Chapter 1

Reorganization of Vegetal Cortex Microtubules and Its Role in Axis Induction in the Early Vertebrate Embryo

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Additional information is available at the end of the chapter

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Abstract

In vertebrate species, induction of the embryonic axis is initiated by the transport of maternally supplied determinants, initially localized to the vegetal pole of the egg, toward the prospective organizer in the animal region. This transport process remains incompletely understood. Here, we review studies involving embryonic manipulations, visualization, and functional analysis of the cytoskeleton and loss- and gain-of-function conditions, which provide insights in this process. Transport of dorsal determinants requires cytoskeletal reorganization of a vegetal array of microtubules, microtubule motors, and an off-center movement of the vegetal cortex with respect to the inner egg core, a so-called cortical rotation. Additional mechanisms may be used in specific systems, such as a more general animally directed movement found in the teleost embryo. Initial polarity of the microtubule movement depends on early asymmetries, which are amplified by the movement of the outermost cortex. An interplay between microtubule organization and axis specification has also been reported in other animal species. Altogether, these studies show the importance of cytoskeletal dynamic changes, such as bundling, force-inducing motor activity, and regulated cytoskeletal growth, for the intracellular transport of maternally inherited factors to their site of action in the zygote.

Keywords: microtubules, dorsoventral axis, cortical rotation, zebrafish, *Xenopus*, embryo

1. Introduction

One of the main events that take place during vertebrate development is the establishment of the dorsoventral (DV) axis. This process has been studied in a variety of vertebrate species, in particular in the amphibian *Xenopus laevis* and the teleost fish *Danio rerio*. In these model systems, embryological manipulations show that the ligation of the vegetal pole of the freshly

laid egg results in embryos that lack a primary (dorsal) axis (reviewed in Ref. [1]). However, the ligation of the same vegetal region after the second cell cycle does not have this effect. These manipulations allowed to infer the presence of dorsal determinants initially localized to the vegetal pole of the egg, which following fertilization are transported to a more animal region to specify prospective dorsal cells. These determinants, through mechanisms that have not been fully determined, result in the activation of the canonical Wnt/β-catenin signaling pathway, leading to dorsal gene expression and the induction of the dorsal organizer [2–4]. In *Xenopus*, the inferred transport of these determinants is coincident with the shift of the outer cortex, the "cortical rotation," relative to the entire cytoplasm, a shift that is readily apparent due to pigmentation patterns of granules in the cortex.

It has been shown that the process of transport of dorsal determinants is dependent on the microtubule cytoskeleton in the egg cortex, specifically on the reorganization of vegetal microtubules as long tracks of parallel bundles (**Figure 1**, left and center). In *Xenopus*, this array of aligned microtubule bundles extends 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 suggests that these tracks of microtubules may be acting as a

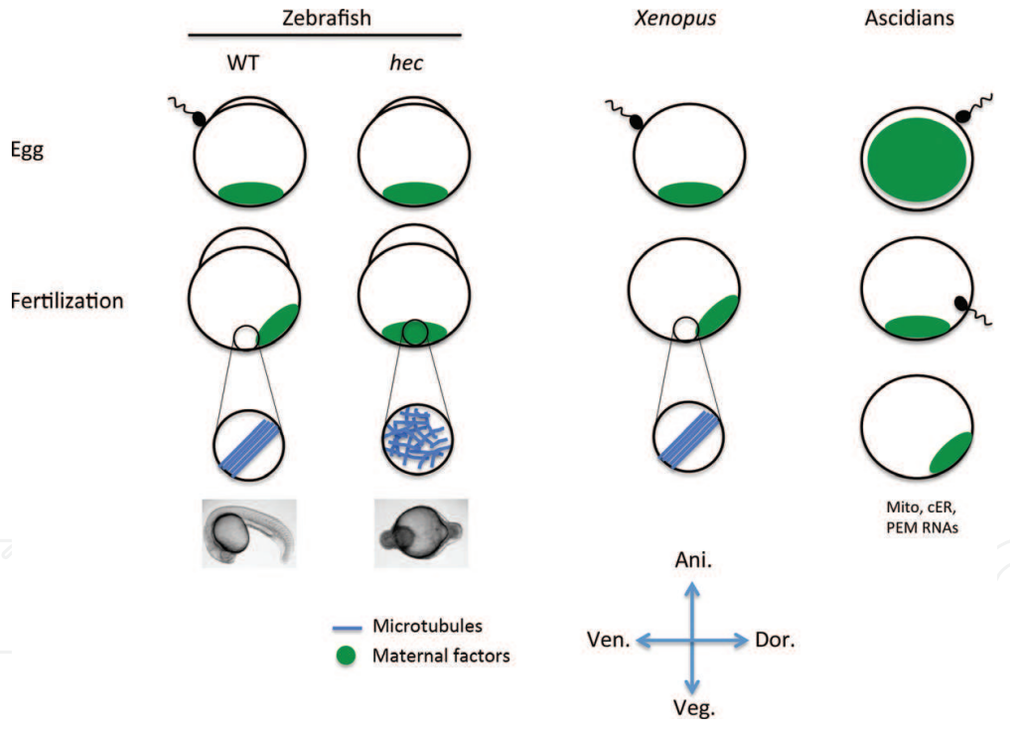

Figure 1. Schematic of early developmental processes in fish, amphibians, and ascidians. Prior to fertilization in zebrafish and *Xenopus* wild-type embryos, maternal factors are localized at the vegetal pole. Upon fertilization, they are transported to the dorsal region via a parallel array of vegetal microtubules. In zebrafish *hecate*/*grip2a* (*hec*) mutants, this vegetal microtubule array is compromised, preventing an initial early off-center dorsal shift of maternal factors, subsequently leading to a ventralized embryo (bottom row, second from left, compared to wild-type at left). A second phase of animally directed transport in zebrafish (not shown) appears to depend on a more general mechanism, independent of vegetal microtubule alignment [26]. In ascidian embryos, first the egg cortex and plasma membrane contract, resulting in the segregation of microfilaments, mitochondria, cER, postplasmic/PEM RNAs, and muscle-forming and endoderm-forming determinants toward the vegetal pole region. These components subsequently move toward the posterior pole through the attraction of a microtubule aster-based center.

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substrate for long-range transport [5, 6]. Early zebrafish embryos do not exhibit an outwardly apparent cortical shift [7], and aligned vegetal microtubule tracks appear to span a more restricted area [6, 8], yet vegetal cortex microtubules may have similar transport functions as in *Xenopus*. Analysis of dynamic changes during microtubule reorganization in the context of the embryo has led to a model in which cortical rotation and microtubule-dependent transport are interdependent processes that together mediate the transport of dorsal determinants (see below) [5]. Forward and reverse genetic approaches in various systems, primarily zebrafish and *Xenopus*, have contributed to our understanding of these processes.

This chapter reviews events involved in the cytoskeletal reorganization required for the movement of determinants leading to axis induction. The outcome of microtubule reorganization in the early embryo is the induction of the dorsal axis, and we first briefly review this process in the zebrafish as well as the amphibian *X. laevis*.

2. Induction signals for axis specification

A primary event in the establishment of the dorsoventral axis in zebrafish and *Xenopus* is the translocation of the normally cytoplasmic protein β-catenin into the nuclei of dorsal blastomeres during cleavage stages (**Figure 2**) [2, 9, 10]. In the absence of Wnt signaling, levels of the cytoplasmic pool of β-catenin are reduced by the activity of glycogen synthase kinase-3 (GSK-3), which promotes β-catenin degradation (reviewed in Ref. [11]). Accordingly, enrichment of β-catenin in dorsal cell nuclei, as well as dorsal axis induction, is blocked by ectopic expression of GSK-3. Activation of the canonical Wnt signaling pathway, resulting in localized inhibition of GSK-3 on the future dorsal side of the embryo, is thought to promote the accumulation of cytoplasmic β-catenin in the prospective dorsal region. Accumulated β-catenin in turn translocates to the nucleus [3, 12], where it can influence gene expression (reviewed in Refs. [13, 14]).

