Supplementary Note

Our experimental results identified a network of functional relationships between the different molecular components that is illustrated in Scheme 1. These interrelationships were based on changes in their junctional localization. To analyse the properties of this network in more detail, we developed a computational model to assess how these relationships help to establish a mesoscopic RhoA zone at the zonula adherens.

Scheme 1

Experimental foundations of the model.

The individual steps in Scheme 1 were based on the following experimental observations:
1. Inhibition of RhoA by treatment with C3-T decreases the amount of ROCK1 found at the ZA (this report, Supplementary Fig S4 b,c).
2. Phosphorylation of Rnd3 by ROCK1 prevents its localization to the ZA (this report, Fig 5).
3. Rnd3 is necessary for p190B to localize to the ZA when myosin or ROCK are inhibited (this report, Fig 4 a and Fig 5 l,m).
4. Junctional RhoA is depleted when p190B localizes to the ZA (this report, Fig 2 f,g,h,i).
5. Blocking ROCK activity reduces junctional Myosin IIA $^{1,2}$. 
6. Myosin IIA is necessary to localize ROCK1 to the ZA (this report, Fig 6 a,b,c).

**Computational model.**

The model employed a set of pairwise stimulation or repression relationships that modulate the concentration of species at junctions using standard Hill function ($\theta$) activation and repression rate equations $^3,4$. Our basic approach was as follows:

Given components $X$ and $Y$, where $X$ stimulates or represses the junctional localization of $Y$, the rate of change of $Y$ at the junctional cortex is given by the equation

$$\frac{dY}{dt} = -aY + b\theta_X^Y \quad \text{(for stimulation)}$$

$$\frac{dY}{dt} = -aY + b(1 - \theta_X^Y) \quad \text{(for repression)}$$

where

$$\theta_X^Y = f(X, K_X^Y) = \frac{X^n}{(K_X^Y)^n + X^n}$$

While interactions may be complex when multiple effectors act on a single molecule, broadly speaking the constants $a,b$ and $K$ correspond to the following:

a) The **decay coefficient** $a$ is the rate at which $Y$ is lost from cell junctions.

b) The constant $b$ is the maximal association rate of $Y$ with the junctional cortex; it is related to the maximal value of junctional $Y$ that can be achieved at steady state.

c) The **coefficient** $K_X^Y$ corresponds to a dissociation constant between $X$ and $Y$ in the original Hill model that can be also interpreted as the junctional concentration of $X$ at which the Hill function $\theta$ has half its maximum value. This can be thought of as the junctional concentration of $X$ at which significant junctional recruitment or junctional dissociation of $Y$ begins to occur. For example, for the case where the presence of $X$ stimulates junctional localization of $Y$, it follows that a large value of $K_X^Y$ corresponds to a greater concentration of $X$ being required at junctions before junctional localization of $Y$ begins to occur.
d) The Hill coefficient \( n \) determines how rapidly the junctional concentration of \( Y \) changes with an increasing junctional concentration of \( X \). A large value of \( n \) corresponds to a near binary step function where association or dissociation of junctional localization of \( Y \) does not occur below concentration \( K_X^Y \), but switches rapidly to its maximum above \( K_X^Y \).

Modeling the system in Scheme 1 using this approach \(^3,^4\) results in 6 first order differential equations that describe the rates of change in junctional concentration of RhoA, ROCK1, p190B RhoGAP (p190B), Rnd3 and NMIIA (Supplementary Note Table 1). Because of its one-dimensional nature and for simplicity, this model deals only with the junctional content of these molecules and does not make explicit reference to their other potential locations in the cell. Matlab \(^5\) was then used to solve the system numerically and obtain time courses for the components using the values for the constants and initial conditions shown in Supplementary Note Table 1.

### Supplementary Note Table 1. Hill (K), maximal association rates (b) and decay (a) constants in the stimulation-repression model shown in Scheme 1 \(^\dagger\).

