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REVIEW

Eph signaling in mitotic spindle orientation: what´s your angle here?

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ABSTRACT

The orientation of the mitotic spindle is a crucial process during development and adult tissue homeostasis and multiple mechanisms have been shown to intrinsically regulate this process. However, much less is known about the extrinsic cues involved in modulating spindle orientation. We have recently uncovered a novel function of Eph intercellular signaling in regulating spindle alignment by ultimately ensuring the correct cortical distribution of central components within the intrinsic spindle orientation machinery. Here, we comment on these results, novel questions that they open and potential additional research to address in the future.

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Eph signaling

Erythropoietin-producing hepatocellular carcinoma (Eph) receptors, the largest family of Receptor Tyrosine Kinases (RTKs), are key regulators of both developmental processes and adult tissue homeostasis, whose misregulation has been associated with different pathologies, including cancer [1–10]. Eph receptors and the Eph family receptor-interacting (ephrin) ligands are highly conserved throughout the animal kingdom. In vertebrates, a large and redundant family of about 16 Eph receptors and a family of 9 ephrin ligands has been characterized, both divided into two subclasses, A and B. Most EphAs bind to glycosylphosphatidylinositol (GPI)-membrane anchored ephrinAs and most EphBs interact to transmembrane ephrinBs, although some class-crossing interactions between both subtypes exist. *Drosophila*, by contrast, has a single Eph receptor and a single Ephrin ligand, the latter being more similar to vertebrate ephrinBs (Figure 1). All Eph receptors, like other RTKs, are transmembrane proteins with an N-terminal extracellular domain containing the ligand-binding motif and an intracellular carboxyl-terminal domain with diverse motifs including the kinase domain [11] (Figure 1). Thus, ephrin-Eph signaling relies mainly on cell-cell direct contact. However, additional mechanisms that are cell contact-independent have been recently reported [12].

Another unique feature of ephrin-Eph signaling is the bidirectional nature of the cell-cell communication: it can be a forward signaling (ephrin-Eph) or a reverse signaling (Eph-ephrin), transduced in the Eph or ephrin expressing cell, respectively [3,13]. As mentioned above, even though Eph receptors were first identified in human tumors [2] and then studied profusely in the context of axon guidance promoting cell-cell attraction/repulsion [3,14], a wide spectrum of developmental processes has been shown to depend on Eph signaling over the past decades. These processes include cell proliferation, cell migration, tissue boundary formation, cell-cell junction dynamics and apoptosis [3,9,15]. We have now uncovered a novel function of the Eph signaling in regulating a fundamental cell process, the orientation of the mitotic spindle, in the *Drosophila* optic lobe neuroepithelial cells [16].

Relevance of mitotic spindle orientation in development and tumorigenesis

Proper orientation of the mitotic spindle is critical as it determines tissue architecture or cell fate specification in the context of a symmetric or an asymmetric cell division. Epithelial cells frequently divide symmetrically, parallel to the anterior-posterior axis of cell polarity, while stem cells/progenitors (for example, *Drosophila* neural stem

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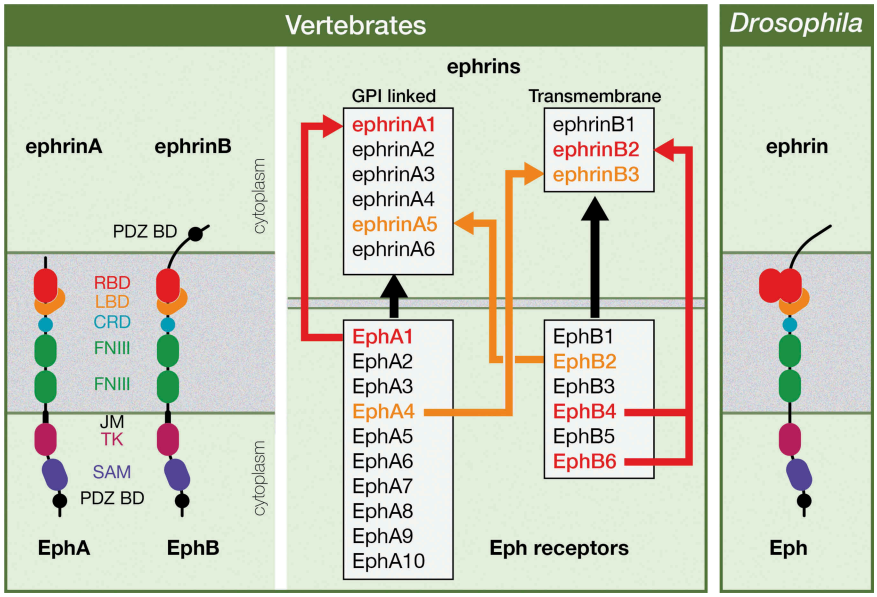

