Advanced Review

Cortical rotation and messenger RNA localization in *Xenopus* axis formation

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In *Xenopus* eggs, fertilization initiates a rotational movement of the cortex relative to the cytoplasm, resulting in the transport of critical determinants to the future dorsal side of the embryo. Cortical rotation is mediated by microtubules, resulting in activation of the Wnt/β-catenin signaling pathway and expression of organizer genes on the dorsal side of the blastula. Similar cytoplasmic localizations resulting in β-catenin activation occur in many chordate embryos, suggesting a deeply conserved mechanism for patterning early embryos. This review summarizes the experimental evidence for the molecular basis of this model, focusing on recent maternal loss-of-function studies that shed light on two main unanswered questions: (1) what regulates microtubule assembly during cortical rotation and (2) how is Wnt/β-catenin signaling activated dorsally? In addition, as these processes depend on vegetally localized molecules in the oocyte, the mechanisms of RNA localization and novel roles for localized RNAs in axis formation are discussed. The work reviewed here provides a beginning framework for understanding the coupling of asymmetry in oogenesis with the establishment of asymmetry in the embryo.

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INTRODUCTION

The establishment of the embryonic body axes from an initially symmetrical egg has intrigued developmental biologists for many years. Historically, this symmetry breaking process has been best understood in amphibian eggs, which undergo cytoplasmic rearrangements resulting in the establishment of dorsoventral polarity prior to the first cell division. In certain species of frogs (e.g., *Rana* spp.) and salamanders, these rearrangements are visible as the conspicuous gray crescent, which forms opposite to the site of the sperm entry point (SEP) and provides a reliable indicator of dorsal fate. Experiments such as Spemann's egg ligations in early 1900s[1] showed that only gray crescent-containing blastomeres could form dorsal axial structures, suggesting that dorsal determinants were associated with the crescent.

A detailed study of gray crescent formation by Ancel and Vintemberger[2] (reviewed in Ref 3) showed that crescent formation likely fixes dorsoventral polarity in the embryo, despite earlier cues, and determines the position of the organizer. Mechanistically, it was shown that the gray crescent forms by the dorsal translocation of the egg cortex over the deeper cytoplasm. This view of dorsoventral polarization in the egg was confirmed and extended during a reinvestigation of axis formation in *Xenopus* by Gerhart, Elinson, and others in the 1980s–1990s.[4,5] At the subcellular level, it is now widely appreciated that cortical rotation, as these rearrangements have been termed, involves the translocation of the cortex and subcortical cytoplasmic determinants to the future dorsal side, along with the transport of dorsal determinants along a parallel array of vegetal microtubules. The main outcome of cortical rotation is the asymmetrical activation of the Wnt/β-catenin signaling pathway and the enrichment of β-catenin (Ctnnb1) in dorsal nuclei.[6,7] β-Catenin is a key effector of Wnt/β-catenin signaling and is required to transcriptionally activate dorsal-specific genes at the midblastula transition (MBT), leading to

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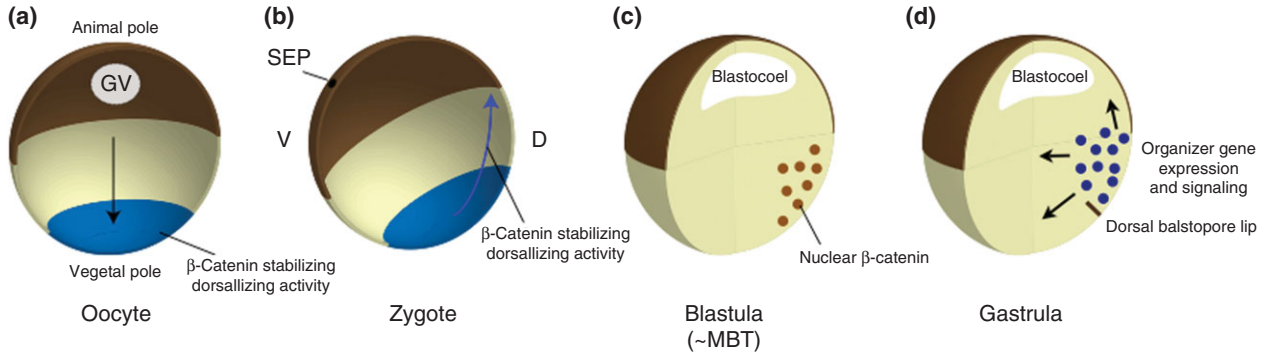

FIGURE 1 | Conceptual model for dorsal axis formation in *Xenopus*. (a) Oogenesis. During oogenesis, β-catenin-stabilizing factors are localized to the vegetal pole. Dorsoventral polarity is not apparent at this stage, and the oocyte and mature egg exhibit axisymmetry about the animal–vegetal axis. (b) Cortical rotation. Following fertilization, the cortex of the egg rotates relative to the inner cytoplasm using a parallel array of microtubules. Movement generally occurs opposite to the sperm entry point (SEP), and transports cortical and subcortical β-catenin-stabilizing dorsalizing activity into the equatorial region of the embryo. (c) β-Catenin stabilization/nuclear localization. During the 16–128-cell stage, β-catenin is stabilized and is enriched in dorsal nuclei, remaining nuclear through the midblastula transition (MBT). (d) Organizer gene expression. At the late blastula/early gastrula stage, β-catenin (along with other proteins, such as Vegt) activates gene expression in the dorsal vegetal and equatorial regions. These targets include Spemann organizer genes *siamois, nodal-related 3, chordin,* and *noggin*, as well as early vegetal *Nodal* homologs, *nodal-related 5/6.* These and other genes regulate axial patterning by antagonizing the function of bone morphogenetic proteins as well as regulating other signaling molecules. D, dorsal side; GV, germinal vesicle; V, ventral side.

organizer formation and overall body plan patterning (Figure 1).[8]

Cortical rotation occurs in anuran and urodele amphibians, chondrostean fish (sturgeons and relatives), and lampreys,[3] and is thus a conserved ancestral feature in vertebrates. By contrast, teleost fish do not undergo cortical rotation[9] but do still exhibit microtubule-dependent cytoplasmic localizations resulting in dorsal activation of Wnt/β-catenin signaling.[10] In birds and reptiles, the localization of maternal factors likely biases axial polarity significantly,[3] although in these animals Wnt/β-catenin signaling is established zygotically during axis formation.[11,12] In mammals, studies in the mouse have suggested (controversially) that maternal localization is not a major factor in axis specification,[12] although whether this is strictly true for all mammals remains to be established.