A key mediator of Wnt signaling, when localized to the nuclei, β-catenin acts as a transcriptional effector to activate dorsal-specific genes such as *bozozok/dharma*, *nodal-related 1*, and *chordin* [13]. Products expressed from these dorsal genes along with those from their targets antagonize ventralizing signals such as bone morphogenetic proteins (BMPs), thus promoting dorsal cell fate specification (reviewed in Refs. [14, 15]). Failure of nuclear β-catenin to localize to the nuclei of dorsal blastomeres results in the ventralization of embryos [16].

The intricacies of the Wnt signaling pathway and its role in vertebrate axis induction have been determined through many studies, including functional manipulation of genes through ectopic expression and knockdown or expression of dominant-negative constructs (reviewed in Refs. [11, 17]). For example, overexpression of β-catenin induces a secondary axis in *Xenopus* embryos [18]. When β-catenin is overexpressed in zebrafish embryos, it is able to induce the expression of target genes such as *goosecoid* and *ntl* [19]. Similar results have been observed with the overexpression of some Wnt ligands [20], including overexpression of Wnt8 and Wnt8b in zebrafish embryos [19].

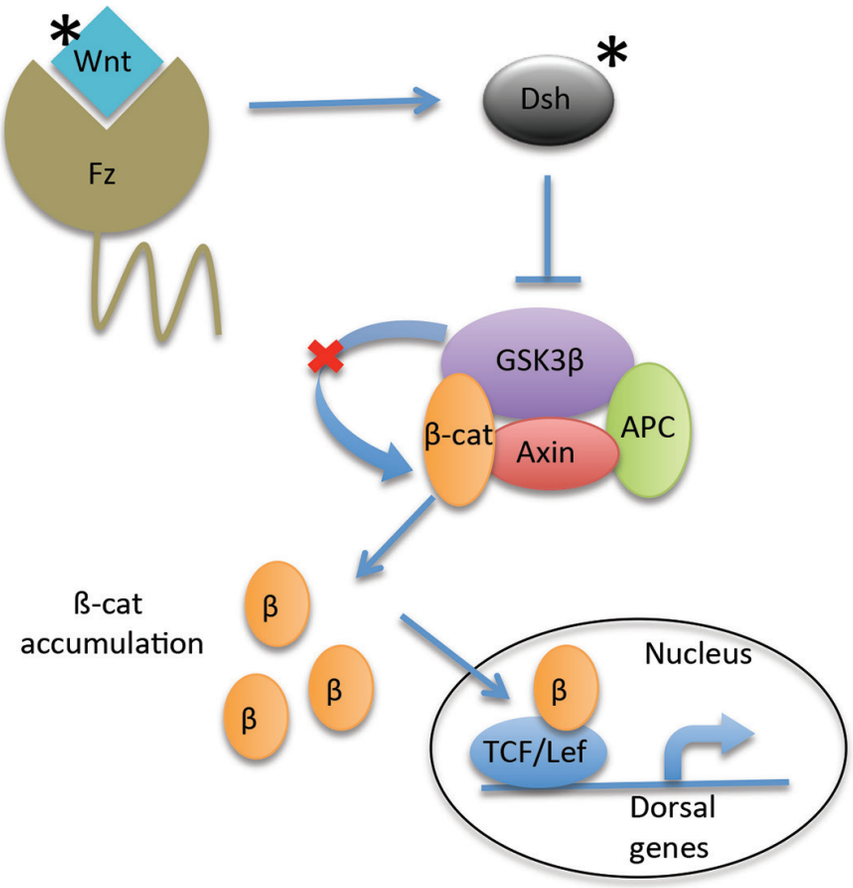

Figure 2. Simplified diagram of Wnt activity involved in axis specification. In the canonical Wnt signaling pathway, extracellular Wnt protein ligands signal through Frizzled transmembrane receptors to activate the cytoplasmic protein dishevelled (Dsh). Dsh in turn inhibits GSK-3 activity. GSK-3 is part of a complex that normally destabilizes β-catenin protein; hence, inhibition of GSK-3 activity results in β-catenin stabilization and its translocation into the nucleus. β-Catenin forms a complex with the transcription factor Tcf/Lef to activate dorsal-specific gene expression. Asterisk denotes factors thought to undergo a translocation to the prospective dorsal site through a process involving cortical rotation and/or microtubule-dependent transport.

Several mutations in zebrafish have allowed for the confirmation of an endogenous role for Wnt signaling pathway in axis induction in the early embryo. An identified recessive maternal-effect mutant in *ichabod* results in defective dorsal organizer formation and severe ventralization and shows impaired nuclear localization of maternal β-catenin protein [21]. This mutation was found to be closely linked to β-*catenin-2* (ctnnb2), a duplicate copy of the β-catenin gene located on a different linkage group from the previously characterized β-*catenin-1* [22]. It was shown that although the *ichabod* mutation does not functionally alter the β-*catenin-2* open reading frame, the level of maternal β-*catenin-2* transcript (but not that of the unlinked β-*catenin-1* gene) is substantially lower in *ichabod* mutant embryos. Reduction of β-*catenin-2* function in wild-type embryos by the injection of a gene-specific morpholino antisense oligonucleotide (MO) results in ventralized phenotypes [22], which are similar to those seen in *ichabod* mutant embryos. In contrast, MOs directed against β-*catenin-1* have no ventralizing effect on wild-type embryos. These data strongly suggest

that the *ichabod* mutation corresponds to the β-*catenin-2* gene, providing genetic evidence for the role of this factor in axis induction. These results indicate that activation of Wnt signaling via the stabilization of β-catenin is essential for proper organization of the embryonic axis.

Activation of the Wnt/β-catenin signaling pathway, as well as its important role in the expression of dorsal genes, has been extensively studied in a number of cellular systems (reviewed in Refs. [11, 17]). However, the identity of the molecules thought to activate the pathway in early vertebrate embryos, referred to as dorsal determinants, remains to be fully elucidated. Wnt11 has been proposed to be a dorsal determinant in amphibian species [23]. In *Xenopus*, *wnt11* mRNA is located at the vegetal pole of the mature egg and, after fertilization, becomes enriched at the future dorsal side of the embryo. Thus, the localization of *wnt11* RNA exhibits the expected behavior of the inferred dorsal determinant, as predicted from transplantation of the dorsal-inducing activity. Additionally, it was shown that depletion of *wnt11* mRNA from oocytes results in embryos defective in dorsal axis induction [24]. Further studies have implicated ubiquitously present Wnt5 as acting together with Wnt11 in Wnt/β-catenin activation [23]. Studies in zebrafish have not implicated Wnt11 or Wnt5 function in axis induction. However, a role for zebrafish Wnt8a has been suggested in this process [25, 26]. Similarly to *Xenopus wnt11*, zebrafish *wnt8a* mRNA is localized to the vegetal pole of the egg and can be observed to translocate after fertilization toward the animally located blastomeres. These studies also indicate that, while Wnt/β-catenin pathway activation may be highly conserved in axis induction across the animal kingdom, there are variations in maternally based mechanisms leading to pathway activation.

Studies in *Xenopus* and zebrafish also showed that the transport of dorsal determinants, which results in the translocation of β-catenin to the nuclei of dorsal blastomeres, requires an array of parallel microtubules originating in the vegetal pole region [6, 27]. Miller and colleagues investigated the mechanisms responsible for the dorsal activation of the Wnt signaling pathway in *Xenopus* eggs and the subsequent specification of dorsal cell fates in the embryo. It was shown that dishevelled (Dsh) protein, a cytoplasmic component of the Wnt pathway that functions upstream of β-catenin [28], is associated with vesicle-like organelles that become enriched in the prospective dorsal side of the egg at the end of the first cell cycle and that the accumulation of Dsh persists through early cleavage stages [27]. Further experiments revealed that when embryos were UV irradiated at the vegetal hemisphere, the distribution of Dsh was blocked, which also blocked dorsal axis formation. Subsequently, when observing the subcellular localization of Dsh fused to GFP, it was revealed that during cortical rotation Dsh-GFP is translocated toward the future dorsal side via the vegetal cortex microtubule array [27]. Together, these data suggest a model in which dorsal-determining factors including *wnt* gene products and Dsh protein are transported via a microtubule-dependent pathway to the future dorsal side of the embryo, leading to the localized activation of the Wnt signaling pathway, the accumulation of β-catenin in dorsal blastomeres, and the induction of dorsal cell fates [27].

