unclear how the embryo compensates for such a localized cortical shift, since there appears to be no observable morphological events that reflect such compensation. Alternatively, it is possible that the coordinated movement of vegetal RNAs and particles interpreted as a cortical rotation is in reality an *en mass* movement of such components along a more static cortex, with microtubule tracks providing a substrate for such transport. Further studies will be required to solve these questions. In spite of ambiguities on the precise mode of transport along the outer cortex, the localization of dorsal factor RNAs in the outermost cortical region is expected to facilitate their asymmetric movement.

The differential localization of dorsal and germ plasm determinant RNAs with respect to cortical depth could originate during oogenesis, at a time in which RNAs become localized to the vegetal cortex. Mechanisms of RNA localization during oogenesis have been studied in greatest detail in the *Xenopus laevis* embryo.[2,37,38] In this organism, localization of vegetal RNAs in the developing oocyte occurs primarily through 2 sequential pathways. An early pathway involves the temporary association to a mitochondrial-rich structure, the mitochondrial cloud or Balbiani body, which migrates toward the vegetal pole of the forming oocyte and mediates the transport of RNAs to this region. At a later stage in

oogenesis, a late pathway results in the localization of additional RNAs to the vegetal pole. Interestingly, RNAs localized via these pathways exhibit differential association to the cytoskeleton: upon egg maturation and activation RNAs localized by the early pathway remain anchored to the cytoskeleton, whereas RNAs localized via the late pathway are released into the vegetal cytoplasm.[39] In zebrafish, *wnt8a*, *grip2a* and *dazl* RNAs localize to the vegetal pole during oogenesis through a mechanism dependent on the mitochondrial cloud[12,13,40,41] and thus potentially analogous to the *Xenopus* early localization pathway. These zebrafish RNAs are temporarily localized through similar dynamics by first temporary enrichment at the mitochondrial cloud and subsequently vegetal cortex localization in the mature oocyte.

Three-dimensional ultrastructural analysis has shown that various components of the *Xenopus* vegetal cortex occupy different subdomains within a larger structure. For example, RNA for the *Xenopus* homolog of zebrafish *nanos*, *Xcat2*, is a component of germ plasm granules, whereas *Xdazl* and *wnt11* RNAs are found in the matrix between germ plasm granules.[42] In the zebrafish vegetal cortex, *wnt8a* and *dazl* RNAs exhibit localization confined to discrete RNP units, whereas *grip2a* RNAs is more diffuse though also exhibiting spatial enrichments. Reminiscent of ultrastructural domains in *Xenopus*, we find that RNAs in the early zebrafish vegetal cortex exhibit mutually exclusive localization. It is possible that differences in the cortical depth localization of these RNAs in the early embryo are established during oogenesis and/or egg maturation via independent sets of particles and localization signals. Alternatively, cortical depth localization differences may arise after egg activation, possibly due to differential anchoring of RNPs to the outermost cortex, reminiscent of RNA release observed in *Xenopus* eggs.[39] Further studies will be required to test the origin of RNA cortical depth differences in the vegetal region of the early zebrafish embryo.

The association of germ cell and axial identity is a common occurrence in animal embryonic development, as exemplified by studies in *Drosophila*,[43,44] *C. elegans*[45] and *Xenopus*.[46,47] Moreover, there appears to be functional overlap between factors involved in these processes. For example the germ cell specification factors *Xenopus dead end* and zebrafish *grip2a* have been shown to be involved in the vegetal cortex microtubule reorganization required for axis induction.[13,48] Such shared localization pathways and functional links may be reflective of an ancestral developmental mechanism to insure the coordination of 2 of the most important decisions in the early embryo, patterning of the main body axis and generation of the germ line.[49] However, this coordinated process also needs to be properly regulated to allow its divergence into separate cell determination pathways in the embryo. Our studies reveal specific RNA localization sub-patterning features at the vegetal cortex, including distinct RNP composition and cortical depth localization, which together with a cortical shift transport system facilitate uncoupling 2 key patterning processes, dorsal induction and germ cell specification.

MATERIALS & METHODS

Animal husbandry and embryo collection: All zebrafish 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). Fish stocks were raised and maintained under standard conditions at 28.5°C. Wild type fish were mated and embryos synchronized by collection within 5 minutes of fertilization, and allowed to develop in E3 embryonic medium (E3).[23]

Drug treatment: Nocodazole was prepared as a 5 mg/ml stock in DMSO and used at 1 μg/ml after dilution in embryonic medium. For nocodazole treatment, embryos were collected and treated by immersion at approximately 5 mpf in the drug solution. Latrunculin A was prepared as a 1 mg/ml stock in DMSO and this stock was diluted to 30 μg/ml in water immediately prior to injection, and 1 nl of diluted drug

solution was injected into one-cell embryos between 7–12 mpf.

Whole mount in situ hybridization (WMISH) and Fluorescent in situ hybridization (FISH): WMISH labeled by the standard blue, visible substrate was performed as previously described,[24] using digoxigenin-labeled antisense RNA probes against *wnt8a*,[25] *dazl*[26] and *grip2a*.[13] For dual-label FISH, embryos were labeled using a digoxigenin antisense RNA probe for the genes *wnt8a* and *dazl* and detected using anti-digoxigenin-peroxidase antibody (1:1000) and Cy3 tyramide (1:50), and a fluorescein-labeled antisense RNA probe for the *grip2a* gene and detected using anti-fluorescein-peroxidase antibody (1:5000) and fluorescein tyramide (1:50). The FISH procedure was carried out as described in ZFIN protocol for multiple channel fluorescent RNA in situ hybridization. Isolation of the vegetal cortex region was carried out by manually dissecting fixed embryos with fine forceps to produce vegetal cortex halves. These vegetal cortex fragments were subsequently processed using the dual label FISH procedure.

Imaging and quantitation: WMISH embryos were imaged using a Leica EC3 camera and analyzed with Leica Acquire software. FISH embryos were analyzed by taking vegetal Z-stack images of labeled cortical fragments using a Zeiss 510 confocal LSM by acquiring 100 slices of 0.09 micron thickness spanning the cortical-most 9 micron section from the surface. The resulting images were analyzed using FIJI software. Two-D images were obtained by Z-projection of all slices. Three-D renderings were generated using the 3D viewer plugin in FIJI. Quantification was obtained by binning sections from the Z-stacks into 3 sub-regions of equivalent thickness according to the distance from the cortex, corresponding to 0 – 2.997 microns (most cortical), 3.087 – 5.994 microns (intermediate) and 6.084 - 9 microns (most internal). Z-stacks for each of these sub-regions were used to generate a partial 3-D rendering, in which particles were manually counted. Particles that spanned partial 3-D renderings for 2 adjacent sub-regions were counted only in the sub-region that contained the greater fraction of the particle, after side-to-side comparisons of the partial 3-D renderings.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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