This general model of axis formation is well supported by evidence from *Xenopus* as well as other vertebrate model organisms. For the most part however, the cellular and molecular mechanisms controlling the microtubule assembly and transport and the identity of the dorsal determinants have not been clearly established. Since the last extensive review of this field,[13] several new players have been identified and novel roles described for known pathways. This review will discuss recent advances in *Xenopus* axis formation, highlighting the roles of localized components in the oocyte and egg in regulating Wnt/β-catenin activity and microtubule assembly in the egg and early embryo. The *Xenopus* egg remains an ideal experimental subject for these studies, as the relevant cell biological and signaling pathways can be readily manipulated and coupled with embryological outcomes. Because the basic mechanisms of axis formation are evolutionarily conserved as well as reiterated in different contexts throughout development, the work reviewed here is likely to be broadly important for understanding many aspects of developmental biology.

CELLULAR AND MOLECULAR MECHANISMS OF CORTICAL ROTATION

The cellular and molecular mechanics of cortical rotation have been studied extensively in frog eggs (*Xenopus* and *Rana*) and the general features and critical cytoskeletal elements involved have been comprehensively reviewed.[5,13,14] Briefly, cortical rotation begins about halfway through the first cell cycle (~45 min at room temperature). Translocation accelerates rapidly to a maximal speed of ~10 μm/min and continues until just prior to first cleavage (~100 min), when microtubule depolymerization occurs and rotation terminates, resulting from maturation-promoting factor (MPF) activation.[15] During this period, the cortex moves as an unit relative to the inner cytoplasmic core. Total movement averages 30° of arc along the animal–vegetal axis and typically is oriented opposite to the SEP.[5,16] Changes in fluid dynamics following egg activation

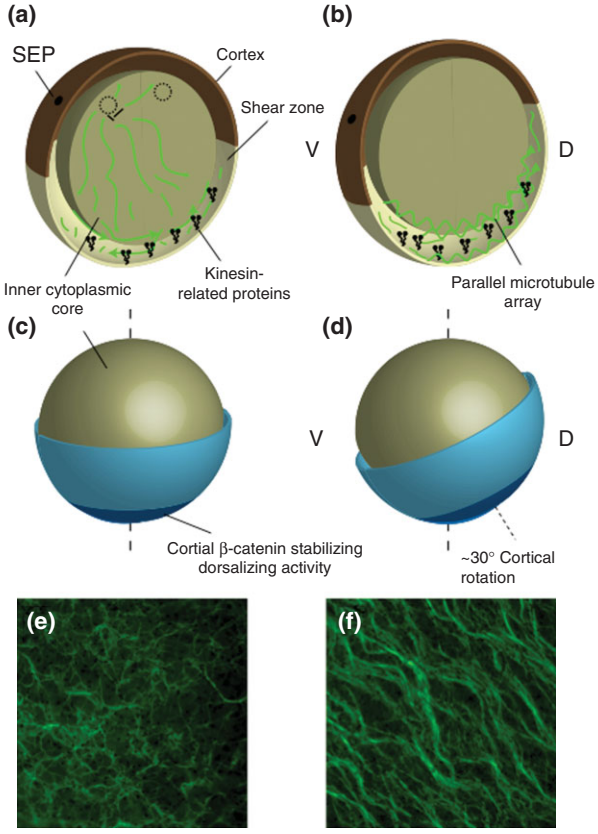

FIGURE 2 | Cellular events of cortical rotation. (a and b) Assembly and alignment of microtubules. (a) Thirty to forty minutes after fertilization. Astral microtubules organized around the sperm centriole and pronucleus grow radially. Microtubules also originate in the cortex/subcortical region and within the inner cytoplasm. Kinesins and related proteins are enriched in the vegetal cortex. Plus ends are indicated by arrows; the relative scale of the shear zone is exaggerated for clarity. (b) Sixty to eighty minutes after fertilization. The egg undergoes cortical rotation. Microtubules in the shear zone are aligned in parallel and oriented toward the future dorsal side. (c and d) Models showing the displacement of the cortex relative to the inner cytoplasm. Only the vegetal cortex is shown (light blue); the region of dorsalizing activity is shaded dark blue. The heavy dashed line indicates the initial animal–vegetal axis; the smaller dashed line indicates the position of the former vegetal pole. (e and f) Immunostaining against α-tubulin. Vegetal views of eggs at 50 min postfertilization (e) and at 80 min postfertilization (f), showing parallel alignment and bundling of microtubules.

are thought to create a low-viscosity cytoplasmic shear zone between the cortex and the dense cytoplasmic core,[16] allowing relative motion between the two regions.

The movements of cortical rotation critically require microtubule assembly and organization (Figure 2). A prominent, transient vegetal microtubule array forms in the subcortical region during the period of rotation[17] and is polarized with the plus ends oriented toward the future dorsal side.[18] Microtubules polymerize and align progressively out of a loose network present at 40 min after fertilization. By 60 min, microtubules are organized into dense parallel bundles, which gradually become finer in structure prior to their disassembly at first cleavage.[17,19] Other cellular components, such as endoplasmic reticulum (ER) and cytokeratin, also align with microtubules during rotation, suggesting an extensive reorganization of the vegetal cortex region occurs in this period.[17] Agents that inhibit microtubule polymerization, but not other cytoskeletal systems, including microtubule-blocking drugs, cold treatment, pressure, and ultraviolet (UV) irradiation of the vegetal pole, all effectively block cortical rotation and ventralize embryos.[17,20–22] Cortical rotation is thus a microtubule-dependent event that is essential for normal dorsal axis formation.

FAST TRANSPORT ALONG THE VEGETAL MICROTUBULE ARRAY

In addition to bulk cortical movement, the cortical microtubule array is thought to mediate a second, rapid transport system critical for the transport of dorsal determinants. Time-lapse microscopy identified dorsally translocating vesicular organelles, pigment granules, injected fluorescent beads,[23] and various green fluorescent protein (GFP)-fusion constructs[24,25] within the shear zone. These particles traveled at speeds of up to 50 μm/min and moved in a saltatory manner in the direction of rotation. Translocation was microtubule-dependent and occurred over >60–90° of arc from the vegetal pole apex, more than the movement of the cortex itself.[23–25] This distribution matches that of a transplantable dorsalizing activity found in eggs (see below), suggesting that this fast transport system may be the main mechanism for transporting dorsal determinants.

The relative importance of these twin transport mechanisms is difficult to discern, since each is sufficient to activate dorsal signaling in the absence of the other. Deuterium (D_2O) stimulates random microtubule polymerization and fast transport and results in hyperdorsalized embryos, although cortical rotation is largely reduced.[26] On the other hand, UV-irradiated eggs, which lack microtubules,[17] can be rescued by tipping them to cause gravity-induced displacement between the cortex and deep cytoplasm.[27] Tipping does not rescue microtubule formation itself[5,28] and normal development can result if at least 9° of relative cortical movement occurs.[29] This result suggests that fast transport is not necessary in an experimental setting, although testing its requirement *in vivo* would require inhibition of

specific transport proteins or microtubule regulators that would not also block bulk cortical rotation, which have yet to be identified.

