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# Rab5-mediated Yolk Cell Endocytosis modulates Zebrafish Epiboly Biomechanics and Tissue Movements

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**Summary**

Morphogenesis in early embryos demands the coordinated distribution of cells and tissues to their final destination in a spatio-temporal controlled way. Topographical and scalar differences in adhesion and contractility are essential for these morphogenetic movements, while the role that membrane remodeling may play remains less clear. To determine how surface turnover may modulate tissue arrangements during embryogenesis we studied epiboly in the zebrafish. Experimental analyses and modeling have shown that the expansion of the blastoderm during epiboly relies on an asymmetry of mechanical tension along the embryo surface. In this scenario, we found that membrane turnover at the external yolk cell syncytial layer (E-YSL) in early zebrafish embryos is mainly directed by the GTPase Rab5ab. Rab5ab was essential for endocytosis, and interference in its expression resulted in reduction of yolk cell acto-myosin contractility, disruption of cortical and internal flows, a disequilibrium in force balance and epiboly impairment. We conclude that regulated membrane remodeling is crucial for directing cell and tissue mechanics and coordinating morphogenetic movements.

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## INTRODUCTION

Membrane turnover can be clathrin-dependent, caveolae-mediated or via macropinocytosis. Clathrin-mediated endocytosis (CME), a mechanism for controlled cargo uptake, is the most general membrane internalization route. It is characterized by the formation of clathrin-coated vesicles (CCV) and the selective internalization of cell-surface components and extracellular macromolecules (for review see [1]). CME depends on dynamin activity for vesicle budding and scission, which leads to trafficking through the endocytic pathway upon clathrin coat disassembling. Caveolae (which incorporate caveolin) are also widely employed by cells for membrane removal. They originate in the Golgi apparatus, associate to cholesterol pools and are involved in the internalization of specific markers and perhaps in transcytosis. Finally, internalization can also take place by macropinocytosis, which usually occurs within highly ruffled regions of the plasma membrane, and is involved in large-scale membrane internalization [2-4]. Macropinocytosis is both Rac1- and actin-dependent and many studies have linked it to the ability to form membrane folds. Importantly, Rab5 (usually implicated in CME [5]) and its effector rabankyrin 5 are also involved in macropinocytosis [6-8]. Whatever their origin, vesicle trafficking through the endosomal route is controlled by specific Rab guanosine triphosphatases (GTPases): Rab5 for internalization and merging into early endosomes; Rab4 for fast recycling towards the plasma membrane; Rab11 for the recycling endosome; and Rab7 for late endosome transport to lysosomes amongst others (reviewed in [9]).

Endocytosis and recycling have been linked to different cellular maturation in several ways (e.g. dendritic arborization in *Drosophila* larvae revealing an important link between microtubule motors and endosomes [10]) or morphogenetic processes (reviewed in [11]) affecting the regulation of surface area [12, 13], tension or contraction [14, 15]. Cellularization and dorsal closure in *Drosophila* and neurulation and apical constriction of bottle cells during gastrulation in *Xenopus laevis* are just some examples of processes demanding a tight control of membrane dynamics [13, 16, 17]. Dynamin and Rab5 are necessary downstream of actomyosin contractility to remove excess membrane. Disrupting endocytosis with dominant-negative Dynamin or Rab5 further perturbs neurulation in *Xenopus*, inhibiting apical constriction in hingepoint cells and resulting in neural tube closure defects [13]. Last, Rab5 mediates the cohesion

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of mesodermal and endodermal (mesendodermal) progenitor cells during zebrafish gastrulation. Endocytosis seems to affect Silberblick (slb)/Wnt11 activity and Rab5c modulates the ability of cells to aggregate via E-cadherin into distinct clusters, which is necessary for the coordinated movement of the prechordal plate [18]. The modulation of the balance between endocytosis and recycling can regulate cellular morphology and tissue deformations and could therefore represent a general mechanism to achieve efficient invagination in embryos.

We aimed to understand the role that membrane remodeling might have during epiboly in zebrafish, a conserved early morphogenetic event. At the onset of zebrafish epiboly (sphere stage), a superficial layer of cells, the enveloping layer (EVL) covers a semi-spherical cap of blastomeres centered on the animal pole of the embryo sitting on a massive yolk syncytial cell. Epiboly consists of the cortical vegetal ward expansion of the EVL, the deep cells (DCs) of the blastoderm and the external layer of the syncytial yolk cell (E-YSL) around the yolk. Epiboly ends with the closure of the EVL and the DCs at the vegetal pole [19-21] (**Figure 1A**). Epiboly progression entails a coordinated series of cellular events. EVL cells and DCs proliferate and exchange neighbours resulting in EVL expansion and DCs intercalation [14, 22]. At the yolk cell, the E-YSL membrane becomes highly convoluted [23] and gradually narrows by localized contraction [24]. As the EVL margin advances, myosin and polymerized actin get progressively confined to a belt at the animal edge of the E-YSL and to the vegetal cap [22, 25]. In the E-YSL, actin is conscripted within and beneath the highly dynamic convoluted ruffles [24]. Actin accumulation is accompanied by myosin phosphorylation [25]. Remarkably, the narrowing of the E-YSL occurs in synchrony with cortical retrograde actin and myosin flows originating at the vegetal pole [26]. Moreover, internally and coupled to epiboly progression, yolk granules sustain stereotyped dynamic movements [24].

It has been suggested that longitudinal and latitudinal tensional forces originating at the E-YSL constitute the major force-generating elements driving epiboly [22, 25, 27, 28]. It has also been proposed that for the vegetal ward movement of the blastoderm some source of tension must be coupled to the contractile E-YSL. Flow-friction motors could generate a pulling longitudinal force through resistance against the retrograde actomyosin cortical flows in the yolk [26]. Alternatively, a positive vegetal ward oriented latitudinal tension gradient at the yolk cell membrane from the EVL margin

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could convey the stress originated by the constriction of the actomyosin ring at the E-YSL [24].

Still, whatever the mechanical means involved are, epiboly must overcome the hindrance that the yolk cell membrane poses to its progression. The EVL does not slide over the yolk cell surface and is firmly attached to it [24, 25]. Thus, as it expands, the yolk cell membrane subsides [14, 22, 27] suggesting that membrane removal at the yolk cell may constitute an essential event enabling epiboly progression [14, 27].

To analyze the role of membrane remodeling in the yolk cell during epiboly we first topographically and temporally characterized its progression as epiboly proceeds. Then, we found that the activity of Rab5ab, a key element of the endosomal route and a mediator of macropinocytosis [9], is essential for yolk cell membrane turnover and epiboly morphogenetic movements. Indeed, it was previously found that knockdown of *rab5ab* produced a striking morphological phenotype during gastrulation [29]. Our results differ from previous reports interfering with Dynamin 2 activity (clathrin-mediated endocytosis) [30], which indicated that the removal of the yolk cell membrane was dispensable for epiboly. We also found that Rab5ab is essential for the proper recruitment of actin and myosin to the E-YSL and its contractile activity affecting EVL cells elongation and internal yolk flows. Impairing Rab5ab expression alters the epiboly's biomechanical landscape decreasing the yolk cell surface tension leading to a reduction on the strength of the E-YSL as a mechanical power source. Altogether, our data show that localized membrane removal in the yolk cell constitutes a necessary step for epiboly progression bridging cellular, geometrical and mechanical constrains.