3. Transport of dorsal determinants in *Xenopus* and zebrafish

3.1. Molecular mechanism underlying cortical rotation

As mentioned above, embryological manipulations showed that, both in *Xenopus* and zebrafish, dorsal determinants are localized to the vegetal pole of the egg at the time of fertilization but have within several cell cycles moved to an animal region where they influence cell fate. Spatial changes that lead to these determinants translocating to the prospective dorsal region appear to be facilitated by two processes: the rotation of the zygote cortex with respect to the core during cortical rotation and the intracellular movement of factors (e.g., *wnt* RNA or Dsh-bound vesicles) along aligned vegetal microtubules. These are likely intertwined processes, as tracks of parallel microtubules appear to be required not only for the movement of vegetal factors to the prospective dorsal side but also for cortical rotation [29]. Treatment of the vegetal portion of embryos to prevent microtubule polymerization, such as exposure to nocodazole, cold shock, hydrostatic pressure, or UV irradiation [30, 31], shows that microtubules are required for cortical rotation in normal conditions [31]. In contrast, cytochalasin D, an inhibitor of actin polymerization, does not interfere with cortical rotation, indicating that microfilaments are not required for this process. Inhibition of protein synthesis with cycloheximide, known to have dramatic effects such as cell cycle arrest [32], also does not inhibit rotation, indicating that the control of cortical rotation is posttranslational and depends on preformed maternal proteins [32].

Failure of cortical rotation results in a ventralized mutant phenotype in the embryo. However, in embryos treated to inhibit microtubules, a cortical rotation can be artificially induced by gravity after immobilizing the embryo in a matrix and physically turning it 90°. This manipulation results in the formation of dorsal structures, albeit delayed [33]. Under these conditions, gravity leads to a rearrangement of the heavier yolk-containing core of the embryo relative to the cortex. This is thought to increase the proximity of vegetally localized cortical signals to internal regions in the more animally located prospective dorsal region. The ability of the entire cortex to move as a whole relative to the embryonic core contrasts with the visualization of moving particles along microtubule tracks. These observations suggest that both transport along cortical microtubules and a cortical shift relative to the embryonic core contribute to the redistribution of signals involved in axis induction during the early embryonic cell cycles. We subsequently address each of these processes.

3.2. Relocalization of RNA determinants during oogenesis and early embryogenesis

The mRNA for the putative zebrafish dorsal determinant *wnt8a* is localized to the Balbiani body during oogenesis. The zebrafish Balbiani body [34] is a mitochondria-rich subcellular structure in the forming oocyte shown to be essential for the creation of animal-vegetal polarity in the oocyte. This structure, thought to be homologous to the early messenger transport organizer (METRO) pathway of localization in *Xenopus* [35], constitutes a crucial component of a vegetally directed transport pathway that entraps mRNAs and other gene products necessary for patterning of the embryo and germ cell formation [34, 35]. Association of *wnt8a*

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RNA with the Balbiani body leads to the localization of this RNA to the vegetal pole of the mature zebrafish oocyte [25]. Thus, fertilized embryos initiate development with *wnt8a* RNA localized to the vegetal pole. However, in wild-type embryos starting at 30 min, this mRNA experiences an asymmetric movement toward a more animal region that will become the prospective organizer region [25, 26].

In addition to *wnt8a*, genetic studies in zebrafish have allowed the identification of other maternally inherited factors involved in the transport of determinants essential for dorsal axis induction, such as *hecate*/*grip2a* mRNA and Tokkaebi/Syntabulin proteins. Molecular characterization of the three independent mutant alleles of the zebrafish maternal effect gene *hecate*/*grip2a* shows that loss of function for its product results in embryos with reduced dorsal gene expression and concomitant defects in forming dorso-anterior structures [26]. Similar effects are caused by a single mutation in *tokkaebi* [36]. Mutations in genes coding for either *hecate*/*grip2a* or *tokkaebi*/*syntabulin* do not interfere with vegetal pole localization of *wnt8a* RNA during oogenesis, but abolish the animally directed asymmetric movement of this RNA that normally occurs after fertilization [25, 36, 37]. Given the proposed role for Wnt8a as the dorsal determinant in zebrafish [25], the postfertilization defect in *wnt8a* RNA asymmetric movement in *hecate* and *tokkaebi* mutants explains axis induction defects observed in these mutants.

Positional cloning of *hecate* shows that this gene encodes glutamate receptor-interacting protein (Grip) 2a, a factor whose *Drosophila* homologue protein is associated with membrane vesicles in postsynaptic neuronal cells, where it acts in the reception of Wnt signals across the synapse [38]. Zebrafish Grip2a protein has four PDZ domains, which are known to interact with membrane-associated factors including members of the Wnt signaling pathway. Mutant alleles in this protein exhibit a range of phenotypes whose severity roughly correlates with the extent of unaffected protein, with the strongest allele causing a premature stop codon that truncates the Grip2a protein, removing all four PDZ domains [26]. The mutation in *tokkaebi* corresponds to syntabulin, which codes for a linker of the kinesin I motor protein [36], and acts as a linker molecule that attaches mitochondria to the kinesin-1 motor, thereby contributing to anterograde trafficking of mitochondria to neuronal processes [39]. The known roles for Grip and syntabulin in the transport of membranous organelles and signaling in neuronal types begin to draw similarities between microtubule-based transport of vesicles in neurons and the transport of dorsal determinants, also thought to at least partially associate with vesicles (as highlighted by Dsh-GFP movement [27]), in early vertebrate embryos.

Consistent with the effect of maternal-effect mutations in *hecate*/*grip2a* and *tokkaebi*/*syntabulin* on the formation of dorsal structures, products of these genes are localized in patterns that likely facilitate the movement of dorsal determinants [26, 36, 40]. In wild-type embryos, *grip2a* mRNA, like *wnt8a*, is localized via a Balbiani body-dependent mechanism to the vegetal pole of the oocyte and early embryo, and following egg activation and fertilization, the localization of this mRNA shifts off-center about 30° from the vegetal pole. During oogenesis, as in the case of *grip2a* RNA, *syntabulin* RNA becomes localized to the vegetal pole of the oocyte via a Balbiani body-dependent pathway, resulting in the localization of both syntabulin mRNA

and protein to the vegetal pole of the egg. After fertilization, as in the case of *wnt8a* RNA and *grip2a* RNA, Syntabulin protein (but not its RNA) exhibits an off-center shift upon egg activation [36]. The off-center shift from the vegetal pole exhibited by *wnt8a* and *grip2a* mRNAs and Syntabulin protein roughly corresponds to a 30° arc offset from the vegetal pole that contains an aligned set of arrayed microtubules in the zebrafish embryo and which has been observed to contain moving subcellular particles [8]. Thus, the movement of these mRNAs roughly corresponds to a region in the teleost early embryo thought to undergo mass movements toward the future dorsal side, reminiscent of the amphibian cortical rotation. The coordinated asymmetric movement of vegetally localized products such as *wnt8a* RNA, *grip2a* RNA, and Syntabulin protein is consistent with the observed mass transport of particles in the vegetal cortex [8], although they may also reflect specialized transport mechanisms involving microtubule tracks, Syntabulin-mediated motor movement, and *wnt8* RNA- and *grip2a* RNA-containing RNPs.

Genetic analysis indicates that the Hecate/Grip2a and Tokkaebi/Syntabulin products are required for the off-center, asymmetric shift of vegetally localized determinants that follows fertilization. *hecate*/*grip2a* mutants show defects in this off-center movement for vegetally localized products such as *wnt8a* RNA and Syntabulin protein, as well as *grip2a* RNA itself [26]. Mutations in *tokkaebi*/*syntabulin* also result in defects in *wnt8a* RNA and Syntabulin protein asymmetric movement [36]. However, in both of these mutants, localization of dorsal factors (*wnt8a* RNA, *hecate*/*grip2a* RNA, and *tokkaebi* products) during oogenesis remains unaffected. Localization of these factors during oogenesis is instead dependent on the function of buckyball [25, 26, 36], a novel protein required for Balbiani body formation [34, 41]. Thus, localization of dorsal factors to the vegetal pole of the oocyte relies on a Balbiani body-dependent pathway, and the asymmetric movement of these factors after fertilization, which is required for axis induction, depends on the subsequent action of *hecate* and *tokkaebi.* As discussed below, these functions rely on microtubule-dependent reorganization and transport processes.