In normal development, cortical rotation and the fast transport system are likely to be redundant and mutually reinforcing, with cortical rotation orienting the direction of fast transport and fast transport further potentiating microtubule polymerization. Fast transport might also provide robustness to the symmetry breaking process by ensuring that determinants are distributed well beyond some minimal limit needed to activate dorsal specification.

WHICH MOTOR PROTEINS REGULATE CORTICAL ROTATION?

Consistent with the orientation of the array and the direction of fast transport, plus end-directed molecular motors and other microtubule-associated proteins are known to have roles in cortical rotation. Inhibition of general kinesin-related protein (KRP) function using pan-KRP-blocking antibodies reduces microtubule organization and cortical rotation,[30] although the specific identity of the required motors is not known. Inhibition of the ubiquitous kinesin 1 alone does not inhibit cortical rotation.[30] Similarly, maternal depletion of messenger RNAs (mRNAs) encoding Xklp1/Kif4a and Xklp2/Kif15 using antisense oligonucleotides did not affect cortical rotation, although defects in other microtubule-mediated events were affected.[31] Dynamitin/p50/Dynactin2 injections suggest that the minus end-directed microtubule motor cytoplasmic dynein also plays a role early in cortical rotation,[32] possibly by sliding microtubules to the cortex. KRPs are suggested to tether microtubules to the cortex, facilitating cortical movement.[32] These results showing dual involvement of KRPs and cytoplasmic dynein are broadly consistent with models of microtubule-based transport in other systems.[33] However, the specific motors required and the regulatory mechanisms controlling motor protein function during cortical rotation remain undefined.

INITIATING CORTICAL ROTATION AND POLARIZING MICROTUBULES

Despite the importance of the vegetal microtubule array, how the array is assembled and polarized is not well understood. Events associated with egg activation are likely candidates to initiate assembly. These include cytosolic calcium release, cytoplasmic pH changes, and other signals. However, as rotation is initiated in pricked eggs or in uniformly activated eggs (e.g., as with calcium ionophore or electrical activation), sperm components are not absolutely required to initiate microtubule polymerization and organization.[29] Importantly, cortical rotation occurs in a single direction in prick-activated eggs, irrespective of the site of pricking. The egg has thus evolved to integrate various inputs to generate a discrete outcome from a general stimulus.

In normal development, sperm entry influences the direction of rotation. The plus ends of sperm aster microtubules, which can extend into the vegetal cortex, might provide an initial directional bias.[34] As sperm entry is usually asymmetric with respect to the animal pole, and microtubule motors are localized vegetally,[18] astral microtubules extending vegetally[35] would tend to drive rotation in the opposite direction. Also, rotation can occur in potentially any direction, so there is also unlikely to be a preexisting asymmetry in the egg. However, Brown and Danilchik[36] have observed that cortical rotation can occur toward the SEP in cases where the egg maturation spot, the site of meiotic spindle assembly and polar body extrusion, is located oppositely, suggesting the existence of some cryptic asymmetry.[36] The nature of this polarity is not known, but it could be related to meiotic spindle formation or pronuclear migration events.

The main mechanism proposed for polarizing the microtubule array is a reciprocal positive feedback loop between cortical movement and microtubule polymerization.[5] In this model, a slight asymmetry in the net force produced by microtubule polymerization in the shear zone would produce a small degree of movement, which would then further promote microtubule polymerization in the direction of rotation, either by shuttling microtubule-stabilizing proteins in the direction of rotation or by a force-based alignment. Longer and more uniformly aligned microtubules would then produce more movement, promoting even more polymerization and alignment toward the dorsal side. Cortical ER could play a role in this process, as ER and microtubules generally colocalize and are functionally interdependent.[37] Consistent with this idea, ER contains plus-end tip-attachment complexes, and ER localization is regulated by plus-end dynamics.[38]

In support of this reciprocal feedback model, rotation can be normally observed before there is an organized microtubule array present.[39] Also, the direction of cortical rotation and microtubule polymerization can be dictated by tipping eggs 90° briefly (~5 min) prior to rotation to initiate gravitational displacement of the inner cytoplasmic core.[5,28] This displacement causes the cortex to rotate to the upper side and will determine the future dorsal

side of the embryo.[27] Cortical rotation is thus a prime example of a self-organizing and self-regulating cellular process in development, in which stochastic asymmetries are amplified and refined to produce a uniform output.

CYTOPLASMIC DORSAL DETERMINANTS

Cortical rotation is proposed to transport putative dorsal determinants from the vegetal pole to the equatorial and animal regions of the egg. The nature of these determinants is not known, although they are proposed to act as activators of β-catenin stabilization (see below). This idea is supported by evidence from elegant cytoplasmic ablation and transplantation experiments showing that vegetal cortical cytoplasm and cortex are each necessary and sufficient for dorsal axis formation.[40–45] Cortical cytoplasm from the vegetal pole of fertilized and unfertilized eggs induces secondary axes when injected ventrally and can rescue UV-irradiated eggs.[40–42] Furthermore, egg equatorial or animal cortical cytoplasm and cortex lacks dorsalizing activity, but gains the activity following cortical rotation.[40,41] The timing and extent of this shift in dorsalizing activity coincides with that of particles moved by the fast transport system during cortical rotation.

The vegetal cortex itself likely contains the activity as well, as implantation of isolated cortex into 8-cell embryos also induces a second axis.[43] Importantly, removal of vegetal cytoplasm along with the cortex early, but not late, in the first cell cycle blocks dorsal axis formation,[44,45] indicating that the required factors are indeed moved away from the vegetal pole. Interestingly, this vegetal dorsalizing cytoplasm is unaffected by UV irradiation of the egg (which primarily inhibits microtubules),[40,41] but is completely inactivated by UV irradiation of the oocyte, although both treatments result in ventralized embryos.[46,47] UV-irradiated oocytes undergo normal cortical rotation, form normal microtubule arrays, and cannot be rescued by tipping,[46] suggesting that the vegetal dorsalizing activity is inactivated, not merely mislocalized as is the case with egg UV irradiation (Table 1).

This phenomenon of changing UV sensitivity is unexplained, but could be related to major changes in egg structure and physiology occurring at maturation, including dissolution of annulate lamellae,[68] changes in the cortical cytoskeleton,[69] the breakdown of the Golgi apparatus and secretory pathway components,[70] and the delocalization of a subset of vegetally localized mRNAs from the cortex, including *vg1/gdf1*.[71,72]

Although most efforts to identify dorsalizing molecules have focused on vegetal factors, equatorial and animal components may contribute as well. It has been hypothesized that the dorsal determinants interact with the equatorial region to cause axis formation, as vegetal cortical implants can only induce secondary axes at high frequency when placed into the equator of 8-cell hosts.[43] This requirement may reflect the fact that the organizer forms in dorsal mesoderm, which is normally induced in the equatorial region. In the animal pole, an uncharacterized dorsalizing poly(A+) RNA fraction has been identified in 16-cell dorsal animal blastomeres.[73] Older morphological studies have identified cytoplasmic localizations within the animal hemisphere that depend on cortical rotation (reviewed in Ref 74). More recent confocal imaging studies found deep cytoplasmic swirling in the dorsal animal hemisphere.[75] To date, however, there are no clues as to the identity of these deep animal–equatorial components or how they would interact with the more predominant vegetal cortical factors.

