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COMMENTARY

Non-muscle myosin II activation: adding a classical touch to ROCK

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ABSTRACT

Non-muscle myosin II molecules are actin-binding proteins with ATPase activity, this latter capacity providing the energy required for actin filament cross-linking and contraction. The activation of these molecular motors relies on direct phosphorylation at conserved sites through different protein kinases, including the Rho-associated coiled coil-containing kinase (ROCK). In the light of some recent results found in our lab, we comment on the necessity of additional regulatory mechanisms to control the subcellular distribution of non-muscle myosin II proteins to ensure their full activation.

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Non-muscle myosin II motor proteins

Non-muscle myosin II proteins are actin-dependent molecular motors with ATPase activity that are expressed by all eukaryotic cells. These proteins are composed of two heavy chains (HCs) that each contains a globular head, a neck, an α-helical rod and a non-helical tail domain, as well as two regulatory light chains (RLCs) and two essential light chains (ELCs). The head region of each heavy chain contains the actin-binding and ATPase motor domain, while the neck domain that links the head to the rod domain binds one RLC and one ELC (Figure 1(a)). The rod domain interacts with other non-muscle myosin II dimers to form bipolar filaments. In these structures, the head domains of each dimer interact with actin filaments to promote their translocation and crosslinking through their ATPase activity (Figure 1(b)). Accordingly, non-muscle myosin II motors can transform the energy stored in ATP into mechanical forces that can be used in multiple cellular processes, including cytokinesis, cell adhesion and cell migration, to induce changes in cell shape and size [1–4].

The regulation of ATP hydrolysis in non-muscle myosin II proteins mainly relies on the reversible phosphorylation of conserved amino acids in the RLCs, specifically at Serine (Ser)-19, phosphorylation that is essential, and at Threonine (Thr)-18, phosphorylation that enhances the ATPase activity [5] (Figure 1). Thus, non-muscle myosin II activation depends on RLC phosphorylation, which favours the myosin ATPase activity in the presence of actin. Numerous kinases are involved in RLC phosphorylation, including the Rho-associated, coiled coil-containing kinase (ROCK) [6–9] that in turn is activated by the small GTPase RhoA [10,11].

Eph signaling triggers Sqh/RLC regulation by activating both Rok and aPKC

We recently unveiled a novel function for the Ephrin-Eph intercellular signaling pathway, activating Rho1 (*Drosophil*a RhoA ortholog)-Rok (*Drosophila* ROCK ortholog) signaling in neuroepithelial cells of the *Drosophila* optic lobe[12]. This activation has two effects, it maintains the phosphoinositide-3-kinase (PI3K)/Akt1 pathway repressed to limit proliferation in this neuroepithelium, while promoting the phosphorylation of myosin II RLC, called Spaghetti Squash (Sqh) in *Drosophila*, as seen in other cell contexts (Figure 2). We observed defects in the apical localization of Sqh/RLC in *Eph* mutant neuroepithelial cells, similar to those observed in different Rho1/Rok loss of function conditions[12]. Intriguingly, a phosphomimetic form of Sqh that simulates its Ser-21 and Thr-20 (Sqh^{E20E21} or Sqh^{EE}) phosphorylation, corresponding to that of the conserved mammalian Ser-19 and Thr-18 residues [13,14], did not suppress the *Eph* mutant phenotype, the severe defects in the apical localization of Sqh^{EE}/RLC^{EE} persisting in this mutant background[12]. Thus, assuming that Sqh^{EE}/RLC^{EE} is a bona fide phosphomimetic form of the endogenous phosphorylated Sqh by Rok, we could conclude that Rok is not sufficient to activate Sqh/RLC in the absence of Eph (see below for a further discussion on this).