## RESULTS

### E-YSL membrane dynamics

To study zebrafish embryo membrane turnover during epiboly we employed fluorophore-conjugated lectins to label its external membranes. Lectins bind to glycoproteins and glycolipids and have already been used to follow plasma membrane dynamics in other teleost embryos [22]. Upon soaking the embryos in fluorophore-conjugated lectin-containing media, both, the whole yolk cell and the EVL external

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membranes were quickly homogeneously decorated. Immediately after, lectin-enriched spots, resembling endocytic vesicles, deposited beneath the yolk cell membrane, accumulating in a circumferential ring ahead of the EVL leading cells (**Fig. 1B** and **Movie S1**). Membrane removal ahead of the EVL can be observed as early as the sphere stage and co-localized with the convoluted E-YSL, where actin and myosin progressively gather [26]. We observed a tight spatiotemporal correlation between the narrowing of the endocytic belt and the reduction of the width of the E-YSL (convoluted yolk cell surface) (**Fig. 1C**), which suggests an intimate relationship between both events. Remarkably, fluid phase endocytosis, reported by the uptake of fluorescent dextran [29, 31], displayed the same pattern as lectin internalization (**Fig. 1D**).

To precisely map and characterize yolk cell membrane turnover, we locally marked the membrane by laser photobleaching and followed its dynamics *in vivo*. Membrane photobleached regions away from the EVL edge remained static, indicating the lack of major lateral diffusion within the yolk cell membrane, up to the time when the photobleached areas of the yolk cell got embedded in the advancing E-YSL. At this time the tagged membrane subdued and became endocytosed (**Fig. 1E** and **Movie S2**). The photobleached area linearly reduced its size and was finally eliminated before contacting the EVL margin (**Fig. 1F**).

The observed dynamics of the yolk cell membrane confirms that the EVL does not slide over the yolk cell [25] and suggests that its progression demands a progressive removal in an animal-vegetal direction of the E-YSL, so that the overall surface of the embryo remains constant.

**Rab5ab-mediated endocytosis is required in the yolk cell for epiboly progression**

We found that the turnover of the yolk cell membrane is topographically associated with the E-YSL proximal domain. The extensive convolution of this area [24] points to non-clathrin dependent macropinocytosis as the mechanism for its removal. Indeed, abolishing Dynamin-2 expression in the yolk cell results in just a partial inhibition of endocytosis not affecting epiboly progression [30].

To fully block endocytosis we set out to interfere with *rab5* expression, a key element for both vesicle internalization and targeting to early endosomes and macropinocytosis

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[9]. To target just the yolk cell without affecting the blastoderm, we performed morpholino (MO) injections into the yolk synctium (YMOs), after its formation at the 512-1024 cell-stage. In this way, injected MOs remained confined to the yolk cell and were not mobilized to the rest of the embryo [32]. Yolk injection of mRNAs or fluorescently-tagged MOs at these stages showed restricted expression in the yolk cell (**Fig. S1**).

In zebrafish, there are five annotated *rab5* genes (*rab5aa*, *rab5ab*, *rab5b*, *rab5c* and *rab5clike*). Of these, *rab5aa*, *rab5ab*, and *rab5c* were known to be ubiquitously expressed, unlike *rab5b*, whose expression is limited to the yolk cell syncytial layer, pronephric duct, and telencephalon [33].

We tested whether inhibition of different *rab5* genes prevented membrane removal by monitoring fluid-phase dextran endocytosis. Three different *rab5ab* morpholinos, Rab5ab YMO1 (ATG), YMO2 (UTR) and YMO3 (UTR-ATG) injected in the yolk caused the same phenotype, while a control mismatched MO or a *rab5c* YMOs [34] did not. It is important to note that MO1 and MO2 had been previously employed to study the role of Rab5ab in nodal signalling during gastrulation. In this context, the effect of both MOs on *gsc* expression was fully rescued by co-injection of a *rab5ab* RNA ensuring their specificity [29].

We found that Rab5ab depletion just in the yolk cell led to deficient membrane removal (**Fig. 2A** and **Movie S3**). Quantitative analysis further showed that the number of internalized dextran-containing vesicles in Rab5ab YMOs was reduced by 84 % (n = 7), while interfering with the expression of Dynamin *2* in the yolk cell just reduced this figure by 48 % [30].

Importantly, dose-dependent Rab5ab yolk cell depletion resulted in a strong early epiboly delay and arrest although other gastrulation and morphogenetic movements (invagination, convergence and extension, and somitogenesis) and head and trunk development seemed mostly unaffected initiating timely (as in age-matched control siblings) (see **Fig. 2B** and **Movie S4**). Rab5ab YMOs displayed a dose-dependent response. Prior to epiboly no apparent phenotype was observed in any case. However, different degrees of delay were observed from dome stage. At a medium dose (4 ng / embryo), *rab5ab* YMOs domed in a timely manner comparable to control embryos but slowed down immediately after, halting at 70 % epiboly. When control injected embryos reached the shield stage, medium dose Rab5ab YMOs had not progressed

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beyond 30 % and when the DCs of controls closed the yolk cell plug, Rab5ab YMOs remained at 60 % epiboly (compare **Fig. 2C** to **2D**; see **Movie S5**). High dose yolk cell-injected embryos (8 ng / embryo) consistently exhibited a strong epiboly delay and never progressed beyond 50 %, when the yolk cell burst (compare **Fig. 2C** to **2E**; see **Movie S6**). Epiboly arrest correlated with a progressive folding of the DC layer that detached from the YSL, and with a major constriction at the surface ahead of the EVL margin. Alongside epiboly delay, Rab5ab YMOs failed to thin the expanding blastoderm, which retracted animalward.

Altogether, these data indicate that *rab5ab* dependent endocytosis is involved in local yolk cell membrane removal at the E-YSL and that this is necessary to enable epiboly progression.

**Rab5ab activity in the yolk cell affects cortical actomyosin and, non-autonomously, EVL shape and yolk granules dynamics**

The epiboly progression defects observed by inhibition of Rab5ab expression in the yolk cell were associated with multiple cellular and structural defects. The local recruitment to the E-YSL of actin and activated myosin was compromised by middle dose interference in yolk cell *rab5ab* expression. The levels of both proteins, detected with phalloidin and an anti phospho-myosin antibody, respectively, were on average strongly reduced in Rab5ab YMOs (**Fig. 3A** and **3B**). These reductions were linked to defects in the retrograde cortical myosin flow that originates at the vegetal pole and sinks into the E-YSL in wild-type embryos [26]. Live time-lapse imaging of transgenic Tg (β-*actin:MYL9L-GFP*) embryos revealed that the magnitude of the yolk cell cortical myosin flows was reduced and their directionality altered (**Fig. 3C** and **Movie S7**). The narrowing of the actin-rich convoluted E-YSL, the major source of force generation during epiboly [24], was also affected. In these YMOs the E-YSL became wider overtime (quantified from surface projections of membrane-GFP tagged embryos - see Experimental Procedures) than in wild type (**Fig. 3D**).

These direct effects of interfering with membrane endocytosis on the yolk cell cortical actomyosin dynamics were accompanied by non-autonomous changes in the shape of EVL cells. EVL cells flattening and elongation (animal to vegetal - AV), which are known outcomes of the accumulation of actin and activated myosin in the E-YSL [25],

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were prevented in Rab5ab YMOs, where the leading EVL cells elongated in the dorsal to ventral (DV) direction (**Fig. 3E** and **Movie S8**). We reasoned that these altered shapes respond to changes in the tension anisotropy within the E-YSL, which as a rule in normal conditions, increases as epiboly progresses [24].

Last, we found that the stereotyped movements of yolk granules, which passively respond to the cortical stresses at the E-YSL [24], became unbalanced upon Rab5ab depletion. Velocity fields, estimated by Particle Image Velocimetry (PIV) from meridional multiphoton microscopy sections, showed that the yolk granules regular toroidal vortices associated with epiboly [24] were completely disrupted (see **Fig. 4A** and **Movie S9**). In Rab5ab YMOs, the yolk granule flows became uncoordinated showing a noticeably slower kinetics.