<table>
<thead>
<tr>
<th>Relation/kinetic equation</th>
<th>a</th>
<th>b</th>
<th>K</th>
<th>Eq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. RhoA(\rightarrow)ROCK1 (stimulation)</td>
<td></td>
<td></td>
<td>(K_{\text{ROCK1}}^{\text{RhoA}} = 0.5)</td>
<td>(Eq. 1)</td>
</tr>
<tr>
<td>(\frac{d\text{ROCK1}}{dt} = -a_1\text{ROCK1} + b_1\theta_{\text{ROCK1}}^{\text{ROCK1}})</td>
<td>(a_1=0.15\dagger)</td>
<td>(b_1=0.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Rock1(\rightarrow)Rnd3 (repression)</td>
<td></td>
<td></td>
<td>(K_{\text{Rnd3}}^{\text{ROCK1}} = 0.5)</td>
<td>(Eq. 2)</td>
</tr>
<tr>
<td>(\frac{d\text{Rnd3}}{dt} = -a_2\text{Rnd3} + b_2(1 - \theta_{\text{ROCK1}}^{\text{ROCK1}}))</td>
<td>(a_2=0.07)</td>
<td>(b_2=0.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Rnd3(\rightarrow)p190B (stimulation)</td>
<td></td>
<td></td>
<td>(K_{\text{Rnd3}}^{\text{p190B}} = 0.5)</td>
<td>(Eq. 3)</td>
</tr>
<tr>
<td>(\frac{dp_{190B}}{dt} = -a_3p_{190B} + b_3\theta_{\text{Rnd3}}^{p_{190B}})</td>
<td>(a_3=0.1)</td>
<td>(b_3=0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. p190B(\rightarrow)RhoA (repression)</td>
<td></td>
<td></td>
<td>(K_{\text{p190B}}^{\text{RhoA}} = 0.5)</td>
<td>(Eq. 4)</td>
</tr>
<tr>
<td>(\frac{d\text{RhoA}}{dt} = -a_4\text{RhoA} + b_4(1 - \theta_{\text{p190B}}^{\text{p190B}}))</td>
<td>(a_4=0.1)</td>
<td>(b_4=0.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5. ROCK1→NMIIA (stimulation)  
\[ \frac{d\text{NMIIA}}{dt} = -a_5 \text{NMIIA} + b_5 \theta_{\text{NMIIA}}^{\text{ROCK1}} \]  
\[ a_5 = 0.1 \quad b_5 = 0.2 \quad K_{\text{NMIIA}}^{\text{ROCK1}} = 0.5 \]  
(Eq. 5)

6. NMIIA→ROCK1 (stimulation)  
\[ \frac{d\text{ROCK1}}{dt} = -a_1 \text{ROCK1} + b_6 \theta_{\text{NMIIA}}^{\text{ROCK1}} \]  
\[ a_1 = 0.15^{\dagger\dagger} \quad b_6 = 0.2 \quad K_{\text{ROCK1}}^{\text{NMIIA}} = 1.7 \]  
(Eq. 6)

\(^{\dagger}\)Unless otherwise explicitly stated in the text, the above constants were used for the stimulation-repression model. An Hill coefficient of \( n=2 \) was used throughout all modeling. In all simulations, initial concentrations were set to 1 unless otherwise stated.

\(^{\dagger\dagger}\)Note that \( a_1 \) correspond to a single decay factor for ROCK1. Thus the net decay of ROCK1 is given by  
\[ \frac{d\text{ROCK1}}{dt} = -a_1 \text{ROCK1} + b_1 \theta_{\text{RhoA}}^{\text{ROCK1}} + b_6 \theta_{\text{NMIIA}}^{\text{ROCK1}} \]  
(Eq. 7)
Implications of the one-dimensional model

As described in the main text, the junctional localization of the components of the network in Scheme 1 exhibits bistable behavior. We also explored the implications of the model by analyzing reduced subsystems in the feedback network, as well as the full network, with a particular focus on testing computationally whether these reduced subsystems were able to generate bistable outcomes for the junctional localization of RhoA.