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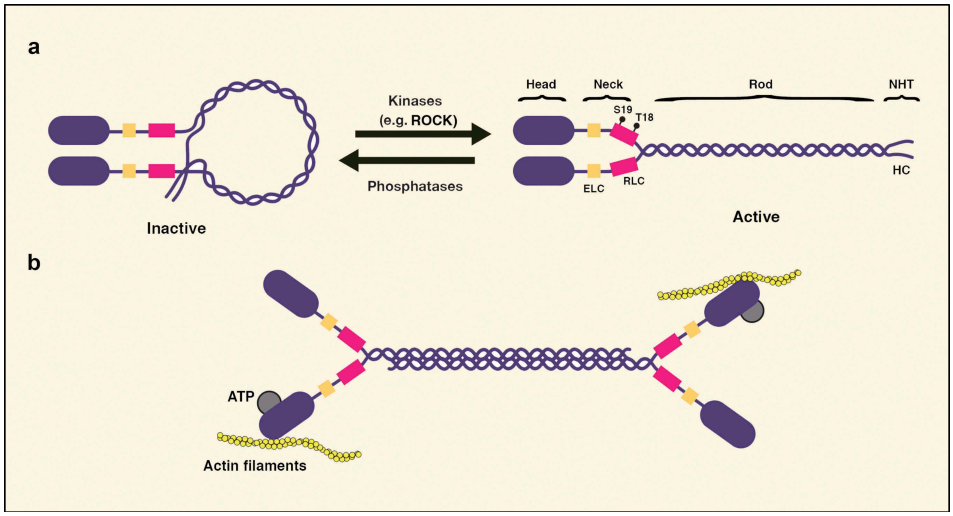

Figure 1. Non-muscle myosin II protein structure and activation. (a) Each myosin II protein is a hexamer composed by two HCs, two ELCs and two RLCs. The HCs contain a globular head, a neck, an α-helical rod and a non-helical tail domain (NHT). Phosphorylation of RLCs, located at the neck region, by different kinases, including ROCK, activates non-muscle myosin II. (b) The rod domains of different non-muscle myosin II proteins interact to form bipolar filaments. In these structures, the head domains of each dimer bind actin filaments to promote their translocation and crosslinking through their ATPase activity, also located at the globular head domain.

Figure 2. Eph signaling regulates spindle orientation and the proliferation of neuroepithelial cells in the *Drosophila* optic lobe: a working model. Forward Eph signaling activates Rok, which has two effects: (1) the repression of the PI3K/Akt1 signaling pathway; and (2), the phosphorylation and activation of Sqh/RLC. Reverse Eph signaling at the SA activates aPKC, which in turn fully activates P-Sqh/RLC, influencing spindle alignment by contributing to drive the correct cortical localization of Cno, Dlg1 and Mud: SA, subapical region; AJs, adherens junctions; BL, basolateral. (Adapted from Franco and Carmena, J. Cell Biol., 2019).

In fact, we found that, in addition to its effect on Rok, Eph signaling is also necessary to activate aPKC, which is in turn essential to 'fully activate' (i.e. to regulate the apical localization) of Sqh^{EE}/RLC^{EE} (Figure 2). Thus, there are clear defects in the apical localization of Sqh^{EE}/RLC^{EE} in *aPKC* mutant neuroepithelial cells, similar to those observed in *Eph* and *Ephrin* mutants[12]. Remarkably, a constitutively activated form of aPKC on an *Eph* and Sqh^{EE}/RLC^{EE} mutant background completely recovered the apical distribution of Sqh^{EE}/RLC^{EE}[12]. This result strongly supports a requirement of aPKC downstream of Eph signaling for a proper function of Sqh/RLC (Figure 2).

We also showed that 'fully activated' Sqh/RLC influences mitotic spindle orientation by contributing to drive the cortical localization of intrinsic spindle orientation regulators such as Canoe (Cno)/Afadin, Discs Large1 (Dlg1) and Mud/NuMA. In fact, both *aPKC* and *Sqh/RLC* mutants develop a similar spindle phenotype as *Eph* mutants, and constitutively activated aPKC on an *Eph* mutant background also expressing Sqh^{EE}/RLC^{EE} suppresses the *Eph* mutant spindle phenotype. Moreover, constitutively activated aPKC reverts the aberrant distribution of Dlg1 in *Eph* mutants in the presence of active Sqh^{EE}/RLC^{EE} but not on an Sqh^{WT} background[12]. Hence, not only is Rock required to activate Sqh but also, in addition to Rock, aPKC activity is essential to fully activate Sqh^{EE}/RLC^{EE}.