In summary, our data indicate that membrane removal at the E-YSL mainly mediated by Rab5ab is necessary for the correct structural organization and activity of the E-YSL. Endocytic activity in the yolk cell, ahead of the EVL, would influence E-YSL contractility, which in turn would non-autonomously affect both EVL cells elongation and the pattern of yolk granules flows.

**Epiboly mechanics in Rab5ab YMOs**

We found that in addition to the structural defects of the E-YSL upon epiboly arrest, the overall geometry of the embryo was affected in Rab5ab YMOs as a result of the impairment of yolk cell membrane removal. The final shape of the embryos was rather an ellipsoid than a sphere with an elongated animal to vegetal (AV) axis (see **Fig. 2** and **Movie S5**). This elongated shape resulted from both the animalward expansion of the blastoderm after 50 % epiboly and the slight elongation of the yolk cell towards the vegetal pole. The altered geometry of these embryos suggests that their global biomechanics was compromised and that the topography and dynamics of stresses during epiboly were disrupted. To explore this possibility, we analyzed the spatio-temporal profile of mechanical power and cortical tension of Rab5ab YMOs by Hydrodynamic Regression (HR) [24]. Briefly, throughout epiboly in wild-type embryos, HR reveals a stereotyped mechanical power density pattern. At the epiboly onset, the active structures mainly map to the blastoderm. Then, once the EVL crosses the equator, the largest mechanical power density localizes in the active, actomyosin-

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rich, E-YSL while the adjacent EVL cells opposes deformation. Further, a gradient of tension pointing towards the vegetal pole progressively develops at the yolk cell surface [24].

To define the biomechanical make up of Rab5ab YMOs versus controls we employed experimental 2D velocity fields obtained by PIV from time-lapse imaging of meridional sections (see above, **Fig. 4A** and **Movie S9**). These analyses nicely showed that the yolk granules' flows were severely impaired (see **Movie S10**). Simulated 3D velocity fields generated from a spherical cortex model were fitted to the experimental velocity fields and dynamic pressure, mechanical power densities and surface tension maps were all inferred by HR [24]. Through this analysis, we found that while mechanical energy was reduced four-fold, the topographical distribution of mechanical power in Rab5ab YMOs was indistinguishable (**Fig. 4B** and **Movie S11**) from that of wild-type embryos [24]. Conversely, both longitudinal (AV) and latitudinal (CC) surface stresses and the positive vegetalward gradient of tension in the yolk cell surface of wild-type siblings [24] were significantly decreased in Rab5ab YMOs (see **Fig. 4C** and **Movie S12**). We interpret these data to mean that Rab5ab-mediated yolk cell endocytosis does not influence where and when mechanical power builds up during epiboly but is necessary to reach a proper level of cortical tension.

To validate the tensional topology of the yolk cell cortex obtained by HR in Rab5ab YMOs we employed laser microsurgery [35]. The experimental disruption of the yolk cell cortex resulted in immediate recoil with an exponentially decaying speed proportional to the tension present before ablation [36]. We performed laser cuts in the AV direction at the E-YSL of both Rab5ab YMOs and controls. These confirmed as an outcome of impaired membrane removal the reduction of surface tension inferred by HR (**Fig. 5A**). Next, by performing laser cuts parallel to the EVL in the yolk cell cortex at different distances from the EVL margin, we confirmed the predicted absence of a yolk cell vegetalward gradient of tension in Rab5ab YMOs. The averaged recoil velocities for a series of 20 μm laser cuts performed at 65 % epiboly at 20 μm (n = 21) and at 60 μm (n = 9) ahead of the EVL margin showed no statistically significant differences. Conversely, in control wild-type embryos, stress tension (recoil velocity) increased by 60 % vegetalward between equivalent cuts [24].

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All in all, our data indicate that local membrane removal is essential to strengthen the latitudinal (CC) and longitudinal (AV) forces building up at the E-YSL and for the development of an anisotropic gradient of tension at the cortex. These, jointly, would govern epiboly movements.

## DISCUSSION

The function of plasma membrane remodeling during morphogenesis is recently receiving high attention. It has been shown that Rab5-mediated endocytosis is required downstream of acto-myosin contraction to remove excess membrane in bottle cells in *Xenopus* and to promote their coordinated constriction [13]. Likewise, Rab5 is required in the amnioserosa during dorsal closure in *Drosophila* to remove membranes as cells delaminate [17]. In zebrafish, different Rab5 isoforms appear to have different roles, participating in Nodal signalling in early embryos (Rab5ab), or muscle and brain development (Rab5b and Rab5c) [29]. On the other hand, Rab11 mediated recycling seems to regulate epidermal cells elongation and membrane growth and invagination during cellularization in fly embryos [37]. The balance between endocytosis and recycling seems thus to be critical to regulate cell morphology and tissue deformations in multiple morphogenetic processes.

The contractile capabilities and gradual change of dimensions of the E-YSL along the dissimilar elastic properties of the EVL and the yolk cell surface minimally account after crossing the equator for epiboly kinematics and mechanical behavior [24]. The proximal E-YSL exerts an isotropic contractile force that generates stress at its adjacent structures, the EVL and the yolk cell cortex, which have different mechanical properties. The EVL is easily deformed by this pulling force and passively expands. On the opposite, the thin yolk cell cortex cannot stretch in response to contraction. In this scenario, we propose that membrane (surface area) removal becomes essential for effective movements towards the vegetal pole (**Fig. 5B**). The non-convoluted yolk cell cortex, would accordingly be progressively recruited to the adjacent E-YSL as this is eliminated.

Endocytosis of the E-YSL surface was previously suggested to contribute to epiboly

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[14, 27]. However, knock-down of Dynamin 2 in the yolk cell had little effect on epiboly progression [30]. This suggested that Dynamin-dependent endocytic removal of yolk cell membrane is dispensable for epiboly progression. Yet, we found that depletion of Rab5ab and the subsequent inhibition of endocytosis in the yolk cell lead to epiboly arrest. These opposite results let to discarding clathrin-mediated endocytosis as the main mechanism involved in membrane trafficking in the E-YSL, pointing instead to macropinocytosis. Macropinocytosis is characterized by large non-selective membrane internalization and by the presence of actin cytoskeleton protrusions (ruffles) and has been previously proposed as a plausible mean for membrane remodeling [2-4]. Tipically, the vesicles formed by macropinocytosis are much larger than endosomes (around 0,2 to 0,5 µm) and they seem to be uncoated.

A common underlying feature of endocytic membranes, as opposed to other regions of the plasma membrane, is their high curvature [11]. This curvature is somehow linked to the presence of a specific set of regulatory proteins, many of them necessary for curvature generation [38]. Further, endocytic membrane curvature also appears to be influenced by cytoskeleton motor proteins such as myosin [39]. In addition to pushing forces mediated by actin polymerization (helping, e.g., to push neck membranes closer together), the cytoskeleton may also provide pulling forces to keep vesicle necks under tension [40]. Our data indicate that actomyosin contractility may be necessary at the E-YSL for membrane folding and be initially required to fold the E-YSL membrane into ripples.

While endocytosis of the E-YSL appears to be key for proper epiboly progression, we found that in the absence of Rab5ab, 1) the overall power and the longitudinal and latitudinal stresses (and the shear stress) were severely disturbed, and 2) the gradient of tension along the φ axis of the yolk cell surface was extremely weakened (**Fig. 4C**). As a result the yolk cell cortical tension, which we validated by laser microsurgery, suffers a significant reduction (**Fig. 5A**). Yet, significant acto-myosin contractile capability persisted and actin and myosin remained localized in the E-YSL cortex in Rab5ab YMOs. Thus, primarily, membrane endocytosis during epiboly appears to follow actomyosin contractility implementing a positive regulatory loop.