Figure 1. Ephrin and Eph receptor family members in vertebrates and in *Drosophila*. The structure of Eph receptors is similar in vertebrates and in *Drosophila*, while *Drosophila* Ephrin is more similar to vertebrate ephrinBs; PDZ BD: PSD95/Dlg1/ZO-1 Binding Domain; RBD: Receptor Binding Domain; LBD: Ligand Binding Domain; CRD: Cysteine-rich domain; FNIII: Fibronectin type III; JM: JuxtaMembrane; TK; Tyrosine Kinase; SAM: Sterile Alpha Motif. Most vertebrate EphA and B receptors bind promiscuously to any ephrin A or B ligands, respectively (black arrows), with some exceptions, in which the Eph receptor only binds particular ephrin ligand (red arrows). Some class-crossing interactions, in which Eph receptors bind ephrin ligands from a different subtype, also exist (orange arrows). In *Drosophila*, there is a single Eph receptor and a single Ephrin ligand.

cells, aka neuroblasts), can divide asymmetrically, along the apical-basal axis of cell polarity, to generate another stem cell and a daughter cell committed to differentiate (Figure 2) [17–21]. Thus, failures in spindle alignment could cause loss of tissue organization or an increased number of self-renewal stem cells in detriment of differentiated cells, both features being potential tumor-promoting events [22–24]. However, the defects in tissue architecture and cell proliferation that might imply a misregulated mitotic spindle positioning are normally counteracted by different mechanisms that have evolved to avoid such lethal phenotypes. For example, in the *Drosophila* wing disc epithelium, basal cell extrusion and apoptosis are used to eliminate those cells with misorientated spindles [23,25]. In other epithelia, such as the *Drosophila* follicular epithelium, misplaced cells due to defects in the planar orientation of the division, are reintegrated into the epithelium, a process promoted by the adhesion molecules Fasciclin 2 and Neuroglian [26]. Some human tumors have been associated with particular mutations in genes such as APC or E-Cadherin, which are known spindle orientation regulators [27–34]. This is very suggestive but it does not strictly prove a cause-and-effect relationship between the spindle phenotype and the tumor development [35]. Moreover, in *Drosophila* neuroblasts, single mutations in core components of the spindle orientation machinery are not enough to induce tumor-like overgrowth in the larval brain, at least in part because of the high redundancy in the regulation of the whole process [36]. The spindle

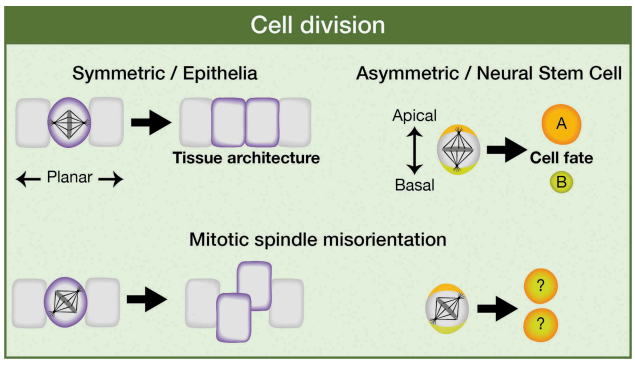

Figure 2. Relevance of mitotic spindle orientation. The orientation of the mitotic spindle is essential as it is going to influence tissue architecture or cell fate specification in the context of a symmetric or an asymmetric cell division, respectively.