Additional studies have shown that, as in *Xenopus*, zebrafish vegetal cortex microtubules become reorganized into parallel bundles (**Figure 3**) [6, 8]. The studies paint a picture of translocation of dorsal axis determinants that is remarkably similar to that of the known *Xenopus* cortical rotation. However, transport of dorsal determinants in zebrafish appears to use a dual system, in which microtubule alignment initiates an off-center shift, and other cytoskeletal processes mediate long-range transport (see below). In spite of observed differences, these studies show that microtubule-dependent transport of dorsal determinants plays an essential role in canonical Wnt pathway activation and dorsal axis determination in teleost embryos, as in amphibians.

Interestingly, the RNA for the *grip2* homologue in *Xenopus*, *XGRIP2* is, like its zebrafish homologue *grip2a*, localized to the mitochondrial cloud (the Balbiani body in zebrafish) during *Xenopus* oocyte development and subsequently to the vegetal pole of the mature oocyte. However, in contrast to zebrafish *grip2a* RNA, *Xenopus XGRIP2* RNA does not have an apparent role in axis induction, and after fertilization its RNA becomes localized to germplasm masses that coalesce in the embryo (see below) [42–44].

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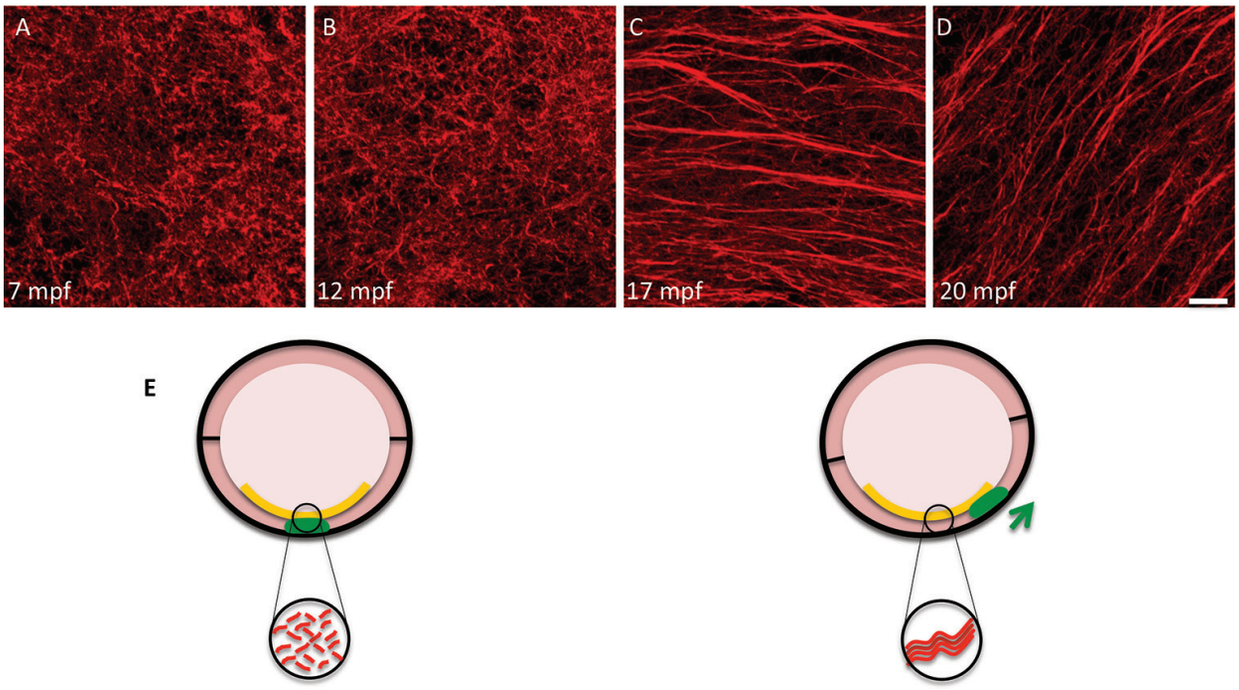

Figure 3. Alignment of microtubules at the vegetal cortex in wild-type zebrafish embryos. (A–B) Between 7 and 12 min postfertilization (mpf), microtubules at the vegetal cortex start to become reorganized to form parallel bundles. (C–D) Around 17 mpf, microtubules become organized into parallel bundles. This organization facilitates the movement of dorsal determinants from the base of the vegetal pole to the dorsal region. Scale bar in D represents two microns for all panels. (E) Diagrammatic representation of dorsal determinants (green) with respect to more internally located determinants, such as vegetally localized germplasm determinants in the zebrafish (orange), depicting microtubule reorganization (red lines), before (left) and after (right) cortical rotation.

Altogether, these studies indicate key roles for RNA localization pathways during oogenesis leading to the localization of factors required for axis induction to the vegetal pole of the egg. Initially localized to the vegetal pole through the action of the mitochondrial cloud during oogenesis, after fertilization and egg activation these factors exhibit an off-center shift dependent on the function of vegetally localized factors, such as Grip2a and the kinesin motor adaptor protein Syntabulin.

3.3. Reorganization of microtubules during cortical rotation

At least in the case of *Xenopus*, it is clear that the rotation of the cortex facilitates the relocation of dorsalizing factors from the vegetal pole to the presumptive future dorsal side or to a more animal (equatorial in the case of *Xenopus*) region, where they act to initiate gene expression programs corresponding to the body axis, at a signaling center known as Spemann's organizer [45]. Following fertilization in the *Xenopus* egg, the cortex rotates an average of 30° within the first cell cycle, relative to the inner cytoplasm [29, 46], a rotation mediated by an array of aligned microtubules beneath the vegetal cortex [47]. At the same time, these microtubules become aligned in a parallel arrangement with plus ends directed toward the direction of cortical translocation [48], a reorganization that coincides with the initiation of cortical rotation [30].

In the early *Xenopus* embryo, microtubule nucleation occurs deep within the animal hemisphere [49] by the sperm-derived centriole near the site of sperm entry. These microtubules extend through the cytoplasm toward the vegetal pole, where they contribute to the formation of the vegetal microtubule array (**Figure 4**, top left) [50]. Thus, in *Xenopus*, rotation (and dorsal site formation) typically occurs away from the sperm site of entry. On the other hand, the orientation of the vegetal microtubule array can occur in potentially any direction with respect to the cleavage site [5, 29], so that it is unlikely that there is an intrinsic preexisting dorsal asymmetry in the egg with respect to the site of cellular cleavage, itself determined by the orientation of the spindle [51, 52]. Studies have also shown that cortical rotation can occur toward the sperm entry point in specific cases, such as when the sites of meiotic spindle assembly and polar body extrusion are oppositely located [53], suggesting the existence of additional unknown variables that influence the orientation of the vegetal microtubule array. In *Xenopus*, cortical rotation is halted right before the first cellular cleavage occurs [47], when the microtubules of the vegetal microtubule array are depolymerized under the influence of M-phase-promoting factor [54].