How does aPKC impinge on Sqh^{EE}/RLC^{EE}?

aPKC forms part of the highly conserved aPKC-Par6-Par3/Cdc42 complex. During *Drosophila* oogenesis, the apical localization of Rok restricts active myosin Sqh^{EE}/RLC^{EE} to the apical cortex of the follicular epithelium[15]. In this context, aPKC, along with the Par complex, is not required for the initial activation of myosin II/Sqh but rather, to anchor active myosin Sqh^{EE}/RLC^{EE} at that apical position. In other systems, such as the mouse MTD1-A polarized epithelial cell line, aPKC also does not affect the initial activation of myosin II but it is necessary to counteract the centripetal contractile forces induced by myosin II on the actin cables in each cell, thereby facilitating the formation of belt-like adherens junctions[16]. An interesting candidate to mediate the effect of aPKC on active myosin II Sqh^{EE}/RLC^{EE} is Lethal (2) giant larvae (L(2)gl/Lgl), given that aPKC, in both *Drosophila* and vertebrates, phosphorylates and represses this polarity protein, which in turn directly binds to and inhibits myosin II/Sqh activation [17–21]. Thus, the activation of aPKC by Eph signaling in optic lobe neuroepithelial cells might promote full Sqh^{EE}/RLC^{EE} activation in the subapical domain by repressing L(2)gl at this site. Accordingly, it is evident that the spatial distribution of myosin II in the cell is critical for its proper function and thus, it must be tightly regulated.

Apart from ROCK, other kinases like MLCK, ZIPK, the Citron kinase or MRCK also phosphorylate myosin II/RLC at Ser19 and/or Thr18[4]. In *Drosophila*, it was recently shown that the localization of one of such kinases, Stretchin-Mlck (Strn-Mlck), to the apical cell cortex in the junctional region is driven by Yorkie (Yki), and that this is required to fully activate Sqh/RLC[22]. In the future, it would be interesting to

Figure 3. Sqh^{EE}/RLC^{EE} partially rescues the *Rok* mutant phenotype in *Drosophila* optic lobe neuroepithelial cells. (a, a') The apical localization of Sqh^{WT}/RLC^{WT} is disrupted (white arrows) after overexpressing a dominant negative form of *Rok* (*UAS-Rok*^{CATKG} under the c855 neuroepithelial Gal4 driver) in all neuroepithelia analyzed (n = 8). (b-c') Sqh^{EE}/RLC^{EE} rescues the *Rok* phenotype (b, b'; 6 out of 9 neuroepithelia analyzed) while fails to rescue the phenotype in some cases (c, c'; 3 out of 9 neuroepithelia; white arrows in c'). NE: neuroepithelium (Franco and Carmena, unpublished results).