alignment regulator Pins/LGN is an intriguing case. On one hand, *pins* mutant neuroblast lineages in the larval central brain do not show overgrowth (rather they are smaller and sometimes without any neuroblast) [37], similar phenotype shown by neuroblast lineages mutant in other core components of the spindle orientation machinery, such as Mud/NuMA, Canoe/Afadin or Dlg1, even though in these latter cases it is frequent the presence of ectopic neuroblasts within the mutant clone [36,38,39]. On the other hand, pieces of *pin*s mutant larval brain implanted into the abdomen of adult hosts originate big tumoral masses after some weeks and can even metastasize into other organs [22]. No such GFP-labeled allograft transplants have been performed with mutant tissue from other spindle regulators. It would be interesting to determine whether Pins is an exceptional case or whether the microenvironment in which the mutant cells grow makes all the difference[36]. Considering all this, we can conclude that mitotic spindle misalignment alone cannot induce cancer development. However, a proper orientation of the mitotic spindle is a relevant process to have into account as it might constitute a sensitized condition that, in combination with other eventual tumor suppressor mutations or oncogene activation, could trigger tumorigenesis [35,40,41].

Mechanisms that regulate spindle orientation: intrinsic and extrinsic cues

Given the relevance of correctly aligning the mitotic spindle during development and tissue homeostasis, it is not unexpected the existence of multiple mechanisms that ensure a robust regulation of this process. Particularly, autonomous or intrinsic cues underlying spindle orientation have been extensively covered in different systems, and recent publications have reviewed these increasingly growing number of autonomous factors in detail [17,40–50]. Among the intrinsic factors, the Pins/LGN-Gαi-Mud-NuMA complex stands at the core of the regulatory network of spindle positioning. However, even some of these molecules, such as Pins/LGN-Gαi, have been shown to be dispensable in some systems, while Mud/NuMA stays as an essential component in all of them [51–53]. Thus, a widespread notion in the field is that the regulation of spindle orientation is highly context-dependent, with variations in the spindle alignment machinery depending on the cell type and organism.

In addition to the extensive analyses of the autonomous cues involved in modulating spindle orientation, over the past few years the role of different extrinsic cues and, importantly, their link with the intrinsic spindle orientation machinery is being uncovered. For example, E-cadherin-mediated intercellular signaling, which affects the orientation of the spindle in multiple systems including mammalian epithelia and *Drosophila* germline [27,29–32,34], has been recently linked to LGN/Pins both in vitro and in vivo [54,55]. In one of these studies, authors show that E-Cadherin performs an instructive role in spindle orientation by recruiting LGN, which binds to E-Cadherin cytosolic tail through the TPR domain at interphase. At metaphase, NuMA is released from the nucleus and competes with E-Cadherin for binding the TPR repeat domain of LGN, which remains attached to the membrane through the Gαi subunit at the adherens junction region. In this way, astral microtubules can contact this region and the spindle is correctly orientated[54]. In the other study, performed in mouse prostate luminal cells, authors also show a direct interaction between E-Cadherin and LGN. In addition, they found that the polarity protein Scribble (SCRIB) forms a ternary complex with them and it is essential for their interaction. At the same time, this complex allows a precise location of the mitotic spindle by linking polarity proteins with spindle orientation cues[55]. Planar cell polarity activated by Wnt extracellular signals has been shown to modulate spindle alignment in different contexts [56–58]. However, like in the case of E-Cadherin, the mechanism by which this pathway directly affects the orientation of the spindle was revealed only some years ago[53]. In this work, a conserved direct interaction between the Wnt effector Dishevelled (Dsh) and the intrinsic cue Mud/NuMA was established both in *Drosophila* and zebrafish. Finally, intercellular Semaphorin-Plexin signaling was recently shown to regulate spindle orientation by controlling the activity of Cdc42, a known regulator of mitotic spindle, in mouse spinal cord and during mouse kidney development and repair [59,60].