A morphologically apparent cortical rotation, observed through changes in the position of an outer cortex relative to an inner core as observed in *Xenopus*, is not readily apparent in the zebrafish embryo. However, studies have indicated the existence of processes in the zebrafish embryo that share similarities with the amphibian cortical rotation. Early studies showed that fluorescent polystyrene beads injected at the vegetal pole were transported

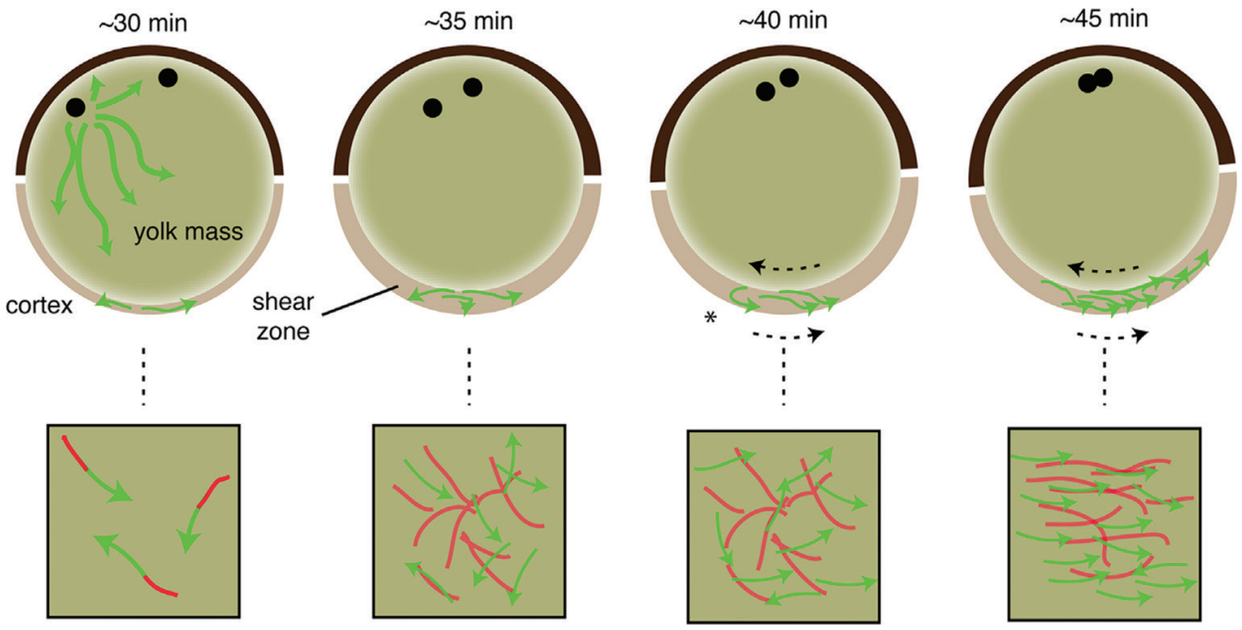

Figure 4. Microtubule dynamics during cortical rotation. In the *Xenopus* embryo, microtubule polymerization is initiated approximately 30 min after fertilization at the vegetal cortex, when astral microtubules derived from sperm components at the animal pole reach the vegetal cortex. Microtubule polymerization also occurs at the vegetal pole (growing microtubule (plus) ends indicated by green arrows and preformed microtubules by red lines). Relative movement between the yolk cell and the cortex (dashed arrows), initiated by the asymmetry conferred by the sperm-derived asters, facilitates the alignment of both growing and preformed microtubules in the direction of movement. Microtubule alignment in turn contributes to cortical movement. Microtubules oriented toward the dorsal side, the direction in which the cortex rotates (Reprinted from Ref. [58], with permission).

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animally along microtubule-based cortical tracks in a microtubule-dependent manner [6] and that this movement had temporal dynamics and functional requirements similar to that of the movement of putative dorsal determinants as defined by embryological manipulations. However, this movement was shown to occur by visualizing injected fluorescent beads, as opposed to an entire cortex, consistent with translocation along microtubule arrays but not necessarily a shift of the outer cortex analogous to a cortical rotation. A cortical rotation process in the zebrafish was later suggested by the observation of coordinated movement of optically visible particles in the vegetal cortex, and that long-term tracking of these particles occurs toward the presumed dorsal side, as expected from a cortical rotation [8].

A cortical rotation-like process is also consistent with differences in the changes in RNP particle distribution at different cortical depths, as visualized by fluorescent in situ hybridization, since RNPs located at the outermost cortex undergo a spatial shift with respect to more internally located RNPs (**Figure 3E**) [55]. To understand the basis of transport for differentially localizing factors at the zebrafish vegetal-most embryonic cortex, double fluorescence in situ hybridization (FISH) was used to detect pairs of RNAs for factors involved in axis induction (*wnt8a* and *grip2a*) and RNAs for vegetally localized germ cell specification factors (*dazl*). Localization of these three factors occurs in different RNPs at the vegetal cortex. Moreover, RNAs for dorsal factors, *wnt8a* and *grip2a*, are enriched in the outermost layer of the cortex, whereas RNPs for the primordial germ cell determining factor *dazl* are present in more internal regions [55]. Although domains containing RNPs for these two sets of vegetally localized factors are both centered at the vegetal pole in the egg, upon fertilization the domain containing the outer cortex RNPs, coding for dorsal induction factors, shifts relative to the more internal domain containing germplasm determinant RNPs. RNPs in the outer cortex have a function in axis determination and need to experience a relative shift to generate an asymmetry in the embryo, facilitated by the cortical rotation-like movement. These observations further add to the finding of bulk particle movement at the zebrafish embryo vegetal cortex [8] and are consistent with a cortical rotation-like process in the early zebrafish embryo. As in amphibians, this teleost cortical rotation-like process may be involved in generating an asymmetry in the location and function of dorsal determinants.

Thus, both in amphibians and teleost, an array of aligned microtubules is associated with the movement of RNA molecules and the vegetal cortex itself with respect to the inner egg core, which altogether mediates the transport of dorsal determinants toward the prospective dorsal site.

3.4. Long-range vs. short-range transport

In both *Xenopus* and zebrafish, the process of cortical rotation appears to be an important part of the mechanism that directs dorsal determinants to their final destination at the animal pole. However, zebrafish and *Xenopus* embryos display some differences in mechanism of animally directed transport. In the *Xenopus* embryo, aligned tracks of microtubules appear to span most if not all of the space between the vegetal pole and the prospective dorsal region. In zebrafish, in contrast, transport with an end point in blastomeres at the animal pole of the

embryo appears to depend on two sequential steps: an initial short-range transport of vegetal localizing factors generating a slight off-center shift toward the animal pole, followed by animally-directed transport via a more general mechanism. The first, off-center asymmetry, is revealed by changes in the distribution of RNAs such as *wnt8a* and *grip2a* in a process that appears to correspond to a cortical rotation-like event. As in *Xenopus*, the initial cortical rotation-like event in zebrafish depends on the alignment of microtubules in parallel bundles at the vegetal cortex. The microtubule reorganization into parallel bundles in turn is dependent on the function of Grip2a (**Figure 1**, left). Short-range shift in vegetal signals is affected in homozygous *hecate*/*grip2a* mutant embryos, evidenced by defective off-center shift of RNAs such as *wnt8a* and other factors [26].

The second step involves a long-range transport along the mediolateral region of the embryo to the base of the blastomeres by a mechanism that is neither restricted to the dorsal side nor dependent on Grip2a function [6, 26]. The presence of such a second transport mechanism can be inferred by the observation that *hecate*/*grip2a* mutants do not exhibit a defect in the long-range animally directed translocation of vegetally injected beads, indicating that animally directed movement occurring in mediolateral regions is independent of *hecate* function. Indeed, injection of beads in opposite sides of the embryo indicates that animally directed travel along the mediolateral region of the yolk cortex occurs in both injected sides, implying that, as opposed to *Xenopus*, the entirety of the zebrafish mediolateral cortex, and not only the prospective dorsal region, is competent for long-range movement [26]. It is possible that the second step in zebrafish depends on a more general transport mechanism associated with animally directed transport in meroblastic embryos, through which other factors with a function unrelated to dorsal axis induction, such as vegetally localized germplasm RNAs [56], need to travel animally toward the forming blastodisc. Thus, both *Xenopus* and zebrafish experience animally directed movement of dorsal determinants facilitated by a microtubule-dependent cortical rotation-like process. However, the *Xenopus* embryo uses a mechanism in which cortical rotation and microtubule alignment into parallel tracks together implement long-range movement of dorsal determinants through an apparently seamless mechanism. In teleost embryos, on the other hand, embryonic-scale differences along the dorsoventral axis are generated by the sequential action of a short-range off-center movement mediated by less expansive vegetal microtubule array, which is subsequently amplified by a more general animally directed system.

3.5. Other factors involved in vegetal microtubule reorganization

Additional factors have been identified to be important for dorsal axis induction. A mutation in the maternal-effect mutant *brom bones*, which has a nonsense mutation in the gene *hnRNP I*, shows egg activation defects, disorganized vegetal microtubule array formation, and subsequently defects in axis formation [16]. Additionally, these mutant embryos display egg activation defects as evidenced by failure of cortical granule exocytosis and chorion expansion. In zebrafish, cortical granule exocytosis is one of the first cellular responses to egg activation and is initiated by a wave of elevated cytoplasmic calcium that is impaired in *brom bones* mutants [16]. It is possible that the defect in vegetal microtubule alignment in *brom bones* is similarly based on the calcium release defects after egg activation, which is required for vegetal microtubule array formation [8].