Wnt SIGNALING AND β-CATENIN FUNCTION IN *XENOPUS*

At the molecular level, cortical rotation and associated dorsal transport mechanisms result in the preferential stabilization and nuclear localization of β-catenin protein in dorsal nuclei, leading in turn to dorsal-specific gene expression and establishment of the organizer. Indications that Wnt/β-catenin signaling is involved in vertebrate axis formation first came from the microinjection of *wnt1* mRNA into *Xenopus* embryos, resulting in the induction of secondary dorsal axes.[76] However, *in vivo* loss-of-function evidence for Wnt activity in axis formation was first obtained by depleting maternal β-catenin mRNA from oocytes using antisense oligonucleotides.[61] Embryos derived from β-catenin-deficient oocytes were ventralized and lacked dorsal structures of all three germ layers, including the neural tube, notochord, and somites. In addition, β-catenin is required for the expression of organizer genes at the late blastula and early gastrula stages.[77] Importantly, β-catenin is enriched in dorsal nuclei in the equatorial region of midblastula embryos,[7] and has even been observed in dorsal nuclei at the 16–32-cell stage.[6] Thus, β-catenin activity is present at the right place and time and is both necessary and sufficient to serve as the dorsalizing output of cortical rotation. The analysis of numerous Wnt components by maternal mRNA depletion experiments has greatly substantiated this idea (Table 1).

TABLE 1 | Summary of Maternal Loss-of-Function Studies Showing Axis Defects

Gene	Phenotypic Information	Cortical Rotation	References
Miscellaneous			
UV-oocyte[1]	Ventralized; inactive cortical cytoplasm; not rescued by tipping	Y	46, 47
UV-egg[1]	Ventralized; active cortical cytoplasm; is rescued by tipping	N	22, 27
Signaling molecules			
Wnt11b	Ventralized; regulates Axin stability in oocytes	n.d.	48, 49
frl1/tdgf1	Ventralized; associates with Wnt11, Wnt5a (not Wnt8)	n.d.	48
wnt5a	Ventralized	n.d.	50
dkk1	Dorsalized; abnormal gastrulation movements	n.d.	50
Receptors			
frizzled7	Ventralized	n.d.	51
lrp6	Ventralized; regulates Axin stability in oocytes	n.d.	49
Other extracellular factors			
ext1	Ventralized	n.d.	52
tpst1	Ventralized; required for Wnt11/5a complex formation	n.d.	52
Intracellular signaling proteins			
frat1	Ventralized	n.d.	53
axin1	Dorsalized; not rescued by dorsal *axin* mRNA injection	n.d.	54
jnk1/mapk8	Dorsalized	n.d.	55
dvl2, dvl3	Decreased JNK activity, partial dorsalization, increased ectoderm marker, convergent extension defects	n.d.	55, 56
plin2	Ventralized, also germ plasm aggregation and PGC defects	N	57
ppp2r5e	Loss of dorsal/organizer gene expression	n.d.	58
trim36	Ventralized	N	59
cyclin Y	Loss of *sia, nr3* expression; loss of Lrp6 phosphorylation; postgastrula lethality	n.d.	60
Nuclear factors			
β-Catenin[2] *(ctnnb1)*	Ventralized; MO injection can ventralize if injected postfertilization, up to 8-cell stage	Y	61, 62
tcf3	Dorsalized; represses dorsally and ventrally	n.d.	63
pygo1	Ventralized	n.d.	64
tcf1	Dorsalized; represses dorsal genes ventrally, activates dorsally	n.d.	65
tcf4	Ventralized	n.d.	65
bcl9	Ventralized	n.d.	66
prmt2	Ventralized	n.d.	67

JNK, c-Jun NH2-terminal kinase; n.d., occurrence of cortical rotation was not determined; Y/N, yes or no for the occurrence of cortical rotation in the study. Genes that show primary dorsal axis defects when inhibited by antisense oligo (DNA or MO) injection into oocytes followed by host transfer are shown.
[1]The effects of UV are shown for comparison, since these are a predominant method of axis perturbation.
[2]*β-Catenin* is the only gene whose function in primary axis formation can be inhibited by postfertilization oligo injection.

Wnt/β-Catenin Signaling Mechanisms

β-Catenin stabilization is a main output of one arm of the Wnt signaling network, and Wnt/β-catenin signaling mechanisms have been extensively studied and reviewed, owing to the involvement of Wnts in many aspects of development, human cancer, and stem cell regulation (reviewed in Refs 78 and 79). Briefly, in non-Wnt-exposed cells, a cytoplasmic protein complex, containing APC (adenomatous polyposis coli), glycogen synthase kinase 3 beta (GSK3β), and Axin1 (the 'destruction complex'), regulates the constant turnover of β-catenin, the key transcriptional effector of Wnt signaling. Axin serves as the primary scaffold for the degradation complex, facilitating the

phosphorylation of β-catenin by GSK3β, and its priming kinase, casein kinase Iα (CKIα/Csnk1a1).[80,81] Phosphorylated β-catenin is then recognized by specific ubiquitylation machinery and targeted for degradation by the proteasome.[82]

The binding of appropriate Wnt ligands to the coreceptor complex, composed of a Frizzled G protein-coupled receptor and a coreceptor of the lipoprotein receptor-related protein 5/6 (Lrp5/6) subfamily, initiates signaling and inhibits the β-catenin degradation system (reviewed in Refs 78 and 79). The initiating mechanisms are not well understood, but are thought to require recruitment of Axin/GSK3β to the phosphorylated cytoplasmic tail of Lrp6, mediated by Dishevelled (Dvl) family proteins, as well as endocytosis. The main biochemical result of Wnt activation is the inhibition of β-catenin phosphorylation, resulting from the disassembly of the destruction complex, and the inhibition of GSK3β activity by Dvl or phospho-Lrp6, Axin degradation, or some combination of the above (reviewed in Refs 78 and 79). Recent data suggest that GSK3β is sequestered into multivesicular bodies following receptor activation,[83] although it is unclear to what extent this mechanism operates during primary axis formation in *Xenopus*.