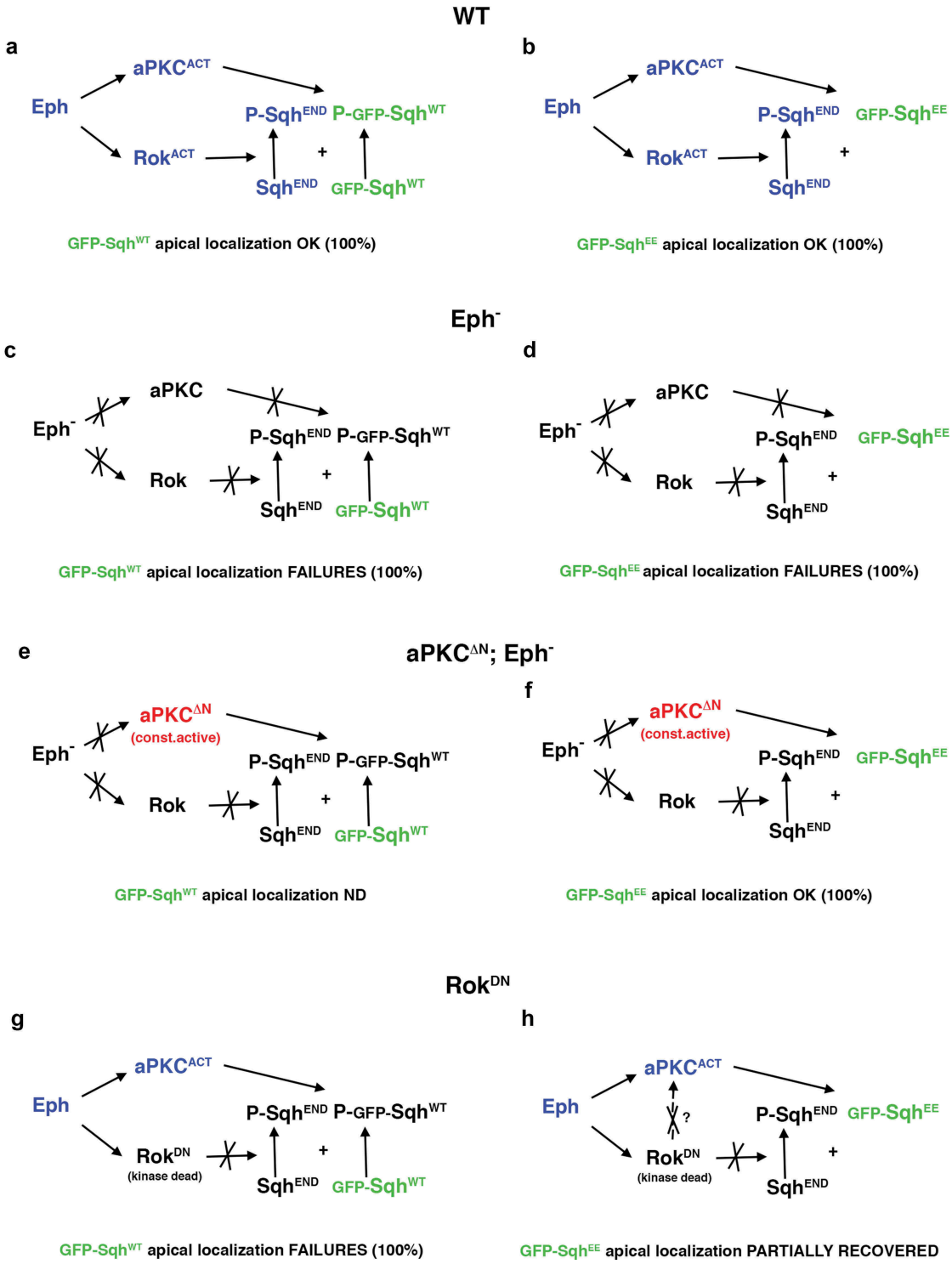

Figure 4. Diagrams representing the requirement of Eph signaling to fully activate Sqh/RLC through both aPKC and Rok. (a-f) The apical localization of Sqh^{WT}/RLC^{WT} and Sqh^{EE}/RLC^{EE} in wild type (WT) neuroepithelia (a, b) is always disrupted in *Eph* mutants (c, d), phenotype that is suppressed by constitutively activated aPKC ($aPKC^{\Delta N}$) in a Sqh^{EE}/RLC^{EE} background (f); the same experiment in a Sqh^{WT}/RLC^{WT} background has not been performed (e) (ND: not determined); see also Franco and Carmena, J. Cell Biol. 2019. (g, h) The apical localization of Sqh^{WT}/RLC^{WT} is completely disrupted after expressing a dominant negative form (kinase-dead) of Rok/ROCK in the neuroepithelia (g), while it is partially recovered in a Sqh^{EE}/RLC^{EE} background (h) (Franco and Carmena, unpublished results; see also Figure 3). *GFP-sqh*^{WT} and *GFP-sqh*^{EE} are transgenes expressed under the endogenous promoter (inserted on the second chromosome) [13,14] in the presence of the endogenous gene *sqh* (*sqh*^{END}) (on X chromosome).

address whether mechanisms that complement the activity of these kinases are also involved in fully activating myosin II/RLC, influencing its spatiotemporal cellular distribution.