1. Effect of removing feedback from ROCK1 to RhoA.

First, we examined how removing feedback from ROCK1 to RhoA (which is mediated by repression of junctional Rnd3 and p190B) affected the capacity of the system to generate bistable junctional localization of RhoA at steady state. Removing this feedback path yields the residual network of Scheme 2, whose component equations are shown in Supplementary Note Table 2.

**Scheme 2**

![Scheme 2](image)

**Supplementary Note Table 2. Kinetic equations for the reduced subsystem shown in Scheme 2.**

\[
\frac{d\text{ROCK1}}{dt} = -a_1 \text{ROCK1} + b_1 \theta_{\text{ROCK1}}^{\text{ROCK1}} + b_6 \theta_{\text{NMIIA}}^{\text{NMIIA}} \tag{Eq.7}
\]

\[
\frac{d\text{RhoA}}{dt} = -a_4 \text{RhoA} + b_4 \tag{Eq.8}
\]

\[
\frac{d\text{NMIIA}}{dt} = -a_5 \text{NMIIA} + b_5 \theta_{\text{ROCK1}}^{\text{NMIIA}} \tag{Eq.9}
\]
This scheme predicts that the steady-state concentration of RhoA at the ZA will be determined by its activation by a GEF (i.e. coefficient $b_4$, see also Extension of the model to 3 dimensions). This computational prediction is consistent with experimental evidence that RhoA must be in its active state to localize to the ZA $^7$ (this report) and, indeed, requires the GEF, Ect2 to localize to the ZA in MCF7 cells$^7$. Of note, the steady-state junctional concentration of RhoA ($RhoA_{s.s.}$, Eq. 10) in Scheme 2 will not be affected by the concentrations of the other species involved as they lie downstream of RhoA. Therefore:

$$RhoA_{s.s.} = \frac{b_4}{a_4}, \text{ where the subscript s.s. denotes steady state (Eq. 10)}$$

Similarly, the solutions for ROCK1$_{s.s.}$ and NMIIA$_{s.s.}$ are given by first substituting RhoA in Eq.7 using Eq.10 and then solving the resulting set of equations (11 and 12) for ROCK1 and NMIIA:

$$ROCK1_{s.s.} = \frac{b_1(\theta_{ROCK1}^{ROCK1})_{s.s.} + b_6(\theta_{NMIIA}^{ROCK1})_{s.s.}}{a_4} \quad \text{(Eq. 11)}$$

$$NMIIA_{s.s.} = \frac{b_5(\theta_{ROCK1}^{NMIIA})_{s.s.}}{a_5} \quad \text{(Eq.12)}$$

As it is known that RhoA is an effective activator of ROCK1 and promotes the junctional localization of ROCK1 (Supplementary Fig S4 b,c), we assume that $RhoA_{s.s.} \gg K_{ROCK1}^{ROCK1}$. Under these conditions, we found that the system also presents a single non-zero solution for ROCK1$_{s.s.}$ and NMIIA$_{s.s.}$. Thus, we conclude that this system can generate a continuous range of values for steady-state junctional RhoA content, which depend on the level of GEF stimulation. However, it does not generate bistability in junctional RhoA content.

**2. Effect of removing feedback from NMIIA to ROCK1.**

To better understand the origin of bistability in the full network, we then used the same approach as above to analyse why the model predicted a loss of
RhoA from the ZA, when feedback from NMIIA to ROCK1 was inactivated (Scheme 3).

According to Scheme 3, the total rate of change of the different species is described in the series of equations in Supplementary Note Table 1 with the difference that the net rate of change for ROCK1 is given by:

\[
\frac{dR\text{OCK}1}{dt} = -a_1 R\text{OCK}1 + b_1 \theta^{R\text{OCK}1}_{\text{RhoA}} \quad (Eq. 13)
\]