β-Catenin Transcriptional Regulatory Mechanisms

The inhibition of β-catenin degradation leads to an overall increase in its steady-state levels, whereupon it translocates to the nucleus and associates with transcription factors of the Lymphoid enhancer-binding factor-1/T cell factor (LEF/TCF) family of high mobility group (HMG)-domain proteins[84,85] to either activate or derepress target gene expression. In *Xenopus*, depletion of maternal *tcf3* mRNA results in ectopic β-catenin target gene activation,[63] suggesting that derepression is sufficient for dorsal gene regulation in early development. *In vivo*, however, β-catenin-dependent transcriptional activation is likely critical as well, as several β-catenin cofactors are required for dorsal gene expression.[64–66] Interestingly, the dorsal axis is most sensitive to manipulation of β-catenin activity during the 16–128-cell stage,[86,87] although major zygotic gene activation does not occur until the MBT, several hours later.[52] Thus, derepression must occur early to prevent dorsal genes from being assembled into repressive chromatin prior to MBT.

Recently, Blythe et al.[67] have provided evidence for this idea. It was demonstrated that β-catenin associates with Wnt target promoters during early cleavage stages, causing epigenetic marking of histone H3 with asymmetric dimethyl arginine. The arginine methyl transferase Prmt2 was identified and shown to be required for normal axis formation through maternal antisense experiments. Furthermore, fusion of Prmt2 with the LEF1 DNA-binding domain could induce secondary axes.[67] Overall, these data suggest that enrichment of β-catenin in dorsal nuclei epigenetically marks dorsal genes for transcriptional activation following MBT. Although this priming function would appear sufficient for dorsal gene activation, the extent to which β-catenin is required for transcriptional activation through Pygo1/Bcl9 recruitment remains to be tested.

ACTIVATION OF Wnt SIGNALING DURING AXIS SPECIFICATION

Despite the requirement for Wnt/β-catenin signaling in dorsal axis formation, exactly how the translocation of dorsal determinants stimulates β-catenin stabilization is less clear. Evidence supports two nonmutually exclusive models: (1) signaling by localized Wnt ligands or (2) the intracellular activation of β-catenin by cytoplasmic dorsal determinants. Recent maternal loss-of-function studies indicate a critical role for extracellular signaling by localized Wnt11b (Wnt11) in *Xenopus* axis formation,[48,50] filling a critical gap in the field. Up until that point, the preponderance of evidence suggested axis formation was Wnt ligand-independent. The extent to which these two possible mechanisms might interact is unknown. It is possible that cytoplasmic determinants could facilitate Wnt11 activity on the dorsal side or that Wnt11 is somehow involved in the production or activation of the dorsal determinants. A model of potential Wnt signaling mechanisms in *Xenopus* axis specification is shown in Figure 3.

Wnt Ligand-Dependent Axis Formation

Ironically, Wnt11 was initially considered as a prime candidate for the 'dorsalizing Wnt'. The mRNA for *wnt11* is maternal and is localized to the vegetal cortex of the oocyte and egg.[88] Also, overexpression assays showed that *wnt11* could induce partial second axes when injected ventrally and could partially rescue UV-irradiated eggs.[88] Other evidence accumulated however, suggesting that Wnt11 might act primarily through 'noncanonical' β-catenin-independent Wnt pathways [Wnt/planar cell polarity (PCP) and Wnt/calcium] to regulate tissue morphogenesis.[89] Arguments against a role for Wnt11 arose out of observations that dominant-negative Wnt11 failed to block *Xenopus* axis formation[90] and that zebrafish maternal/zygotic *wnt11* mutants did not have axis defects.[91]

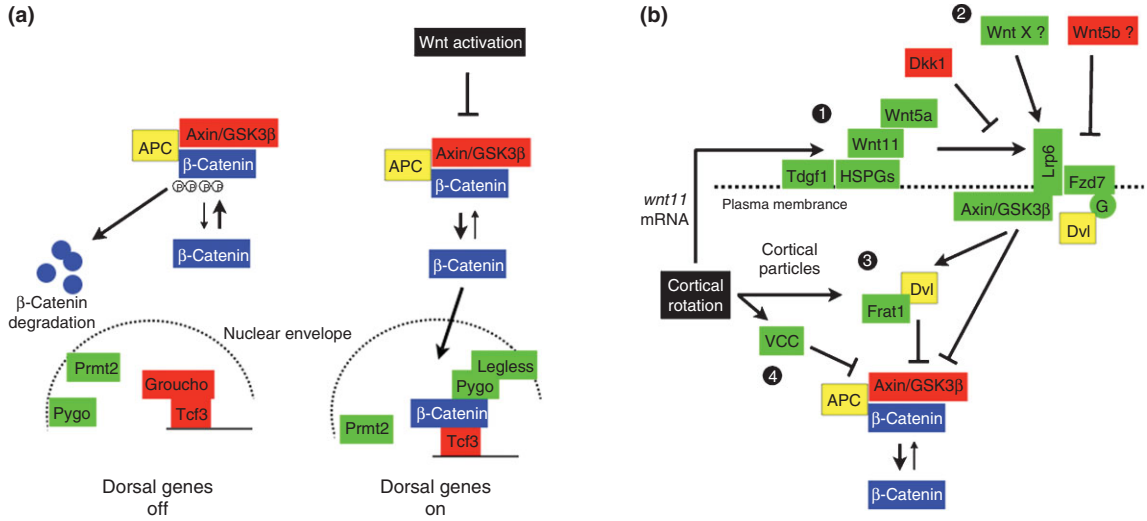

FIGURE 3 | Models for Wnt/β-catenin signaling regulation during *Xenopus* axis formation. (a) Diagram of overall Wnt/β-catenin signaling. In ventral cell, β-catenin is phosphorylated by the destruction complex [Axin/adenomatous polyposis coli (APC)/GSK3β] and targeted for degradation. In those nuclei, Tcf3 represses critical dorsal gene expression. In a dorsal cell, Wnt activation leads to inhibition of destruction complex activity and accumulation of free β-catenin, which can enter the nucleus. β-Catenin is assembled into transcriptional complexes with Tcf3/Pygo/Legless at sites primed by Prmt2 and dorsal genes are expressed. (b) Potential mechanisms of β-catenin activation in the *Xenopus* embryo. (1) Enrichment of wnt11 RNA dorsally, followed by activation by secreted Wnt11/5a/Frl1 complexes or (2) an unknown Wnt ligand; (3) intracellular activation by Frat1/Dvl2 particles or (4) an unknown component of vegetal cortical cytoplasm (VCC). Predominantly positive-acting components are shown in green, negative components in red. Molecules with potentially dual or noncanonical roles are shown in yellow. Arrowed lines indicate positive regulation and 'T' bars indicate negative regulation. The arrangement of the boxes is not meant to convey specific exact binding relationships or stoichiometry.

However, a reinvestigation of maternal Wnt11 function following maternal mRNA depletion in the oocyte found that Wnt11 is indeed required for dorsal axis formation in *Xenopus*.[48] Previous antisense experiments had shown that maternal *fzd7*, a putative Wnt11 receptor, was also required for normal axis formation.[51] In addition, Wnt11 function required extracellular heparin sulfate proteoglycans, as well as the Frl1/Tdgf1 protein, typically considered a Nodal coreceptor.[48] The significance of this interaction with Tdgf1 has not been further examined, but the point was made that secreted proteins were needed for axis formation, in addition to potential intracellular activators.