Mounting evidence points to a direct relation between membrane reservoir and trafficking pathways with tension in the regulation of cell shape changes and movements in morphogenetic processes [15, 41-47]. During morphogenesis, as tissues

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change their shapes and sizes, cell membranes dynamically change their area, composition and links to the cortex. As a consequence, membrane tension is subjected to constant modulation [48, 49]. How membrane tension is integrated with the cell's overall mechanical properties is unknown. In teleosts, pioneering studies in loach uncovered a direct correlation between surface membrane folds and endocytic-rich domains in early eggs. Further, the experimental decrease of loach eggs surface tension by volume reduction was found to lead to a tightly packed folding of their membrane [50]. Alongside, in *Fundulus heteroclitus* embryos, mechanical deformations affect epithelial apical membrane turnover [51]. Yet, these early studies failed to provide a comprehensive view of the links between membrane removal, tension and morphogenetic movements.

We propose that, in the early zebrafish embryo, the surface membrane tension constitutes a mechanical buffering system constantly maintained by endocytosis and contractile activity at the E-YSL that regulates epiboly progression. The rates of removal of E-YSL membrane would vary with time and would be proportional to the tension of the yolk cell surface. Endocytosis will lead to membrane tension anisotropies in the yolk cell surface and these will mechanically feedback to regulate membrane dynamics. This mechanical loop alongside the concerted actions of latitudinal and longitudinal forces at the E-YSL would direct epiboly movements. Mechanical loops set up by membrane remodeling could constitute a common way to coordinate tissue movements in morphogenetic processes.

## EXPERIMENTAL PROCEDURES

### Zebrafish lines maintenance

AB and TL wild type strains were used throughout this study. Membrane-GFP transgenic (Tg (β-*actin:m-GFP*)) fish [52] were provided by Lilianna Solnica-Krezel and Myosin-GFP transgenics (Tg (β-*actin:MYL9L-GFP*)) (unpublished) by Carl-Philipp Heisenberg. Adult fish were cultured under standard conditions and staged embryos were maintained at 28.5 ºC in embryo medium [53].

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**mRNA and Morpholino injections**

A DNA construct encoding for LifeAct-GFP [54] and cloned in a Zebrafish expression vector was provided by Erez Raz. mRNA was *in vitro* synthesized (µMessage Machine kit, Ambion) and injected into the yolk at one- or 512-cell stages (150 pg). To knockdown Rab5ab, morpholino yolk injections (4 ng and 8 ng) were performed at the 512-1024-cell stage.

**Actin, myosin and nuclear stainings**

Zebrafish embryos were fixed overnight in 4 % paraformadehyde at 4 °C, washed in 0.3 % Triton in PBS (PBT) and manually dechorionated. They were then washed in PBT, followed by a 2-hour incubation in blocking solution (1 % bovine serum albumin in PBT). Embryos were then incubated either for 1 hour in blocking solution containing 0.2 µg/µl Phalloidin-TRITC (Molecular Probes, Invitrogen) at room temperature or with an antibody against activated myosin (rabbit anti-phospho-myosin light chain 2 Ser19 (Cell Signalling) at 1:100). DAPI was used for nuclear counterstaining. After incubation, embryos were washed 4 times for 15 minutes in PBT. Immunostained embryos were incubated with a secondary fluorescent antibody and washed in PBT. For imaging, embryos were mounted on dishes with 0.5 % low melting agarose (A9045 Sigma) in PBS medium. Images were acquired on a Zeiss LSM700 confocal microscope with 10 X / 0.3 and 63 X / 1.40 oil objectives.

**Live Imaging and Analysis**

Whole embryo images were collected from non-dechorionated animals aligned in a 1.2 % agarose mold and covered by E3 medium. Images were acquired (4X magnification) every 5 minutes with an Olympus MVX10 Macroscope.

For confocal and spinning-disc microscopy, embryos were mounted in 0.5 % low melting agarose (A9045 Sigma) in E3 embryo medium.

Sagittal sections (350 µm depth from the yolk cell membrane surface) were collected from (Tg (β-*actin: m-GFP*)) embryos using a Leica SP5 two-photon microscope equipped with a mode-locked near-infrared MAITAI Laser (Spectra-Physics) tuned at 900 nm, with non-descanned detectors and with a 25 X / 0.95 water-dipping objective.

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Images were scanned at 200 Hz and frames were averaged three times. Stacks of 30 µm, 10 µm step-size, were acquired every 2 minutes.

Dextran and lectin internalization were monitored from dechorionated embryos previously incubated in 0.05% 10.000 MW Rhodamine B-Dextran (Life Technologies) for 10 minutes [31] or 100µg/ml lectin-TRITC (Sigma L1261) for 5 minutes at the sphere stage, both diluted in E3 embryo medium. The Lectin-TRITC used was from *Helix pomatia* [51], which binds *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine residues of glycoproteins and glycolipids on the cell surface. After treatment embryos were rinsed in E3 medium, mounted in 0.5 % low melting agarose and imaged in a Zeiss LSM700 confocal microscope with a 40 X / 1.3 oil immersion objective. A stack of 20 µm, 0.39 µm step size, was acquired every 4 minutes.

To visualize myosin cortical flows, spinning-disc images were captured from (Tg (β-*actin: MYL9L-GFP*)) embryos on either an Olympus X81 inverted microscope (Andor Technologies), using a 40 X / 0.60 Dry objective or a Zeiss Axiovert 200M inverted microscope (PerkinElmer UltraView ERS) using a 40 X / 1.3 oil DIC objective. Stacks of 16 µm, step size 1 µm, were acquired every 45 seconds.

To visualize the surface of the yolk cell, (Tg (β-*actin: m-GFP*)) embryos were imaged in a Zeiss LSM700 confocal microscope with a 63 X / 1.4 oil objective. A stack of 25 µm, step size of 0.2 µm was acquired. We also used embryos injected with *LifeAct-GFP* at the 512-cell stage and collected the images with a Zeiss Axiovert 200M inverted microscope (PerkinElmer UltraView ERS) using a 100 X / 1.4 oil DIC objective. Stacks of 10µm, step size of 0.45 µm, were acquired every 12 seconds.

For photo-bleaching, selected ROIs were created for lectin-TRITC soaked embryos and bleached using 100 % power of a 555 nm laser with 100 iterations in the selected area (in the YSL at 150 µm from the EVL margin) in embryos at 40 % epiboly. A stack of 4 µm, step size 1 µm, was acquired every 30 seconds.

Most image analyses and processing were performed using Fiji (http://pacific.mpi-cbg.de) and Matlab (Mathworks). To obtain velocity fields we applied the MatPIV software package written by Johan Kristian Sveen for use with Matlab [55]. To measure the width of the wrinkled area, surface projections at different stages were obtained with Matlab and mean width and standard deviations were plotted (Excel, MS Office). To quantify endocytosis, E-YSL dextran-containing vesicles were monitored from

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maximum projections of Z-stack images.

Laser Surgery Experiments, Retraction Analysis and Hydrodynamic Regression (HR) are described in the Supplemental Experimental Procedures.