Based on these equations, the steady state junctional concentrations for this system are:

\[
R\text{OCK}1_{s.s.} = \frac{b_1 (p^{R\text{OCK}1}_{\text{RhoA}})_{s.s.}}{a_1} \quad (Eq. 14)
\]

\[
R\text{nd3}_{s.s.} = \frac{b_2 (1-(\theta^{R\text{nd3}}_{\text{ROCK1}})_{s.s.})}{a_2} \quad (Eq. 15)
\]

\[
p^{190}B_{s.s.} = \frac{b_3 (\theta^{p190B}_{\text{Rnd3}})_{s.s.}}{a_3} \quad (Eq. 16)
\]

\[
R\text{hoA}_{s.s.} = \frac{b_4 (1-(\theta^{R\text{hoA}}_{p190B})_{s.s.})}{a_4} \quad (Eq. 17)
\]

\[
N\text{MIIA}_{s.s.} = \frac{b_5 (\theta^{N\text{MIIA}}_{\text{ROCK1}})_{s.s.}}{a_5} \quad (Eq. 18)
\]
Note that in Scheme 1 (Supplementary Note Table 1), where the full network is present, the steady state value for ROCK is given by:

\[ \text{ROCK1}_{s,s} = \frac{b_6(b_{\text{ROCK1}}^{\text{ROCK1}})_{s,s}}{a_1} + \frac{b_6(b_{\text{NMII}}^{\text{ROCK1}})_{s,s}}{a_1} \]  

(Eq.19)

Comparing Eq. 19 with its equivalent in Scheme 3, Eq. 14, reveals that when feedback from NMIIA to ROCK1 is removed, the expected junctional concentration of ROCK1 decreases by a value of \( \frac{b_6(b_{\text{ROCK1}}^{\text{ROCK1}})_{s,s}}{a_1} \). Note that if the concentration of junctional ROCK1 at steady state is below \( K_{\text{ROCK1}}^{\text{Rnd3}} \), the system will stimulate junctional Rnd3 accumulation and therefore cause RhoA to be lost from the junctions through the action of p190B. Therefore, one explanation for why feedback from NMII to ROCK1 promotes bistability is that it increases the effective junctional concentration of ROCK1, therefore making it better able to antagonize the junctional localization of Rnd3.

We also found that without feedback from NMIIA to ROCK1 (Scheme 3), for the set of parameter values used in our simulations, there is only a single solution for ROCK1 that leads to a single state where there is low junctional RhoA. This is shown in the next figure where eq. 15 and 16 were substituted in Eq. 17 and numerical solutions for this equation together with equation 14 were obtained graphically. Here it can be seen that the reduced network shown in Scheme 3 cannot generate bistable outcomes for junctional RhoA under these conditions.
Of note, it is possible to switch the system to a bistable mode for junctional RhoA by modifying the parameters that determine the amount of ROCK1 at steady state. One of these possibilities, as it was mentioned above, occurs when feedback from NMIIA to ROCK1 is added (Eq. 19). This is shown by the graphical solution for Eq. 17 and 19 shown below. Here it can be seen that adding the loop introduces a discontinuity in equation 19 that limits the system to two possible solutions (represented by the intersections between curves): one characterized by high junctional ROCK1 and RhoA and the other by low junctional ROCK1 and RhoA.

Thus, under these conditions the system evolves either towards a state with high junctional RhoA or one with low junctional RhoA depending on the initial concentrations of the other species in the network. This is shown in the phase diagram in Supplementary Fig. 7b.

Taken with our experimental data, these computational considerations suggest that in cells the amount of ROCK1 that is required to repress junctional accumulation of Rnd3 is not achieved when myosin is inactivated, further implying that junctional accumulation of RhoA in the system is repressed under these conditions.