Eph intercellular signaling as a novel extrinsic cue regulating spindle orientation

In a recent work, we have unveiled a role of Eph signaling as a novel extrinsic mechanism required to regulate the mitotic spindle positioning in the neuroepithelial cells of the *Drosophila* optic lobe[16]. We have shown that Eph signaling activates aPKC and that this is necessary to fully activate P-myosin II/P-Sqh. This, in turn, impinges on spindle alignment by contributing to the proper cortical localization of intrinsic cues located at the core of the spindle orientation machinery, such as Canoe/Afadin, Mud/NuMA and Dlg1 (Figure 3). Based on our results and other published data in vertebrates, we propose that a reverse Eph-Ephrin signaling is responsible for aPKC activation at the subapical region. In the epithelia of Xenopus embryos, it has been demonstrated a competition between ephrinB1 and active Cdc42 for binding to Par-6. Par-6, in a complex with Par-3, must bind active Cdc42 for this in turn to activate aPKC at the tight junctions, which is a domain equivalent to the subapical region in *Drosophila* [61–64]. Thus, a Par-3/Par-6/active Cdc42/aPKC complex is necessary for the formation or/and maintenance of the tight junctions, and a Par3/Par-6/ephrin complex hampers aPKC activation and leads to the disruption of the tight junctions. In these epithelial cells, the phosphorylation of ephrin induced by an Eph reverse signaling prevents the interaction of ephrin with Par-6, allowing the binding of Cdc42, the activation of aPKC and the consequent stabilization of the tight junctions[63]. In *Drosophila Eph* mutant neuroepithelial cells, we found defects in aPKC activation, as a constitutively activated form of aPKC was able to rescue the *Eph* mutant phenotype, including defects in the mitotic spindle orientation as well as failures in the localization of phosphomimetic myosin II/Sqh^{EE} in neuroepithelial cells. Moreover, in *Eph* mutants, the subapical region of the neuroepithelial cells was completely collapsed. This suggests that under normal conditions, and similarly to that observed at the tight junctions of Xenopus epithelia, an Eph reverse signaling leads to the phosphorylation of Ephrin at the subapical region. In fact, constitutively activated aPKC rescued the formation of the subapical region in *Drosophila Eph* mutant neuroepithelial cells[16]. It would be interesting to analyze in these mutant cells the localization of Cdc42, which would be expected to be altered if the hypothesis of the Eph reverse signal at the level of the subapical region is correct in our system.

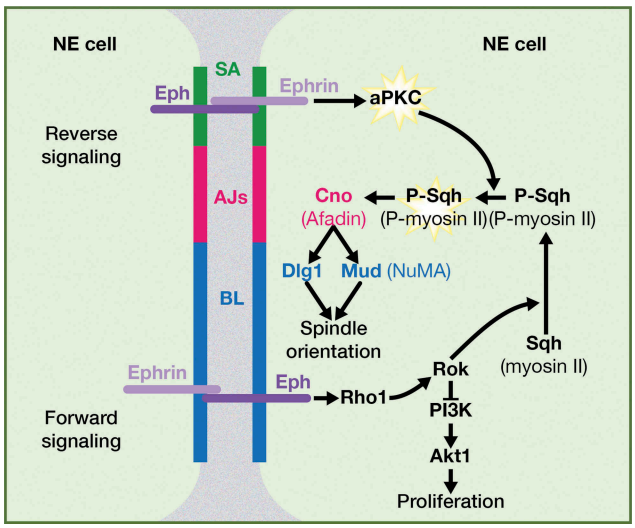

Figure 3. Eph signaling regulates mitotic spindle orientation: working model. Both a reverse and a forward Eph signaling would be operating between neuroepithelial cells in the *Drosophila* optic lobe. A forward signaling activates Rok/ROCK, which (1) inhibits PI3K-Akt1 signaling pathway and hence proliferation and (2) phosphorylates and activates myosin II Sqh/RLC. A reverse signaling at the level of SA activates aPKC, which fully activate P-Sqh/P-RLC and this, in turn, impacts on spindle orientation by contributing to the correct cortical localization of intrinsic cue regulators, such as Cno/Afadin, Dlg1, and Mud/NuMA. SA: SubApical region; AJs: Adherens Junctions; BL: Basolateral region. (Adapted from Franco and Carmena, 2019).