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Studies have also revealed that an ubiquitin ligase, *tripartite motif-containing 36* (*trim36*), is required for vegetal microtubule reorganization mediating axis induction. *Xenopus trim36* is maternally expressed, with mRNA enrichment at the Balbiani body in stage 1 oocytes and localization to the vegetal cortex of stage VI oocytes. *trim36* mRNA is also detectable in the germplasm of fertilized eggs and cleavage-stage embryos [57]. Embryos depleted of *trim36* function by injection of antisense oligos into oocytes exhibit defects in vegetal microtubule reorganization and cortical rotation, leading to reduced organizer formation and severe embryo ventralization at later stages [57]. As expected, injection of *wnt11* mRNA rescues this effect, confirming that Trim36 functions upstream of Wnt/β-catenin activation. Recent studies have shown that Trim36 attenuates the growth of plus ends of vegetal microtubules during array formation (see below) [58], indicating a role for this factor, possibly through the mediation of protein degradation, in the regulation of microtubule dynamics essential for array formation.

The mRNA for *dead end*, which codes for an RNA-binding protein initially shown to be essential for the development of the germ line [59–68], has been shown to have a role in vegetal microtubule array formation. In *Xenopus*, the mRNA for *dead end1*, like that of *trim36*, is localized to the vegetal pole of the oocyte [61]. Early embryos depleted of dead end exhibit an unexpected defect in the formation of arrays of parallel vegetal microtubules and consequently axis specification [69]. This requirement appears to depend on the function of Dead end protein to directly bind *trim36* mRNA and anchor it to the oocyte vegetal pole, likely increasing Trim36 protein local concentration in this region [46].

Thus, a variety of factors are required for the reorganization of vegetal cortex microtubules leading to dorsal determinant transport. In some cases, these factors are important for general processes essential for the microtubule reorganization, such as in the case of *hnRNP I* and dependent calcium signaling. In other cases, these factors begin to delineate a pathway for microtubule reorganization, as in the case of *dead end* and *trim36*, involved in the regulation of vegetal microtubule growth.

3.6. Mechanism of microtubule alignment during cortical rotation

Even though it has been shown that microtubule-dependent cortical rotation is important for axis formation, the molecular mechanisms underlying the organization and orientation of cortical microtubule have not been fully elucidated. The process of cortical rotation is highly conserved, and it likely requires the embryo to use a significant amount of energy. Weaver and Kimelman [70] asked the question that if dorsal determinants can travel along microtubules, then what is the purpose of the cortical rotation? As described above, cortical rotation might directly contribute to the overall animally directed movement of the dorsalizing activity. However, studies have also suggested that cortical rotation might serve to facilitate aligning the polymerizing microtubules into parallel bundles and orienting their plus ends toward the dorsal side. One favored model for the orientation of the microtubule array is a positive feedback mechanism where initial random asymmetry in microtubule growth is amplified by continuous movement of the cortex [31, 58].

Microtubules that form the vegetal microtubule array appear to arise from several sources [70]. Some are nucleated by the centriole of the sperm, which acts as a minus-end microtubule-organizing center, others extend toward the periphery from unknown sources deep in the cytoplasm and bend into the vegetal shear zone, and, finally, some arrays appear to polymerize spontaneously in the vegetal shear zone [49, 50]. As the vegetal microtubule array begins to form, it becomes progressively stabilized by movement of the cortex during cortical rotation, which provides an amplifying loop for microtubule alignment [58]. The precise manner by which this cortical movement contributes to microtubule alignment and stabilization is not fully understood. Suggested mechanisms, described below, include a combing process mediated by cortically anchored kinesin-related proteins [54, 70] or the stabilization of microtubules by membrane compartments such as the endoplasmic reticulum and vesicles [58].

Vegetal microtubules originally appear with their plus ends in a random orientation yet subsequently become aligned in parallel arrays with plus ends directed toward the dorsal side (**Figure 4**) (reviewed in Ref. [70]; see also Ref. [58]). Marrari and colleagues suggested how microtubules could become aligned through cortical motor proteins and the process of the cortical rotation [54] (reviewed in Ref. [70]). They proposed that cortically anchored plus-end-directed motor proteins, such as kinesins, move toward microtubule plus ends, generating a cortical displacement with respect to the inner core [47, 54, 71]. The attachment of plus ends to the moving cortex mediates aligning of microtubules in the same direction. Thus, the movement and action of these kinesin-related proteins could potentially align the microtubules as well as generate the pulling force that is needed to translocate the cortex relative to the cytoplasm [54, 70]. This positive feedback loop also allows amplifying an original small asymmetry into the observed prominent array of parallel microtubule bundles.

Marrari and colleagues also investigated the role of kinesin and dynein motors in the formation of the cortical microtubule array as well as their role in the translocation of the vegetal cortex [47, 54, 71]. The function of kinesin was inhibited using an antibody against a highly conserved peptide of the kinesin motor domain, LAGSE. Anti-LAGSE antibodies block spindle elongation in semi-in vitro systems [47, 54, 71, 72] and successfully interfere with kinesin function [47, 54, 71]. The function of dynein was inhibited by microinjection of p50/dynamitin beneath the vegetal cortex [54]. In *Xenopus* egg extracts as well as cells, excess dynamitin inhibits processes dependent on dynein function by disrupting the dynactin complex [73].

Inhibition of kinesin-related function results not only in expected defects in mitosis and cell cleavage but also in disruptions in the array of vegetal microtubules and cortical rotation [71]. On the other hand, inhibition of dynein causes an inward shift in the distribution of microtubules with respect to the cortex, indicating that dynein functions to move microtubules outward, into the vegetal subcortical layer [47]. Moreover, these experiments showed that the formation of the vegetal microtubule array (and therefore cortical rotation) is sensitive to dynein inhibition prior to array formation, but that cortical rotation remains sensitive to inhibition of kinesin function throughout the normal period of rotation [47]. Together, these data suggest that kinesin and dynein motors have different functions during cortical rotation (**Figure 5**) [47]. In this model, dynein motors anchored to internal

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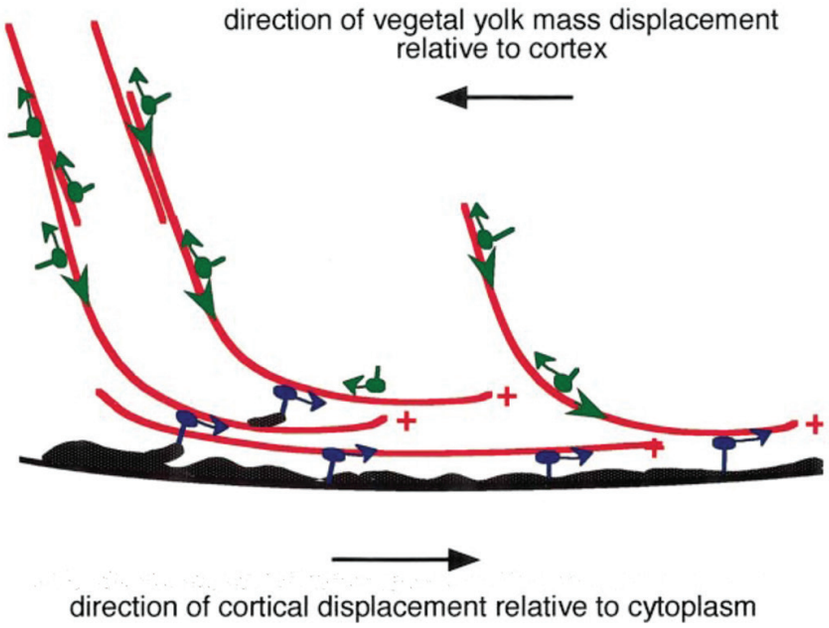