Sqh^{EE}/RLC^{EE}: a phosphomimetic form of Sqh?

Martin´s and Sellers´ groups showed recently that phosphomimetic Sqh alleles (i.e. Sqh^{EE}/RLC^{EE}) do not fully mimic the phosphorylated state or the activity of endogenous Sqh/RLC phosphorylated at Ser-21 and Thr-20[23]. They found that this Sqh^{EE}/RLC^{EE} allele can bind to actin but it has reduced motor activity (about 30% of the functional Sqh activity)[23]. In fact, even though Sqh^{EE}/RLC^{EE} has been shown to partially suppress the *Rok*/*ROCK* mutant larval lethality in *Drosophila* (4% of mutant flies survive to adulthood) [14], Sqh^{EE}/RLC^{EE} does not revert the cell contractility defects evident in *ROCK* mutants or following ROCK inhibition [24–27].

Based on these novel findings[23], we wondered whether Sqh^{EE}/RLC^{EE} would be able to suppress the *Rok* mutant phenotype in our system. Very recent unpublished results from our lab (Franco and Carmena) showed that the apical localization of Sqh^{EE}/RLC^{EE} was altered in some neuroepithelia (3 out of 9) that are overexpressing a dominant negative form of Rok (Rok^{CATKG}), although it showed a clear improvement in most of the neuroepithelia analyzed (6 out of 9); the apical localization of Sqh^{WT}/RLC^{WT} in Rok^{CATKG} always showed defects (n = 8) (Figures 3 and 4(g,h)). Hence, in our system, Sqh^{EE}/RLC^{EE} can partially overcome the loss of Rok activity. Conversely, as mentioned above, the localization of Sqh^{EE}/RLC^{EE} in *Eph* mutant neuroepithelial cells is completely disrupted[12] (see also Figure 4(b,d)). One possibility is that the localization mechanism of Sqh^{EE}/RLC^{EE} in response to Eph requires a dynamic activation/inactivation regulation, which cannot be achieved in this constitutively phosphorylated form. However, this does not seem to be the case, as the localization defects of Sqh^{EE}/RLC^{EE} on an *Eph* mutant background are completely suppressed by constitutively activated aPKC, strongly supporting the relevance of aPKC activity downstream of Eph[12] (Figure 4(f)). Related to this latter result, at least another question still remains: why are the Sqh/RLC localization defects that appear after overexpressing a dominant negative form of Rok (Rok^{CATKG}) only partially suppressed by Sqh^{EE}/RLC^{EE}? (i.e. in this genetic background Eph-aPKC should be active) (Figures 3 and 4(h)). A potentially significant difference between these genetic backgrounds (Figure 4(f,h)) is the state of activation of aPKC, constitutively activated (Figure 4(f)) or activated under the regulation of Eph signaling (Figure 4(h)). We could also speculate that, in addition to a direct requirement of Eph for aPKC activation (Eph reverse signaling, Figure 2), Eph-dependent Rok activation (Eph forward signaling, Figure 2) might be also somehow impinging on aPKC activation (Figure 4(h)); at least, the localization of aPKC is affected in different Rho1 and Rok mutant conditions[12]. In this regard, it would be interesting to determine whether the localization defects of Sqh^{EE}/RLC^{EE} in cells that are expressing a dominant negative form of Rok (Rok^{CATKG}) (Figure 4(h)) are suppressed by constitutively activated aPKC, and also which is the activity state of aPKC in different Rok mutant backgrounds. Future work will help to clarify all these questions.

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Disclosure statement

No potential conflict of interest was reported by the author.

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