## ACKNOWLEDGEMENTS

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## AUTHOR CONTRIBUTION

MM and AHV performed all biological tests; PAP designed the modeling and the regression analysis and EMB designed the study, analyzed the data and wrote the paper. MM and AHV contributed equally to the study. All authors discussed the results and commented on the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## FIGURE LEGENDS

Figure 1

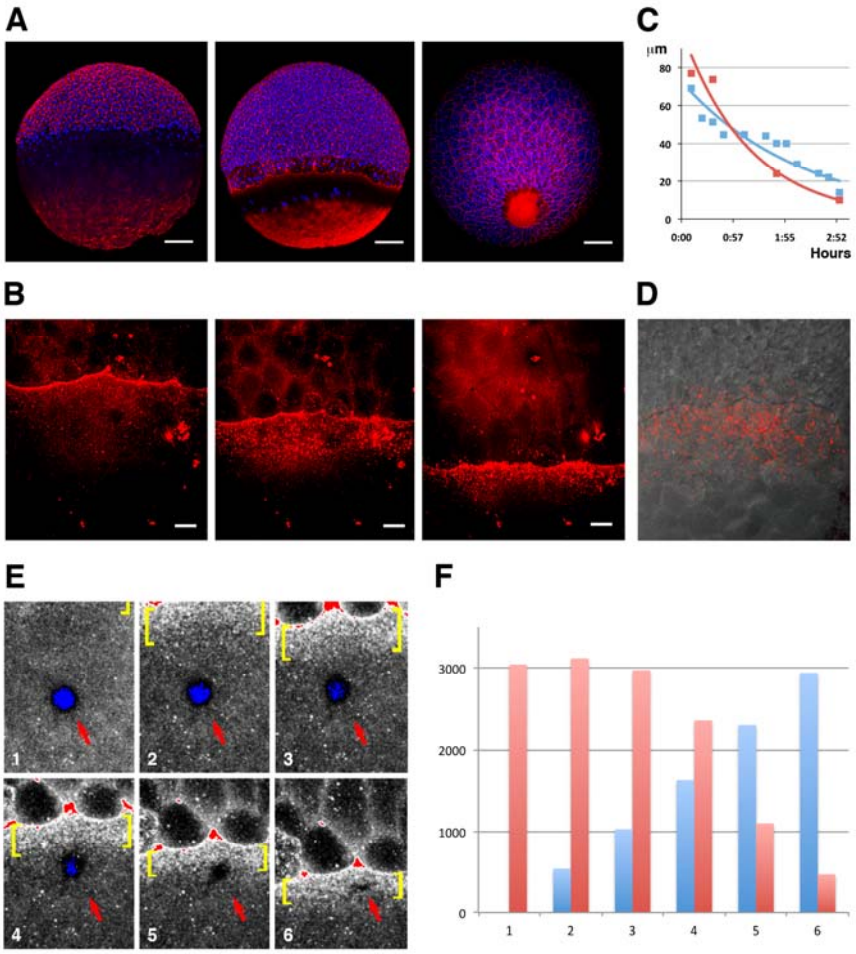


**Figure 1. Yolk cell membrane endocytosis at the E-YSL**

**A)** Blastoderm expansion during epiboly. At sphere stage epiboly has not yet begun (left). F-actin accumulates at the periphery of all cells as well as in the yolk cell, mainly at the vegetal cap. At 70 % epiboly (middle), the blastoderm has crossed the equator and will decrease its margin until closure. A belt of actin develops at the E-YSL ahead of the EVL and an actin-free zone separates this belt from a vegetal actin-rich patch. At 90 % epiboly (right), the E-YSL and the vegetal actin-rich patch merge. Embryos were

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stained with phalloidin-TRITC (red) and DAPI (blue). Scale bars 100 µm. **B**) Sequential images of a confocal time-lapse video of a wild type embryo soaked in lectin–TRITC for 5 minutes at sphere stage. The lectin binds to the membrane of both the yolk cell and the EVL cells and gets internalized accumulating in vesicles in the E-YSL just ahead of the EVL margin (from **Movie S1**). Scale bar 25 µm. All confocal images are maximum projections. **C)** Parallel reduction during epiboly progression of the width of the convoluted domain (blue) and of the area undergoing membrane removal (red) of the E-YSL. X and Y axes represent hours after 50 % epiboly and width in µm, respectively. **D)** Uptake of fluorescent dextran (red) at the E-YSL just ahead of the EVL margin at 65 % epiboly. **E**) Snapshots of time-lapse images (from **Movie S2**) of a lectin-TRITC soaked embryo showing a circular photobleached area (red arrow) in the yolk cell away from the EVL leading edge. The photobleached membrane is removed and endocytosed only upon its enclosure within the advancing E-YSL (yellow brackets). All confocal images are maximum projections. Scale bar 25 µm. **F**) Membrane internalization dynamics. Histograms depicting the normalized relationship between the photobleached areas (red) and the progression of the leading EVL front (blue) at regular sequential time points (from 1 in **E**). The removal of the photobleached membrane shows a precise linear relationship with the speed of advancement of the leading front.

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Figure 2

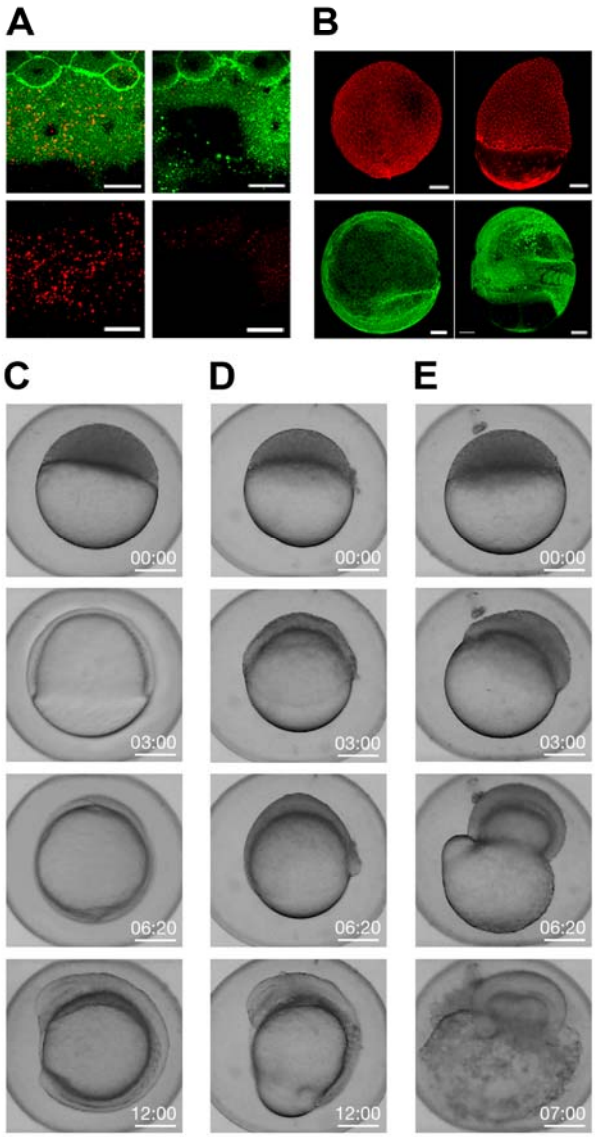


**Figure 2. Endocytosis and Epiboly are impaired by Rab5ab depletion**

**A**) Rhodamine B-Dextran internalization was reduced at doming stage in membrane-GFP (Tg (β-*actin:m-GFP) rab5ab* (right) compared to sibling controls (left) YMOs. Top panels show dextran (red) and membrane staining (green) overlays. Images are confocal maximum projections. Scale bar 25 µm. See also **Movie S3**. **B**) Yolk cell depletion of Rab5ab results in epiboly delay without affecting other gastrulation movements [Control (left) and Rab5ab YMOs (right)]. Top panels show Phalloidin-

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TRITC (red) staining of a control YMO at the end of epiboly and a sibling medium dose Rab5ab YMOs of the same age. Lateral views. Bottom pannels show membrane-GFP (Tg (β-*actin:m-GFP*) YMOs at 1 day post fertilization. Rab5ab YMOs present an open back phenotype but succeed in other gastrulation movements leading to somite formation (see also **Movie S4**). Images are confocal maximum projections. Scale bar 100 µm. **C-E**) YMOs show a dose dependent epiboly delay. Macroscopic bright field images of sibling controls (**C**) and medium (4 ng) (**D**) and high (8 ng) dose (**E**) Rab5ab YMOs (from **Movies S5** and **S6**). Medium and high dose YMOs remained at 70 and 40 % epiboly respectively when control siblings have already closed. Embryos were imaged in their chorion. Scale bar 250 µm.