Other questions that arise from our work/model are: 1) what is exactly the meaning of "fully activated" P-myosin II/P-Sqh and 2) how aPKC is mediating this state? "Fully activated" would imply that apart of being phosphorylated by the kinase Rok/ROCK, myosin II/Sqh would require aPKC for regulating its apical localization. And an obvious candidate downstream of aPKC involved in the activation state of myosin II/Sqh is the myosin II/Sqh repressor Lethal (2) giant larvae (L(2)gl). aPKC phosphorylates L(2)gl blocking its binding to myosin II/Sqh in different systems; as a consequence, L(2)gl cannot inhibit myosin II/Sqh [65–71]. Hence, we might expect an ectopic apical activation of L(2)gl in *Eph* mutant neuroepithelial cells, in which aPKC is inactive, and the consequent mislocalization of myosin II/Sqh.

In addition to the aPKC activation through a reverse Eph-Ephrin signaling, we propose that an Ephrin-Eph forward signaling also functions between

neuroepithelial cells to trigger Rho1/RhoA-Rok /ROCK activation (Figure 3). A good candidate for mediating the link between the Eph receptor and Rho is the Rho-guanine nucleotide exchange factor (GEF) Ephexin, which physically interacts with Eph and activates Rho1/RhoA in response to Ephrin-stimulated Eph in neuronal growth cones, both in *Drosophila* and in vertebrates [72–74]. Another potential connection between Eph and Rho activation is Dsh/DVL, the main effector of Wingless/Wnt-Frizzled signaling pathway [75,76]. In Xenopus, Xdsh forms a complex with EphB1/B2 and ephrin-B1, regulating RhoA activity in both a forward and a reverse Eph signaling[77]. In our working model, the activation of Rho1/RhoA-Rok/ROCK signaling by Eph has two main consequences: 1) the phosphorylation and activation of myosin II/Sqh and 2) the inhibition of the PI3K-Akt signaling pathway, a major regulator of cell proliferation. In fact, *Eph* mutant optic lobe neuroepithelia show overgrowth[16]. It is important to remark that this overproliferation phenotype is the result of the upregulation of Akt signaling in *Eph* mutants and not a consequence of spindle misorientation. Actually, the overproliferation phenotype of *Eph* mutants is suppressed by specifically downregulating in the neuroepithelia either Akt or PI3K[16]. As mentioned before, different mechanisms have evolved to avert the fatal consequences that failures in the orientation of the spindle could imply. In the *Drosophila* optic lobe neuroepithelium, it operates the same adhesion molecule-mediated protective mechanism as in the follicular epithelium to reintegrate misplaced cells into the neuroepithelium [26]. As a result, we do not observe an increase in the multilayering/width of the neuroepithelium, as it would be expected. On the contrary, the reintegration process plus the defects in myosin II/Sqh distribution present in *Eph* mutants lead to narrower, simple organization neuroepithelia by late third instar larvae. It would be interesting to analyze the consequences of mutating those adhesion molecules on an *Eph* mutant background. We expect a very strong tissue disorganization, given the misorientation of the spindle along with the increase in proliferation observed in *Eph* mutants.

Finally, one relevant question that emerges from our work is how conserved is the function of Eph signaling as a spindle orientation regulator in other *Drosophila* and vertebrate epithelia. As mentioned above, the mechanisms that control spindle alignment are very diverse and highly context-dependent. For example, aPKC is not essential for spindle planar orientation in the chicken neuroepithelium, neither in the *Drosophila* wing disc and follicular epithelium [51,78,79]. Thus, we could speculate that these epithelia would be independent of Eph signaling for regulating spindle orientation, given that aPKC activation stands central downstream of Eph signaling in the optic lobe neuroepithelium. However, we cannot completely discard a function of Eph in those epithelia, as Eph might operate through alternative mechanisms in those tissues and, in addition, it still might be required for regulating Rho1/RhoA-Rok /ROCK signaling. Further work will be necessary in the future to solve all these questions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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