Subsequent maternal mRNA depletion studies found that the Wnt11 likely acts through the Wnt coreceptor Lrp6.[49] Recent data also implicate *Xenopus* Wnt5a in axis formation, acting in complexes with Wnt11.[50,92] The formation of these multi-Wnt complexes required sulfation of conserved tyrosines in Wnt11/5a, a Wnt modification not previously reported.[92] Interestingly, Lrp6 and Wnt11 signaling appears to regulate steady-state β-catenin and Axin levels in stage VI oocytes, prior to maturation or fertilization. Depletion of either Wnt11 or Lrp6 causes a decrease in β-catenin abundance and a concomitant increase in Axin1 abundance in oocytes and cleavage-stage embryos.[49]

These experiments raise the issue of the timing of Wnt11 signaling. It is possible that manipulation of Wnt11 signaling in oocytes signaling skews the initial stoichiometry of regulatory components, such as increasing Axin1 levels, which could then result in ventralization. Axin has been proposed to be limiting in the β-catenin destruction complex,[93] thus small changes in Axin may have profound changes in β-catenin abundance. Also, in oocytes, Lrp6 is phosphorylated at a serine implicated in receptor activation (S1490), whereas this phosphorylation is removed in fertilized eggs, providing additional evidence for the importance of oocyte signaling.[60]

Alternatively, Wnt11 could act during the cleavage stages, as has been proposed, since Wnt11 protein is enriched dorsally.[94] The mechanism for this enrichment is unclear as both overall dorsal enrichment of *wnt11* RNA transcripts[48] and dorsal-specific polyadenylation and translation of *wnt11* mRNA[94] have been reported using differing methodologies. In either case though, the enrichment would depend on cortical rotation.

Wnt11 and Wnt5a appear to act together in axis formation; however, other Wnts and Wnt receptors are expressed maternally in *Xenopus* and their roles remain to be characterized. These include *wnt5b*, *wnt7b*, and *wnt8b*, and many *frizzleds* (reviewed in Ref 95), as well as both coreceptors.[96] In zebrafish,

maternal effects mutations in *wnt5b (pipetail)* have reduced calcium release and are dorsalized, resulting from disinhibition of maternal β-catenin signaling.[97] A similar ventralizing Wnt/calcium pathway is proposed to exist in *Xenopus*,[98] although this pathway has yet to be tested by maternal loss-of-function analyses in *Xenopus*.

Wnt Ligand-Independent Axis Formation

Separate from the issue of Wnt11, evidence indicates that the transplantable dorsalizing activity in eggs functions like an intracellular activator of β-catenin stabilization as opposed to a Wnt ligand.[99] Notably, injection of cortical cytoplasm into animal caps could mimic Wnt activation, causing β-catenin accumulation and ectopic expression of direct β-catenin target genes, *siamois (sia1)* and *nodal-related 3 (nr3.1)*.[96] Additional support for ligand independence came from experiments showing that injection of dominant-negative Dvl2 (Xdd1[100]) and the secreted Wnt antagonists *dkk1*[101] and *frzB*[102,103] into fertilized eggs could inhibit zygotic Wnt activity and that of exogenous injected Wnts, but did not block endogenous axis formation.

The identity of the putative Wnt activator in the vegetal cortical cytoplasm remains undetermined. Depletion of β-catenin RNA from the donor egg did not affect dorsalizing activity of transplanted cytoplasm, ruling out β-catenin itself as the active component, although β-catenin protein had been reported to translocate with the vegetal microtubule array during cortical rotation.[6] Epistasis experiments comparing the activity of vegetal cortical cytoplasm with that of known Wnt activators implicated APC or a negative regulator of APC as possible dorsalizing components.[99] However, the role of APC in *Xenopus* is still unclear[104,105] and loss-of-function experiments have not been reported. APC has been shown to have a positive role in Wnt/β-catenin signaling by promoting Axin degradation,[106] in addition to its traditional role in facilitating β-catenin degradation. APC is an interesting candidate as it regulates microtubule dynamics[107] and associates with microtubule plus ends,[108] suggesting it could be transported during cortical rotation.

Dishevelled and GSK binding protein/frequently rearranged in advanced T-cell lymphomas 1 (GBP/Frat1),[25,53] an inhibitor of GSK3β that is necessary and sufficient for axis formation in *Xenopus*,[53] are the main candidates for the vegetal dorsalizing factors. Dvl and Frat1 GFP-fusion proteins localize to punctate particles in eggs,[24,53] which were thought to be vesicles based on their resemblance to $DiOC_6(3)$-stained organelles. Also, these fusions are translocated dorsally by the fast transport mechanism during cortical rotation.[24,53] Interestingly, overexpressed Frat1 can reduce GSK3β protein levels, mimicking a reduction in cortical GSK3 levels that was observed following cortical rotation.[109] By contrast, Wnt and Dvl overexpression reduces GSK3β-specific activity.[109,110] In addition, Dvl2 can bind to Frat1 and the two molecules synergize in the activation of Wnt signaling in overexpression assays,[111,112] suggesting that complexes of Dvl2/Frat1 might act as dorsal determinants in the embryo.[13]

Although this model is compelling, several observations question the extent of the roles of Dvl/Frat1 in cortical cytoplasm. First, the specificity of the transport of Dvl2-GFP and Frat1-GFP and the nature of these particles is unclear. Because different types of structures can move dorsally during cortical rotation, including vesicular organelles, pigment granules, and exogenous fluorescent beads,[23] it is possible that any sufficiently large complex might undergo dorsal translocation. The punctate structures formed by Dvl2 could be vesicles,[113] Lrp6 signaling endosomes,[114] or assemblies of Dvl protein polymer.[115] It is unclear at present what the biological significance of these puncta are or which of these the Dvl2 particles in *Xenopus* eggs represent.

Second, loss-of-function studies suggest Dvl2 has an inhibitory role in axis formation. Partial depletion of maternal *dvl2* mRNA using antisense oligos reduced maternal JNK1/Mapk8 activation, a condition that tends to dorsalize embryos.[55] A more comprehensive study of maternal Dvl2 and Dvl3 function also failed to indicate a role in axis formation, finding instead ectodermal and convergent extension defects.[56] The depletion strategies used, however, failed to fully eliminate cortical Dvl, which could represent a sequestered pool of proteins necessary for dorsalizing activity.