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**Figure 3**

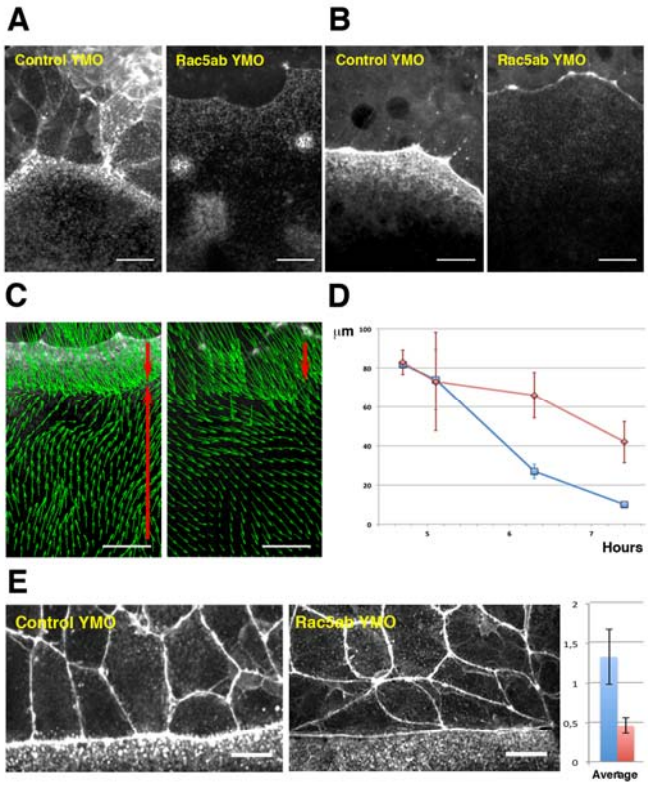


**Figure 3. Cytoskeleton dynamics and EVL leading cells shapes are affected by Rab5ab depletion**

**A)** Actin fails to accumulate at the E-YSL in Rab5ab YMOs versus controls (C YMO). Time-lapse snapshots of two LifeAct GFP injected sibling embryos. **B)** Myosin fails to accumulate at the E-YSL of Rab5ab YMOs versus controls (C YMO). Time-lapse snapshots of two Myosin-GFP transgenic (Tg (β-*actin:MYL9L-GFP*)) sibling embryos. Notice the delay in the progression of the EVL and the weaker accumulation of actin and myosin in the Rab5ab YMOs. **C**) Myosin cortical retrograde flows. PIV of time-lapse snapshots of Tg (β-*actin:MYL9L-GFP*) embryos at 40 % epiboly (from **Movie S7**). Notice the vegetalward movement of cells and E-YSL (red top arrows) and the retrograde animalward cortical flow from the yolk cell vegetal pole sinking at the E-

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YSL (red bottom arrows). Scale bar 25 µm. **D**) E-YSL contraction is delayed in Rab5ab YMOs. Between 4.5 to 7.5 hours post fertilization, the E-YSL width is reduced from 80 to 10 µm as an average in controls (blue), but from 80 to 40 µm in Rab5ab YMOs (red). Data collected form surface projections of membrane-GFP (Tg (β-*actin:m-GFP*) *rab5ab* and sibling YMOs (see **Movie S8**). **E)** Compared to control siblings at 70 % epiboly (C YMO - left), leading EVL cells of Rab5ab YMOs flatten and elongate latitudinally (right). Actin was stained with phalloidin-TRITC. Scale bars 25 µm. All confocal images are maximum projections. Quantification of the shape ratio (animal to vegetal vs latitudinal - Y axis) of leading EVL cells (far right) show that control siblings (red) are extremely elongated on the animal vegetal direction while leading EVL cells in Rab5ab YMOs (blue) flatten at their front and elongate latitudinally. Standard deviations are shown.

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Figure 4

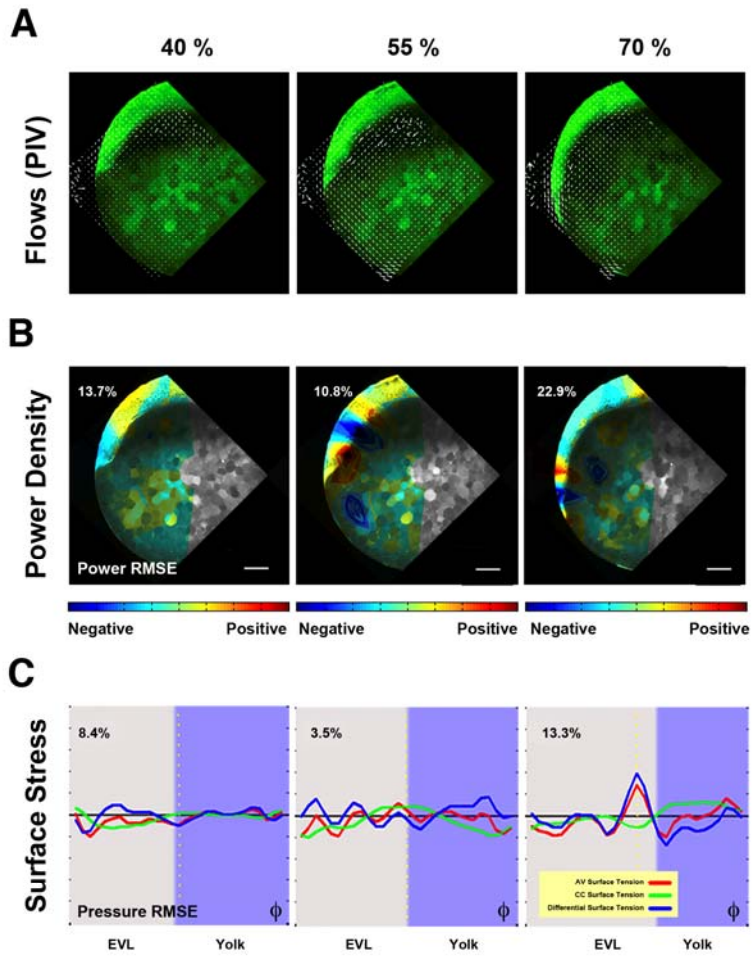


**Figure 4. Biomechanics of yolk cell endocytosis impaired embryos**

**A**) Yolk granules flows patterns are altered in Rab5ab YMOs. PIV of time-lapse snapshots imaged by two-photon microscopy of a Tg (β-*actin:m-GFP*) Rab5ab YMO (from **Movie S9**; see also **Movie S10**). From epiboly onset, yolk granules flows are uncoordinated in Rab5ab YMOs. The internal toroidal vortices characteristic of epiboly progression [24] do not form or are severely reduced and the laminar flows become asymmetrically distributed. Animal (A) and vegetal (V) poles are indicated. Scale bar 25 µm. **B**) Mechanical power density maps over time obtained by HR analysis in in

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Rab5ab YMOs (from **Movie S11**). The Relative Mean Square Error (RMSE) of the inferred power maps is shown as a percentage expressing the fitting accuracy. Qualitatively, Rab5ab YMOs display no differences with wild type embryos in the spatial and temporal distribution of mechanical work [24] but exhibit an overall decrease of power of about four fold. **C**) Longitudinal along the AV axis (red) and latitudinal or circumferencial - CC (green) stresses and their differences (blue) along the embryo cortex in a membrane-GFP transgenic (Tg (β-*actin:m-GFP*)) Rab5ab YMO (from **Movie S12**). Stresses were plotted as a function of the φ angle from animal to vegetal (40 %; 50 %; 65 % epiboly). The equator - dotted yellow line -, yolk cell surface - purple shadow - and the RMSE of the dynamic pressure (fitting accuracy) as a percentage for each time point are displayed. The latitudinal stress does not steep up from animal to vegetal until 60 % epiboly, while the longitudinal stress oscillations are sustained.