The above arguments lead to the following explanation for why the stimulation-repression model depicted in Scheme 1 evolves into a single state or bistable states for junctional RhoA. According to the Hill function, the stimulation coefficient \( K_{NMIIA}^{ROCK1} \) can be understood as the dissociation constant between
ROCK1 and NMIIA (Fig 7c). When the interaction between these proteins has a high apparent affinity (lower values of $K_{NMIIA}^{ROCK1}$), small increases in junctional NMIIA cause a transient increase in junctional ROCK1; in the cellular context, this represents the increase in cortical ROCK1 that occurs where NMIIA is localized. This process favors an increase in the local concentration of ROCK1 and consequently efficient antagonism of junctional Rnd3 localization. On the other hand, when the interaction between ROCK1 and NMIIA has a low apparent affinity (higher values of $K_{NMIIA}^{ROCK1}$), the recruitment of ROCK1 to the cortex is inefficient, even when the amount of NMIIA may be high, and therefore the local concentration of ROCK1 is not high enough to antagonize the junctional recruitment of Rnd3. Thus, the system becomes inactivated irrespective of the initial concentration of NMIIA. It is important to consider that the value of $K_{NMIIA}^{ROCK1}$ at which the system exhibits bistability will also depend on the factors that determine the local concentration of NMIIA and ROCK1 at the ZA: the apparent binding constant of NMIIA to the cortex (which determines the amount of myosin present at the cortex) and the affinity between RhoA and ROCK1. Together, these properties and our experimental evidence showing that NMIIA and ROCK1 are capable of interacting, determines the local concentration of ROCK1 at the cortex and whether or not this is sufficient to effectively antagonize junctional Rnd3 accumulation.

3. **Effect of adding negative feedback from Rnd3 to ROCK1.**

It was previously reported that Rnd3 could also inhibit ROCK1 activity. To investigate in silico how this additional interaction might affect the properties of the minimal regulatory network that we have identified, we included an additional inhibitory step from Rnd3 to ROCK1 as is shown in red in Scheme 4.
This inclusion adds an additional repression equation to the system of the form

\[
\frac{dROCK1}{dt} = -a_1 ROCK1 + b_7 \left(1 - \theta_{RND3}^{ROCK1}\right)
\]  
(Eq. 20)

where

\[
\theta_{RND3}^{ROCK1} = \frac{RND3^2}{(K_{RND3}^{ROCK1})^2 + RND3^2}
\]  
(Eq. 21)

We solved the system numerically for different initial concentrations of RhoA and Rnd3 to analyse the effect of increasing the strength of this negative feedback on: 1) the capacity of the system to exhibit bistability; and 2) junctional RhoA content at steady state. We performed this by varying the coefficient \(K_{RND3}^{ROCK1}\), the concentration of Rnd3 at which significant junctional dissociation of ROCK1 occurs, while maintaining the coefficient \(b_7\) constant and equal to 0.15.

We found in our numerical analysis that the addition of this negative feedback loop from Rnd3 to ROCK1 did not alter the behaviour of the network, which still is able to exhibit bistable properties (Supplementary Fig S7c). However, increasing the strength of this negative feedback loop causes the system to be more sensitive to inhibition by Rnd3, i.e. lower concentrations of Rnd3 are sufficient to switch the network to a low RhoA content state.

We can see the origin of this last effect by analysing the steady state concentration for ROCK1 for the network structure in Scheme 4, which is given by
It can be deduced from this equation that increasing the strength of the negative feedback from Rnd3 to ROCK1 reduces the effective concentration of ROCK1 at the junctions. This implies that higher concentrations of RhoA or NMIIA are required to maintain the system in a state with high RhoA content, a result that is consistent with our simulations (Supplementary Fig 7c). Moreover, in the presence of a strong negative feedback, it is possible that the amount of ROCK1 falls below the corresponding $K_{\text{NMIIA}}^{\text{ROCK1}}$. In the extreme case, it is expected the system will always evolve towards a state of low RhoA content, irrespective of the values for the rest of the parameters. Our experimental observation that cells also achieve a steady-state with high junctional RhoA content (Fig 7d) implies that this extreme level of negative feedback is unlikely to be common.
Extension of the stimulation/repression model to 3 dimensions

We then extended this computational analysis to a 3-dimensional model in order to test how spatial localization of myosin II (analogous to what we have observed at the ZA) might affect the spatial patterning of RhoA.

1. Derivation of the explicit form for the equations used in the spatial model.

To extend our model from one to three dimensions we re-wrote the kinetic equations that describe the cortical (junctional) localization of proteins in Scheme 1 and in Fig. 7a as adsorption/desorption reactions to the cell cortex that can be stimulated or repressed by accessory proteins.