Figure 5. Proposed role of microtubule-dependent motors on the rotation of the vegetal cortex, as suggested by inhibitor studies [47, 54, 71]. A pushing force from the minus-end-directed microtubule motor dynein (green; green arrows show direction of motor movement relative to microtubules) helps translocate microtubules (depicted in red) from the inner cytoplasm outward onto the cortical surface (green arrowheads indicate direction of microtubule movement). Plus-end-directed microtubule motors such as kinesins (blue) anchor microtubules to the cortex and facilitate cortical movement relative to the yolk mass (blue arrows show direction of motor movement relative to microtubules). (Reprinted from Ref. [71], with permission. The original image has been rotated horizontally for a better comparison to others in this chapter.)

elements generate an outward force to facilitate bringing microtubules from the inner egg core region to the vegetal cortex. Kinesins, on the other hand, are thought to act by tethering microtubule plus ends to the cortex, thus generating a pulling force on microtubule arrays, mediating the rotation of the cortex itself, and favoring further parallel alignment of microtubules within the array. It is important to note that, after the vegetal microtubule array has formed, further microtubule alignment and cortical rotation can occur independent of dynein function, but motors of the kinesin-related protein family are needed for the movement of the cortex [47]. Thus, kinesin motor function appears to be essential for *Xenopus* cortical rotation, whereas the role of dynein appears to be more indirect. Altogether, these data suggest that both motor proteins interact early in the process of vegetal microtubule array, followed by a period in which kinesin-dependent translocation is sufficient to generate cortical movement.

Olson and colleagues performed experiments that would characterize microtubule plus-end dynamics in *Xenopus* oocytes and eggs, identified changes in microtubule stability and plus-end flux during the oocyte to egg transition, and characterized behaviors that are present at the onset of cortical rotation (**Figure 4**) [58]. They showed that the initial phase of microtubule assembly is between 25 and 35 min post egg activation. During this time, microtubules are short and dynamic with a low initial density that increases rapidly [58]. In the second phase of assembly, microtubules polymerize rapidly from sites within the vegetal cortex. Microtubules became thinner or less bundled, and the entire network appears to sink deep

into the cytoplasm. At this time the microtubule array is referred to as exhibiting a "fine-combed" appearance, which is thought to be the result of the continual action of cortical kinesin-related proteins that straighten microtubules as the cortex moves along them [47, 58, 70]. At the same time, at approximately 36 min post activation, the cortical shift in relation to the egg core becomes apparent [58]. These studies also reveal that microtubule-directed growth, occurring after the initial cortical microtubule alignment, has an important contribution to the formation of the vegetal array of parallel microtubules, which powers cortical rotation.

It was previously noted that the direction of microtubule polymerization in cultured cells depends on the arrangement of elongated tubes of endoplasmic reticulum [74]. Endoplasmic reticulum, vesicles, and tubes possess kinesin-like microtubule-associated proteins that associate with microtubules during transport and elongation, and it is possible that similar membrane organelles are attached to the vegetal cortex and facilitate kinesin-mediated anchoring of microtubules during cortical rotation [31]. A precedent for this is the association of cortical ER with aligned microtubules in early ascidian embryos (see below) [75]. Further studies will be required to address a potential role for membrane organelle attachment in *Xenopus* vegetal microtubule array formation and cortical rotation, such as membrane organelle sliding between membrane organelles and microtubules, or associations of ER extensions with growing microtubule tips [76].

Studies in zebrafish are consistent with mechanisms for cortical microtubule array formation and alignment as detailed in amphibians, including the presence of early internal microtubules, increase in cortical microtubule polymerization concomitant with microtubule alignment and bulk movement of the cortex, and the aligned orientation of microtubule plus ends toward the prospective dorsal site [8].

Altogether, these studies suggest that the formation of the vegetal microtubule array is dependent on the orchestration of various influences, including dynein-dependent outward translocation of existing microtubules, kinesin-dependent vegetal anchoring of cortical microtubules, and microtubule polymerization at the vegetal cortex. Vegetal microtubule and cortical rotation are interdependent and enhance each other, resulting in the alignment of preexisting and new microtubules and allowing dorsal determinant transport.

4. Cortical rotation and cytoskeletal dynamics in invertebrate and protovertebrate systems

As described above, a cortical rotation process has been described in amphibians, and a related process proposed in teleosts. However, other studies have described processes of cytoskeletal reorganization that serve a similar purpose as the cortical rotation, namely, the early distribution of cellular determinants that will help pattern the egg or embryonic axis. We briefly discuss three such examples below, in ascidians (a chordate protovertebrate), the nematode *Caenorhabditis elegans*, and the dipteran *Drosophila melanogaster*, highlighting similarities with cortical rotation-like processes in lower vertebrates. For a more in-depth description of these processes, the reader is referred to Refs. [77–81] .

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4.1. Ascidians

In ascidians, gastrulation and neurulation involve cellular rearrangements that are comparable to those in vertebrates, with the exception that ascidians are composed of just a few hundred cells, while vertebrate embryos contain thousands of cells [82]. In fact, the very first classical evidence that localized determinants control cell fate specification was found in ascidians [82, 83].

The ascidian egg undergoes dramatic cytoplasmic and cortical reorganizations between fertilization and the beginning of the first cleavage, a process that has been referred to as ooplasmic segregation [83–85]. Ascidian ooplasmic segregation occurs in two major phases (**Figure 1**, right). The first phase occurs shortly after fertilization. The first consequence of fertilization is that a calcium wave is initiated from the site where the sperm and egg fuse [86]. Upon fertilization, the sperm activates the stage IV oocyte, which was arrested in metaphase I of meiosis, resulting in the contraction of the egg cortex and the plasma membrane as a wave that travels across the egg in the animal to vegetal direction. It was suggested early on that an oocyte actomyosin cortical network can only contract in a general animal to vegetal direction regardless of the sperm entry site, because of it being less dense around the animal pole, in a basket-like arrangement [86, 87]. This animal-to-vegetal contraction in turn causes the segregation of cortical and subcortical components including microfilaments, mitochondria, and the cortical endoplasmic reticulum (cER) [77, 88, 89].

Unfertilized eggs after the first phase of ooplasmic segregation are radially symmetrical along the animal-vegetal (A-V) axis. This symmetry is broken in the second phase of reorganization after the movement of cortical and subcortical components from the vegetal pole toward the posterior pole occurs, generating an anteroposterior asymmetry, and eggs become bilaterally symmetrical [77]. In this second ooplasmic segregation phase, a number of cellular organelles such as the ER and mitochondria are brought toward the future posterior pole [90]. These organelles also anchor specific RNAs, termed postplasmic/PEM, which are important for muscle determination and the specification of the posterior cell fate, in particular the germ line [91]. Other factors involved in endoderm formation and gastrulation do not move toward the future posterior pole and instead expand their distribution to the vegetal hemisphere (see **Figure 1**) [77]. Reminiscent of asymmetry development in *Xenopus*, it has been suggested that also in the ascidian egg, reorganization of plus-end-directed motors attached to the ER could provide the major force to move the vegetal cortex dorsally to a more equatorial location [48, 92].

Ascidian embryonic polarity is directed by a posteriorly located centrosome, introduced through sperm entry in this region [77, 93, 94]. In contrast to the first phase which is driven by microfilaments, and where the sperm triggers a cortical contraction [88], the second phase is mediated by anchoring one of the centrosomes of the bipolar spindle to the vegetal posterior cortex, resulting in the posterior asymmetric localization of germ line-determining components. Spindle pole posterior anchoring also results in the eccentric, posteriorly located placement of the spindle, which in turn (because of the influence of the spindle midzone on furrow induction) [51, 52], results in asymmetric division [75, 77]. In this manner, the embryo generates sets of smaller posterior cells fated to become the germ line.

Thus, in both *Xenopus* and ascidians, microtubule-dependent function results in the redistribution of embryonic determinants just before the onset of embryonic mitoses, the

posterior-specifying cytoplasmic components such as the cER-mRNA and myoplasm domains being displaced posteriorly in ascidians and dorsalizing factors being translocated toward the future dorsal side in *Xenopus* [89, 95].