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**Figure 5**

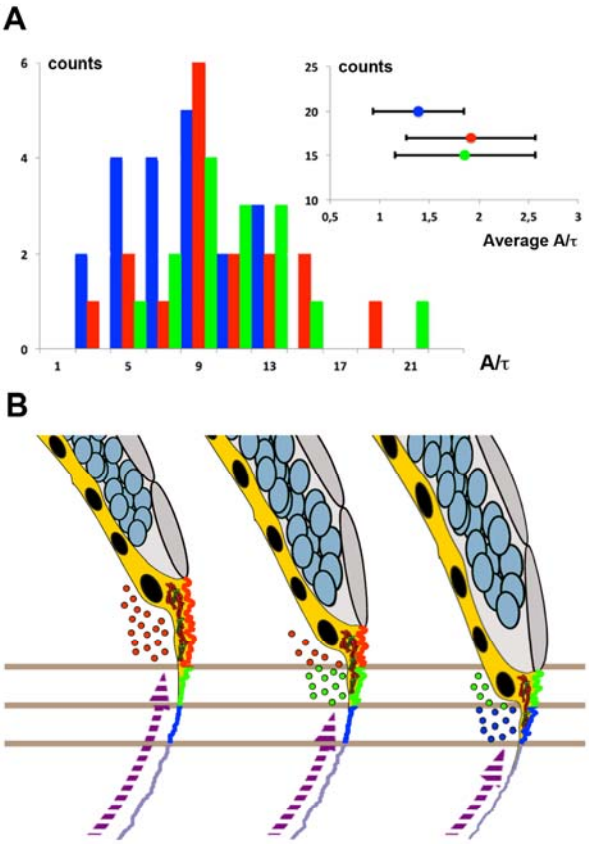


**Figure 5. Membrane cortical tension and endocytosis at the E-YSL are necessary for epiboly progression**

**A)** The distribution (counts) of instant retraction velocities (A/□) after laser surgery of the actomyosin cortex of Myosin-GFP (Tg (β-*actin:MYL9L-GFP*) Rab5ab YMOs at 55% epiboly (blue) shows a significant reduction (Wilcoxon test $p<0.01$) versus wild type (red) and control YMOs (green). The instant velocity estimate was extracted from the exponential fit of the distance between fronts (see Supplemental Experimental Procedures). The averaged instant retraction velocity (A/□) for Rab5ab YMOs was 1.39 ± 0.46 □m, while control YMOs reach 1.86 ± 0.71 □m and wild type embryos 1.92 ± 0.65 □m (inset). Counts represent the number of analyzed laser cuts for each condition.

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**B)** Proposed model of epiboly progression. The contractile E-YSL and the imbalance of stiffness between the EVL and the yolk cell surface account for epiboly progression. Rab5ab-mediated yolk cell localized endocytosis (color coded dots) accounts for the reduction of the yolk cell surface coupled to the progression of EVL (grey) and DCs (blue) towards the vegetal pole. Membrane removal associates to the convolution and contraction of the E-YSL surface and the recruitment of actin and myosin (purple dashed arrow) from vegetally located pools. Three chronological time points are shown. Different sequential zones on the surface of the E-YSL are color-coded. Actin and myosin are diagramatically illustrated in red and green within the YSL (yellow).

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## SUPPLEMENTAL EXPERIMENTAL PROCEDURES AND INFORMATION

Supplemental Experimental Procedures

Supplementary Figure Legends

- Figure S1

Supplementary Movies Legends

- Movie S1, Related to Figure 1
- Movie S2, Related to Figure 1
- Movie S3, Related to Figure 2
- Movie S4, Related to Figure 2
- Movie S5, Related to Figure 2
- Movie S6, Related to Figure 3
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- Movie S9, Related to Figure 4
- Movie S10, Related to Figure 4
- Movie S11, Related to Figure 4
- Movie S12, Related to Figure 4

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Morpholinos

Morpholinos (MOs) were purchased from Gene Tools and designed against selected regions (ATG or UTR) of the *rab5ab* gene (Accession Number ENSDARG00000007257) (Gene Tools): MO1-ATG (5-TCGTTGCTCCACCT-CTTCCTGCCAT-3), MO2-UTR (5-GACCCAAAACCCCAATCTCCTGTAC-3), MO3-UTR-ATG (5-ACCTCTTCCTGCCATGACCCAAAAC-3) and a mismatch MO (5-TCcTTcCTCgACCTCTTCgTcCCAT-3) (mispaired nucleotides in lower case). Interference with *rab5c* (ENSDARG00000026712)] was performed with the following MO: 5-CGCTGGTC-CACCTCGCCCCGCCATG-3 provided by C.P. Heisenberg [34].

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For all experiments, a group of embryos was injected with a Standard Control MO (5-CCTCTTACCTCAGTTACAATTTATA-3).

**Hydrodynamics Regression (HR)**

HR is based in fitting analytically modeled velocity fields to experimental velocity fields in and outside a cortex. Considering that in deforming tissues, stresses at the fluid/cortex boundary are continuous (boundary condition), HR can estimate cortical stresses and retrieve the complete dynamic pressure distribution in the fluid and at the fluid-cortex interface. From these, HR also infers at each time point the cortex shear stress at each point of the surface and the mechanical power density. HR is performed independently at each time point to retrieve the overall spatio-temporal distribution of all these mechanical quantities [24].

In our analyses, experimental 2D velocity fields were estimated by PIV from time-lapse imaging of meridional sections of zebrafish embryos. Second, simulated 3D velocity fields generated from a spherical cortex model (SC) with Stokeslets pairs distributed on a single spherical shell were fitted to the experimental velocity fields. Last, knowing the fluid deformation rates, it is possible to calculate the local values of the cortical surface tension, the cortical mechanical power density and the spatio-temporal evolution of both cortical stresses and mechanical power density maps (analytical codes are available on [24].

**Laser Surgery Experiments and Retraction Analysis**

Laser surgery of the actomyosin cortex was performed with a pulsed UV laser (355 nm, 470 ps per pulse) by inducing plasma-mediated ablation as described before [36]. To compare the cortical tension in the longitudinal direction at the E-YSL a 20 μm-laser line containing 50 pulses was scanned 5 times at a frequency of 500 Hz, parallel to the EVL front, centering the cut at a distance of about 20 μm, through a 63 X / 1.2 W objective lens. Fluorescence imaging was performed through a custom spinning Nipkow disc unit equipped with a 488 nm laser line and a Hamamatsu ORCA CCD camera, acquiring at 1.5 frames per second. Transmission and fluorescence imaging was performed by alternated illumination with two out-of-phase mechanical shutters blocking the 488 nm laser and the halogen bright field lamp.

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We followed the accepted assumption [36] that the tension present in the actomyosin cortex before the laser cut is proportional to the outward velocity of the immediate recoil. Retraction analysis was performed through a customized kymograph analysis along the retraction axis (perpendicular to the cut), with ImageJ (http://rsbweb.nih.gov/ij/). Kymograph processing included subtraction of the intensity minimum and normalization to the maximum, both measured in the position of the cut, to ensure stable edge detection by intensity threshold across the whole sequence. The front-to-front length, during the retraction phase (until reaching a plateau), was fitted to an exponential function (Igor Pro 6.0, Wavemetrics) to evaluate the slope at the origin. The function used was:

$$F(t) = y0 + A\,(1- \exp(-t / \tau))$$

and the slope at the origin was derived from its derivative:

$$dF(0) / dt = A / \tau$$

The width of photo bleaching (about 1µm) introduced by the UV laser was subtracted to the measured length L. This method was applied to the comparative analysis of YMOs conditions.