Based on this understanding, we could express the rate of RhoA translocation to the cortex with the following equation:

\[
\frac{d[RhoA_{\text{cortex}}]}{dt} = -k_{\text{off}}^{RhoA_{\text{cortex}}} [RhoA_{\text{cortex}}] + k_{\text{on}}^{RhoA_{\text{cortex}}} [RhoA_{\text{cyt}}] \quad (\text{Eq. 23})
\]

where

\[
[RhoA_{\text{cortex}}] = \text{concentration of RhoA to the cell cortex}
\]
\[
k_{\text{off}}^{RhoA_{\text{cortex}}} = \text{kinetic dissociation constant of RhoA from the cell cortex.}
\]
\[
k_{\text{on}}^{RhoA_{\text{cortex}}} = \text{kinetic association constant of RhoA to the cell cortex.}
\]

Note that this equation does not explicitly address the nucleotide-loaded status of Rho. However, we have shown experimentally that inhibiting RhoA with C3-transferase causes it to be lost from the ZA, which implies that at steady-state RhoA must be in its GTP-loaded, active state to optimally localize to the ZA. Accordingly, the model can be simplified to treat RhoA as a single species that is able to translocate to the cell cortex when it is biologically active (exactly comparable to the simplification that we used to develop the 1-dimensional model). This simplification allows us to express the relationship between

\[
k_{\text{off}}^{RhoA_{\text{cortex}}} \text{ and } k_{\text{on}}^{RhoA_{\text{cortex}}} \text{ as}
\]

\[
k_{\text{on}}^{RhoA_{\text{cortex}}} = K_a^{RhoA_{\text{cortex}}} \cdot k_{\text{off}}^{RhoA_{\text{cortex}}} \quad (\text{Eq. 24})
\]

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where \( K_{RhoA_{cortex}} \) is the intrinsic association constant of RhoA for the cell cortex.

Similarly to our one-dimensional activation/repression model, the modulation of RhoA translocation by a RhoGEF and a RhoGAP (p190B,\(^7\) and present work) was executed by introducing Hill type functions \( \theta \) that switch from 0 to 1 depending on the concentration of these species\(^3,4\).

Combining (Eq.23) and (Eq.24) we then obtain,

\[
\frac{d[RhoA_{cortex}]}{dt} = -k_{RhoA_{cortex}}^{RhoA_{cortex}}[RhoA_{cortex}] + \theta_{RhoA_{p190B}}^{RhoA_{p190B}} \cdot (1 - \theta_{RhoA_{p190B}}^{RhoA_{p190B}}) \cdot K_{RhoA_{cortex}}^{RhoA_{cortex}}.
\]

where

\[
\theta_{RhoA_{p190B}}^{RhoA_{p190B}} = \frac{[p190B_{cortex}]^n}{(K_{RhoA_{p190B}}^{p190B})^n + [p190B_{cortex}]^n}
\]

and

\[
\theta_{RhoA_{Ect2}}^{RhoA_{Ect2}} = \frac{[Ect2_{cortex}]^n}{(K_{RhoA_{Ect2}}^{Ect2})^n + [Ect2_{cortex}]^n}
\]

We then used this structure to write differential equations (Supplementary Note Table 3) for the cortical accumulation of the rest of species involved in the network (based on the same experimental foundations as used for the one-dimensional model).

**Supplementary Note Table 3. Kinetic equations for the stimulation-repression model in 3 dimensions**

\[
\frac{d[ROCK1_{cortex}]}{dt} = -k_{ROCK1_{cortex}}^{ROCK1_{cortex}}[ROCK1_{cortex}] + \theta_{ROCK1_{ROCK1}}^{ROCK1_{ROCK1}} \cdot \theta_{ROCK1_{ROCK1}}^{ROCK1_{ROCK1}} \cdot k_{ROCK1_{cortex}}^{ROCK1_{cortex}}.
\]

\[
\theta_{ROCK1_{ROCK1}}^{ROCK1_{ROCK1}} = \frac{[ROCK1_{cortex}]^n}{(K_{ROCK1_{ROCK1}}^{ROCK1_{ROCK1}}) + [ROCK1_{cortex}]^n} \quad ; \theta_{ROCK1_{NMIIA}}^{ROCK1_{NMIIA}} = \frac{[NMIIA_{cortex}]^n}{(K_{NMIIA}^{ROCK1_{NMIIA}}) + [NMIIA_{cortex}]^n}
\]