4.2. *Caenorhabditis elegans*

In the nematode *C. elegans*, a role for PAR proteins in anterior-posterior (AP) axis specification is well documented [96]. In contrast, dorsal-ventral (DV) patterning in this system is less understood. It was recently reported that the so-called cytokinetic midbody remnant (MBR), a thus-far poorly studied organelle, acts as a polarity cue to define the *C. elegans* DV axis [97]. The MBR is an organelle that forms from the cytokinetic midbody when the fully constricted actomyosin furrow embraces the condensed material of the spindle midzone [98, 99]. To understand the role of the MBR in DV axis specification, Singh and Pohl [100] analyzed the pattern of segregation and the movements of the MBR during the first divisions of the *C. elegans* embryo. The AP axis of the *C. elegans* embryo is established by the asymmetric distribution of PAR proteins during the P0 division producing an anterior AB and a posterior P1 blastomere. Subsequently, the DV axis is established in the transition from the two-cell to the four-cell stage [101]. During prophase of the second cell division in the P1 cell, a 90° rotation of the nucleus-centrosome complex relative to the AP axis takes place, and is regarded as a key event in DV axis formation [102, 103]. It was not clear as to what generates this movement, which has long been a point of interest. The authors showed that the MBR was displaced toward the ventral side of the embryo and that it acts as a positional cue for mitotic spindle rotation in the P1 cell, thereby establishing DV axis patterning. Importantly, the authors demonstrated that ventral displacement of the MBR is directed by myosin II cortical flow [97, 100]. In this system, again microtubules together with coordinated actomyosin regulation are important for symmetry-breaking events in the embryo.

4.3. *Drosophila melanogaster*

In *D. melanogaster*, the transition from a round to an elongated egg is driven by the rearrangement of the polar arrays of microtubules [80, 81], a process that is again facilitated by the actomyosin cytoskeleton [81]. As in *C. elegans* and ascidians, this reorganization results in the segregation of cell determinants to the posterior pole of the egg, except that in the case of *Drosophila*, these changes occur during oogenesis and not early embryogenesis.

Altogether, these studies show that the microtubule cytoskeleton, and in some cases the actomyosin cortex, is used to generate axis asymmetry in various organisms, although the precise details of the interactions, and whether microtubules act as tracks that mediate transport or attraction centers, are specific to different species [97, 104].

5. Relationship between axis induction and germ cell specification

As mentioned above, in addition to dorsal determinants, anuran and teleost embryos contain other vegetally localized factors, particularly RNAs that become associated with the germplasm. The germplasm, also referred to as nuage, is a maternally inherited cytoplasmic

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structure containing RNPs present in some animal species. Through a mechanism referred to a preformation, inherited germplasm determines the germ cell fate [105]. Evidence for preformation mechanism for PGC induction in anurans was originally shown by the inheritance of electron-dense cytoplasm, corresponding to germplasm, into the primordial germ cells of this organism [106]. This electron-dense cytoplasm was later shown to contain specialized mRNAs involved in germ cell specification [107]. Similarly, RNAs involved in germ cell development in zebrafish, such as for the gene *vasa* [108] and subsequently other mRNAs [56, 59, 109], were shown to localize in electron-dense particles and become segregated to primordial germ cells.

Maternally inherited germplasm in *Xenopus* and zebrafish contains shared sets of factors for primordial germ cell specification, such as *deleted in azoospermia-like* (*dazl*) and *Xcat2*/*nanos*. Zebrafish and *Xenopus* additionally share similarities in the way in which germplasm masses are assembled and segregated, including the gradual condensation of germplasm masses from smaller particles, the formation of four germ masses, and their asymmetric segregation during cell division in the cleavage stages [110, 111].

Recent studies in these systems have begun to suggest a functional connection between axis induction and germ cell determination. As described above, during oogenesis both dorsal determinants are transported to the vegetal pole of the egg through the mitochondrial cloud in *Xenopus* and its equivalent structure, the Balbiani body, in zebrafish [111, 112]. Moreover, during early embryogenesis, genes acting in dorsal induction functionally overlap and share localization patterns with genes involved in germ cell determination. For example, the germplasm component *dead end*, which has been well characterized as a germplasm-specific transcript both in *Xenopus* [61] and zebrafish [59] and is known to function in germ cell migration and survival, has been shown in *Xenopus* to have an unexpected role in axis induction [46]. *Xdead end* RNA localizes to the vegetal pole in oocytes beginning at the early stage III to stage VI, when it becomes transported to the vegetal pole via the late RNA transport pathway [61]. It has recently been shown that maternal XDead end plays a role in vegetal microtubule reorganization required for dorsal axis induction [46]. When XDead end function is disrupted, the expression of dorsal-specific genes is reduced, and embryos become ventralized, due to the disruption in vegetal microtubule reorganization [46]. As mentioned above, this requirement appears to be due to a role for XDead end function in the vegetal cortex anchoring of the RNA for the Trim36 ubiquitin ligase [46], itself needed for growth regulation of the vegetal microtubule array [58].

Conversely, factors known to be involved in dorsal axis induction also function in germ cell development. One example is maternal Syntabulin, which as mentioned above is important for vegetal microtubule array reorganization and axis induction in both zebrafish and *Xenopus* [36, 113]. Recently, *syntabulin* mRNA has been shown to localize in *Xenopus*-cleaving embryos to clusters near the cleavage furrow on the vegetal hemisphere of the early embryo, consistent with germplasm localization and colocalization with *Xpat* RNA, a germ cell marker, during later stages [113]. *Xenopus* Syntabulin is also expressed in scattered cells localized along the posterior endoderm, presumably primordial germ cells [113]. These data suggest that, in addition to a role in DV patterning, Syntabulin may have a role in germ cell development.

Similarly, *grip2a*, which as mentioned above is required for vegetal cortex microtubule reorganization in zebrafish [26], has gene homologues involved in germ cell development in the *Xenopus* embryo [43, 44], suggesting a potential scenario in which an ancestral *grip* gene had a role in both processes. Altogether, these findings suggest that there is functional overlap between factors involved in germplasm segregation and axis induction. Whether this functional overlap is caused by evolutionary history or convergent evolution remains to be determined.

It is important to note, as stated above, that there is a difference with respect to cortical depth between the factors that are localized to the vegetal pole. Those that are important for microtubule reorganization, and thereby patterning the embryonic axis, namely, *grip2a* and *wnt8a*, are located toward the outermost region of the cortex. This allows them to be transported from the vegetal to the prospective dorsal region of the egg and embryo through a cortical rotation-like process. Those factors that are important for germ cell specification, such as *dazl* RNA, are localized deeper within the embryo and are transported via the actin cytoskeleton to the animal pole, where they become localized to the aggregating zebrafish germplasm [55]. Thus, RNA localization at the cortex reflects transport mechanisms consistent with the function of the localized product.

These set of studies highlight commonalities between processes and factors involved in axis induction and germ cell specification. Factors such as Dead end, Grip2, and Syntabulin may form a core gene set with a current or ancestral function in both axis induction and germ cell determination.

6. Conclusion: challenges and future directions

The cytoskeleton plays an essential role in axis specification, through its role mediating the movement of maternal factors within the early zygote. Studies have shown that the reorganization of the microtubule cytoskeleton is important for the transport of factors from the vegetal pole of the embryo to the future dorsal side in both zebrafish and *Xenopus*, in a process associated with the shift of the outermost cortical layer of the embryo—a cortical rotation. This cytoskeletal reorganization allows for the asymmetric transport of localized dorsal determinants, involved in the specification of the main embryonic axis. Precise mechanisms for microtubule reorganization remain incompletely understood, although are known to involve microtubule dependent motors and a positive feedback loop in which an early asymmetry and microtubule alignment triggers the rotation of the cortex, which in turn amplifies and stabilizes the incipient cytoskeletal rearrangement. Anurans and teleosts show similarities in the use of microtubule arrays and a cortical rotation-like mechanism, although they also exhibit differences in the spatial extent implemented by these coordinated processes, variations that may be related to the different cleavage type of these embryos. Components of the germplasm, which also become localized to the vegetal pole of the fertilized embryo, may escape cortical rotation by virtue of differential localization in more internal regions of the embryo. A comparison of early cytoplasmic segregation events in other species, such as ascidians,

nematodes, and dipterans, highlights the importance of microtubule- and other cytoskeletal-dependent processes in the generation of early asymmetries in the embryos. Further studies will allow better understanding for mechanisms of microtubule generation, bundling, and alignment that drive the movement of cellular determinants in the early vertebrate embryo and their relation to similar processes in other animal lineages.

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