Third, although maternal *frat1* is required for endogenous dorsal axis formation,[53] genetic studies in mice found that triple knockouts of all three *Frat* genes were normal phenotypically and cells treated with exogenous Wnt showed normal responses.[116] Frat1 could thus be required for Wnt signaling specifically in *Xenopus*, possibly downstream of Wnt11, or it could have a role outside of Wnt/β-catenin signaling. Recent data from cell culture experiments indicate that Frat1 interacts with the ankryin repeat protein Diversin/Ankrd6 in both Wnt/β-catenin and Wnt/PCP pathways.[117]

It is notable that other proposed dorsalizing molecules in *Xenopus*, such as Dvl, Fzd7, and Wnt11, also act in both β-catenin-dependent and -independent

Wnt signaling. It is unclear how Wnt/PCP signaling might be required for axis formation, as this pathway is typically thought to antagonize Wnt/β-catenin. Both pathways might be necessary in parallel or axis formation might require dynamic modulation of signals between the two. Wnt11 regulates both the Wnt/β-catenin and Wnt/PCP pathways during foregut differentiation and morphogenesis, providing evidence for this idea.[118]

SETTING THE STAGE: LOCALIZED mRNAs IN *XENOPUS*

Many of the molecules critical for *Xenopus* axis formation are localized to the vegetal cortex of the oocyte and egg. There are two main mechanisms for localizing molecules to this region during oogenesis (Figure 4). The first is the translocation of the mitochondrial cloud (Balbiani body) to the future vegetal pole during early oogenesis.[119] The mitochondrial cloud is a prominent feature of previtellogenic stage I oocytes and contains numerous mitochondria, ER, and the germ plasm[120,121] itself composed of germinal granules and associated localized mRNAs.[122] The cloud fragments during stage II and disperses to the future vegetal pole. By stage III, the cloud material occupies a limited area within the future vegetal cortex. Fragmentation continues during the remainder of oogenesis so that the germ plasm is dispersed into numerous cortical islands centered on the apex of the vegetal pole.[119] The forces that drive the disruption and cortical localization of the cloud are not known and will likely be difficult to identify as the process occurs over an extended period of time.

A number of RNAs essential for germline determination localize to the mitochondrial cloud in stage I oocytes and translocate vegetally with the cloud material. These include *xcat2/nos1*,[72] *dazl*,[123] and *deadend homolog 1/dnd1*,[124] supporting the idea that localized RNAs are critical for germ plasm function. Interestingly, the final distribution of germ plasm in the oocyte and egg roughly matches the distribution of the vegetal cortical cytoplasm, suggesting that the germ plasm may contain dorsalizing components as well.

The second vegetal localization mechanism begins around stage III of oogenesis and transports a subset of localized RNAs including *vg1* and *vegt* to the cortex of the vegetal hemisphere.[122] Prior to this stage, these RNAs are uniformly localized. By stage III, vitellogenesis begins and animal–vegetal polarity is apparent. The so-called late pathway of RNA localization follows a subdomain of ER from the germinal vesicle to the vegetal pole.[125] The previous localization

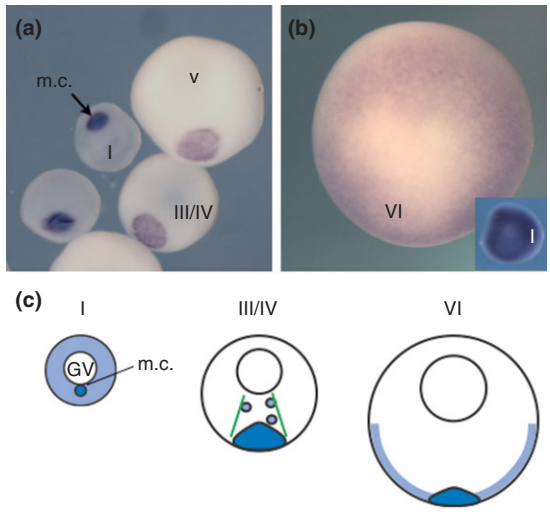

FIGURE 4 | Localized messenger RNA (mRNA) in *Xenopus*. (a) Expression of a germ plasm-localized mRNA during oogenesis, showing restriction to the mitochondrial cloud (m.c.; arrow) in stage I oocytes. Expression in later stages (stage III–VI) is localized to a compact domain at the vegetal apex. (b) Expression of a late pathway mRNA in a stage VI oocyte (vegetal view). Expression is broadly distributed throughout the vegetal hemisphere. Inset shows nonlocalized expression in a stage I oocyte. Images in (a) and (b) are not to scale. (c) Diagram of mRNA localization pathway in *Xenopus* oocytes. Germ plasm-localized mRNAs are indicated in dark blue, late pathway mRNAs are in light blue. Microtubules involved in late localization are in green. GV, germinal vesicle. Oocyte stages are indicated above each diagram.

of the mitochondrial cloud may establish this domain as it moves vegetally. Late pathway function is dependent on microtubules, but not on other cytoskeletal elements.[126] General RNA localization mechanisms have been reviewed elsewhere and involve the binding of *trans*-acting proteins to repeat-containing localization elements in the 3′ untranslated regions (3′UTRs) of mRNAs.[127] Several of these *trans*-acting proteins, hnRNPI/Ptbp1,[128] Vg1rbp/Vera/Igf2bp,[125] and Staufen1/Stau1,[129] are conserved regulators of RNA localization in many organisms. These proteins interact with the *cis* elements on RNAs and are also likely involved in coupling ribonucleoprotein complexes to kinesin motors.

Interestingly, although the majority of microtubules in the oocyte are oriented with minus ends toward the cortex,[130] a subpopulation of microtubules exists with plus ends oriented toward the vegetal cortex along the vegetal localization path.[131] It is possible that dorsal determinants could use these transport mechanisms either directly or indirectly as part of the germ plasm to achieve vegetal cortical localization during oogenesis.

Recent evidence points to roles for germ plasm-localized mRNAs in different aspects of axis

formation. As previously discussed, *wnt11* localizes early to the mitochondrial cloud/germ plasm and is required for Wnt signaling during axis specification. Additional work has identified two germ plasm-localized RNAs with potentially novel roles in regulating cortical rotation, *fatvg/perilipin 2* (*plin2*[57,132]) and *tripartite motif containing 36* (*trim36*[59]).

Embryos depleted of *plin2* mRNA (*fatvg*[132]) were ventralized owing to a failure of cortical rotation, and also showed precocious germ plasm aggregation.[57] The mechanisms of Plin2 function in cortical rotation are unclear, and status of the microtubule array was not investigated. Plin2 is a conserved component of lipid droplets, the main lipid storage organelles in the cell (Box 1).[133] Consistent with this localization, immunostaining and electron microscopy showed Plin2 protein localized to the surface of lipid droplets in the vegetal cortex of *Xenopus* oocytes.[57] Plin2 could possibly regulate lipid droplet lipid metabolism, which might be critical for cortical rotation, an energy-intensive process. Alternatively, Plin2 might regulate lipid droplet transport via microtubules, which could help facilitate cortical rotation or dorsal transport. Supporting this idea, a perilipin homolog in *Drosophila* mediates directional transport of embryonic lipid droplets, which also depends on coordinated dynein and kinesin motor activity.[134] Unfortunately, except for their transport mechanisms, lipid droplet function has not been extensively studied in early development.