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**SUPPLEMENTAL FIGURE**

**Figure S1**

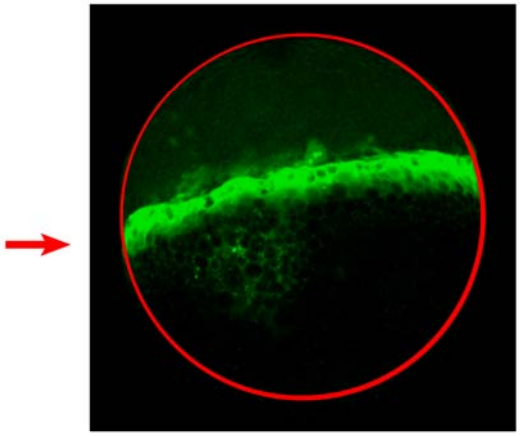

**Figure S1. Yolk Cell-Specific Morphants (YMOs)**

Fluorescent image of an embryo injected at the 512 cells stage with a fluorescently labelled Morpholino (FITC) observed at 40 % Epiboly. The red arrow points to the fluorescence in the yolk cell, accumulating at the E-YSL.

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## SUPPLEMENTAL MOVIES LEGENDS

### Movie S1. Endocytosis

Confocal video time-lapse of a wild type animal soaked in lectin–TRITC for 5 minutes at sphere stage. 20 µm width stacks were captured every 4 minutes. Note the cumulative lectin-TRITC internalization at the E-YSL domain ahead of the advancing EVL.

### Movie S2. Endocytosis at the E-YSL

HiLo LUT code (Fiji) processed confocal video time-lapse of a lectin-TRITC soaked embryo showing a circular photo bleached ROI in the yolk cell 60 µm away from the EVL leading edge. 4 µm width stacks were captured every 30 seconds. Maximum projection is shown. Scale bar 25 µm. The photo-bleached membrane (blue circle) is not removed or endocytosed and remains unchanged up to its enclosure within the advancing E-YSL.

### Movie S3. Endocytosis impairement in Rab5ab YMOs

Confocal video time-lapse of control and Rab5ab YMOs (medium dose) soaked in Rhodamine B-Dextran for 5 minutes at sphere stage. 20 µm width stacks were captured every 4 minutes. Note the reduced dextran internalization ahead of the advancing EVL.

### Movie S4. Specific arrest of epiboly movements in Rab5ab YMOs

Confocal video time-lapse of a membrane-GFP (Tg (β-*actin:m-GFP*) Rab5ab YMOs (medium dose) that slowly proceeds throughout epiboly arresting at 70 % presenting an open back phenotype but succeeding in other gastrulation movements leading to somite formation.

### Movie S5. Medium dose Rab5ab YMO macroscopic phenotype

Macroscope video time-lapse of control and Rab5ab YMO sibling embryos (at a middle dose) are shown. Bright field images were captured every 5 minutes from sphere stage to 16 hours post fertilization. Note that the Rab5ab YMO at a middle dose is at 60 %

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epiboly at the time when the control embryo closes (yellow arrow). The Rab5ab YMO continues its elongation in the animal-vegetal direction but remains open.

**Movie S6. High dose Rab5ab YMO macroscopic phenotype**

Macroscope video time-lapse of control and Rab5ab YMO sibling embryos (at a high dose) are shown. Bright field images were captured every 5 minutes from sphere stage to 16 hours post fertilization. Note that Rab5ab YMO at a high dose halt before reaching the equator and burst.

**Movie S7. Cortical Myosin flows on the E-YSL in control versus Rab5ab YMOs**

Spinning-disc video time-lapses of zebrafish transgenic (Tg (β-*actin:MYL9L-GFP*)) embryos from 50% epiboly onwards (control sibling (top) and a Rab5ab YMO (bottom). The movements of cortical actin were analyzed by Particle Image Velocimetry (PIV). 9 µm width stacks were captured every 12 seconds. Maximum projections are shown. Scale bar 25 µm. The retrograde animalward cortical myosin flows from the vegetal pole of the yolk cell towards the E-YSL are impaired in Rab5ab YMOs.

**Movie S8. EVL marginal cells elongate in the CC direction in Rab5ab YMOs**

Confocal video time-lapse of control and *rab5ab* yolk cell membrane labelled [Tg (*β-actin:m-GFP*)] zebrafish transgenic YMOs (medium dose). While control embryos marginal EVL cells elongate in the animal/vegetal direction (left), Rab5ab YMOs marginal cells flatten and elongate in the circunferencial direction. The speed of progression of the EVL is reduced and this eventually stalls.

**Movie S9. Yolk granules flows in Rab5ab YMOs**

Flow trajectories from PIV measurements of a two-photon video time-lapse of a membrane-GFP (Tg (β-*actin:m-GFP*)) zebrafish transgenic Rab5ab YMO at the middle plane, 350 µm deep inside, throughout epiboly. Scale bar 100 µm. Flows in the Rab5ab YMO are partly arrested.

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**Movie S10. Yolk granules kinematics in Rab5ab YMOs**

Flow lines from PIV measurements of two-photon excitation stitched video time-lapses of membrane-GFP (Tg (β-*actin:m-GFP*)) zebrafish transgenic control (left) and *rab5ab* (right) YMO siblings at the middle plane, 350 µm deep inside, throughout epiboly. Scale bar 100 µm. The Rab5ab YMO shows epiboly delay and disrupted yolk flows.

**Movie S11. Power Density Maps in Rab5ab YMOs**

Two-photon excitation video time-lapse of a membrane-GFP (Tg (β-*actin:m-GFP*)) Rab5ab YMO at the middle plane, 350 µm deep inside, throughout epiboly (left). Mechanical power density maps were calculated by HR from the velocity fields, color-coded at an equal scale for each time point (right) and overlaid on the fluorescence images (negative - blue; 0 - green; positive - red) (middle). The relative mean square error for the Power is displayed as a percentage for each time point. Scale bar 100 µm. As in the wild type [24], during doming, most of the energy supply is generated by the blastoderm. After 50 % epiboly, the E-YSL constriction becomes the main motor of epiboly, while the EVL cells resist their stretching. Thus, Rab5ab YMOs display no qualitative differences with wild type embryos in their mechanical activity distribution but, importantly, they exhibit an overall decrease of power of about four fold.

**Movie S12. Longitudinal and latitudinal stresses in Rab5ab YMOs**

Main surface stresses were calculated by HR of velocity fields obtained from stitched two-photon excitation video time-lapse of a membrane-GFP (Tg (β-*actin:m-GFP*)) Rab5ab YMO embryo at the middle plane, 350 µm deep inside, throughout epiboly . Longitudinal (red) and latitudinal (green) stresses and their differences (blue) were plotted as a function of the φ angle from animal to vegetal. Time points were every 40 minutes. The equator - dotted yellow line -, yolk cell surface - purple shadow - and the relative mean square error of the pressure as a percentage for each time point are displayed. In these YMOs, the longitudinal and latitudinal stresses are equal at the poles. Contrary to wild type embryos, the latitudinal stress does not steep up from animal to vegetal. Their difference (longitudinal minus latitudinal) shows a defined profile at the EVL margin after 50 % epiboly, becoming positive in the EVL and negative at the E-YSL.