Trim36 was identified in a microarray screen for vegetal cortex-enriched transcripts and encodes a single RING-finger-type ubiquitin ligase.[59,135] Depletion of *trim36* in oocytes by antisense oligo injection also produced ventralized embryos that failed to assemble microtubules and undergo cortical rotation.[59] In rescue experiments, a mutant construct deficient in ubiquitin ligase activity failed to rescue the effects of *trim36* depletion on microtubule assembly, suggesting that Trim36-dependent ubiquitylation might normally promote microtubule organization or stability.[59] In human Opitz syndrome, mutations in a related ubiquitin ligase, TRIM18/MID1, result in abnormal accumulation of protein phosphatase 2A (PP2A) specifically on the microtubules, causing microtubule abnormalities.[136] Similarly, Trim36 could target the microtubule pool of a protein critical for stabilizing microtubules during cortical rotation. Unlike Trim18, Trim36 does not bind to α4, suggesting that Trim36 regulates targets other than PP2A.[137] Interestingly, ubiquitin has been found to colocalize with microtubules in cultured cells,[138] suggesting a general importance of ubiquitylation to microtubule assembly.

trim36-Depleted embryos also failed to stabilize β-catenin and to express Wnt target genes.[59] Inhibition of microtubule assembly during cortical rotation using UV irradiation results in ectopic localization of β-catenin[7] and of Wnt targets (*sia1, nr3.1*) to the vegetal pole.[139,140] Although it is difficult to compare the broad effects of UV with molecular perturbations, it is possible that the transport and activation of Wnt components along microtubules are more interrelated than previously appreciated. Alternatively, Trim36 could regulate proteins that control both microtubule assembly and Wnt signaling.

The linkage of germ plasm RNA localization during oogenesis with the transport of dorsal determinants is interesting from the evolutionary perspective as well. Teleost fish, such as medaka and zebrafish, do not undergo cortical rotation,[9] but do depend on microtubule-based transport of determinants in the vegetal pole of the zygote following fertilization.[141,142] Blocking microtubules at this stage produces ventralized embryos.[141,143] Aligned microtubules form during the cleavage stages, extending from the marginal blastomeres to the vegetal pole for the yolk cell.[144] Transport of cellular lipid droplets and fluorescent beads has been visualized moving

BOX 1

LIPID DROPLETS

Lipid droplets are ubiquitous organelles in the cell that are responsible for the storage and regulation of neutral lipids and various sterol esters and di- and triacylglycerols (reviewed in Ref 133). They are composed of an inner core of lipids surrounded by a phospholipid monolayer. Proteins coating lipid droplets include perilipin family members and lipid metabolic enzymes (e.g., fatty acyl coA transferases). Lipid droplets are thought to bud from the ER, and are trafficked in the cell by microtubule motors. One of the main functions of lipid droplets is to regulate lipid storage and mobilization for metabolic activities. Lipid droplets interact with a variety of other organelles, such as mitochondria and endosomes, and are thus likely to have many additional functions in the cell. The control of lipid droplet formation and function are not well understood. Abnormal lipid accumulation in lipid droplets has been implicated in metabolic diseases such as atherosclerosis, diabetes, and obesity. Understanding lipid droplet biology is thus central to understanding basic cell physiology and its underlying role in human disease.

along these arrays, suggesting that determinants are moved from the dorsal side of the yolk cell to the dorsal blastomeres and yolk syncytial layer.[144,145] β-Catenin subsequently becomes localized to nuclei in this region[7] and activates dorsal gene expression in the embryonic shield.[10]

A recent analysis of the ventralized tokkaebi (*tkk*) mutant zebrafish identified a role for the syntabulin (*sybu*) gene product in dorsal transport.[146] Syntabulin is a linker protein for kinesin 1 and has been implicated in presynaptic protein localization and mitochondrial localization in neurons.[147] Strikingly, *sybu* mRNA is localized to the mitochondrial cloud/Balbiani body of zebrafish oocytes, which mediates subsequent *sybu* localization to the vegetal pole.[146] Sybu protein is enriched asymmetrically in zygotes in a microtubule-dependent manner and is required for direct Wnt target gene expression in blastulae.[146] These data suggest that Sybu might be transporting dorsal determinants and are consistent with the idea that dorsal determinants and dorsal transport factors preferentially use the mitochondrial cloud to achieve vegetal localization in the zebrafish. It would be interesting to learn whether *plin2* and *trim36* are localized in zebrafish oocytes and play roles in microtubule regulation or to what extent these genes only function in *Xenopus* or in embryos that undergo a typical cortical rotation.

CONCLUDING REMARKS

Cortical rotation is essential for dorsoventral axis formation in *Xenopus* and is one of the best-characterized examples of the asymmetric distribution of developmental signaling molecules. Despite this importance, the mechanisms regulating microtubule assembly and transport of molecules during cortical rotation are unknown. The potential involvement of lipid droplets and the ubiquitin pathway suggests that much more needs to be learned about the basic cell biology of cortical rotation. Recent advances in the imaging of fluorescent molecules might render the regulation of microtubule dynamics more experimentally tractable. How vegetal localization pathways in the oocyte are coordinated with the events of cortical rotation also remains an open question. In addition to localized products being important, it is tempting to speculate that molecules used in vegetal transport might be reiteratively used for cortical rotation.

The nature of the cytoplasmic dorsal determinants has also defied explanation. These determinants are thought to facilitate Wnt/β-catenin signaling, but the mechanism is unknown. Indeed, the general mechanisms of Wnt signaling initiation are only partially understood. Elucidating the process of endogenous Wnt activation in a living organism such as the *Xenopus* embryo, as opposed to cultured cells, will be important for understanding Wnt biology *in vivo*. Also, work in *Xenopus* has shown that different Wnt ligands can interact to enhance signaling. As many tissues coexpress multiple Wnts in normal development (the mammalian blastocyst expresses all of them[148]), it will therefore be important to determine how signaling from different Wnt ligands is integrated within the cell.

Overall, this work on cortical rotation has broad implications for understanding important aspects of cell and developmental biology. The transport of critical signaling molecules along microtubules occurs in a variety of other cell types, including stem cells and neurons. Also, polarized microtubule arrays are common in epithelial cells and neurons, but it is not clear to what extent various signaling pathways depend on these structures. In general, the coupling of signaling activities with underlying cell biological mechanisms is a little studied but an important area of research. Also, embryonic cytoplasmic localization and β-catenin asymmetry are required for endoderm formation and gastrulation in many bilaterian animals (reviewed in Refs 149 and 150). Understanding the polarization of β-catenin-stabilizing activities will therefore shed light on the evolution of animal development.

Thus, understanding the mechanisms of cortical rotation in *Xenopus* will likely have broad implications for understanding vertebrate evolution as well as the connection between microtubules and Wnt signaling in human disease.

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