(Eq.25)
\[
\frac{d[Rn3_{\text{cortex}}]}{dt} = -k_{\text{off}}^{Rn3_{\text{cortex}}} [Rn3_{\text{cortex}}] + (1 - \theta_{ROCK1}^{Rn3}) \cdot K_a^{Rn3_{\text{cortex}}} \\
\times k_{\text{off}}^{Rn3_{\text{cortex}}} [Rn3_{\text{cyr}}] \quad \text{(Eq.26)}
\]

\[
\theta_{ROCK1}^{Rn3} = \frac{[ROCK1_{\text{cortex}}]^n}{(K_{ROCK1}^{Rn3})^n + [ROCK1_{\text{cortex}}]^n}
\]

\[
\frac{d[p190B_{\text{cortex}}]}{dt} = -k_{\text{off}}^{p190B_{\text{cortex}}} [p190B_{\text{cortex}}] + \theta_{Rn3}^{p190B} \cdot K_a^{p190B_{\text{cortex}}} \\
\times k_{\text{off}}^{p190B_{\text{cortex}}} [p190B_{\text{cyr}}] \quad \text{(Eq.27)}
\]

\[
\theta_{Rn3}^{p190B} = \frac{[Rn3_{\text{cortex}}]^n}{(K_{Rn3}^{p190B})^n + [Rn3_{\text{cortex}}]^n}
\]

\[
\frac{d[RhoA_{\text{cortex}}]}{dt} = -k_{\text{off}}^{RhoA_{\text{cortex}}} [RhoA_{\text{cortex}}] + \theta_{RhoA}^{RhoA} \cdot \left(1 - \theta_{p190B}^{RhoA}\right) \cdot K_a^{RhoA_{\text{cortex}}} \\
\times k_{\text{off}}^{RhoA_{\text{cortex}}} [RhoA_{\text{cyr}}] \quad \text{(Eq.28)}
\]

\[
\theta_{RhoA}^{p190B} = \frac{[p190B_{\text{cortex}}]^n}{(K_{RhoA}^{p190B})^n + [p190B_{\text{cortex}}]^n}, \quad \theta_{RhoA}^{RhoA} = \frac{[RhoGEF_{\text{cortex}}]^n}{(K_{RhoA}^{RhoGEF})^n + [RhoGEF_{\text{cortex}}]^n}
\]

\[
\frac{d[NMIIA_{\text{cortex}}]}{dt} = -k_{\text{off}}^{NMIIA_{\text{cortex}}} [NMIIA_{\text{cortex}}] + \theta_{ROCK1}^{NMIIA} \cdot K_a^{NMIIA_{\text{cortex}}} \\
\times k_{\text{off}}^{NMIIA_{\text{cortex}}} [NMIIA_{\text{cyr}}] \quad \text{(Eq. 29)}
\]

\[
\theta_{ROCK1}^{NMIIA} = \frac{[ROCK1_{\text{cortex}}]^n}{(K_{ROCK1}^{NMIIA})^n + [ROCK1_{\text{cortex}}]^n}
\]
As is shown in Eq. 25, the association between ROCK1 and the cell cortex was modelled as a single adsorption process, which is in principle modulated independently by NMIIA and RhoA, as there is no direct evidence for a ternary complex between RhoA, ROCK1 and NMIIA.

Expressions for the steady state concentration and the possible maximal concentration of each species at the cell cortex (Supplementary Note Table 4) were obtained by solving:

\[
\frac{dx_{\text{cortex}}}{dt} = 0,
\]

with \(X = \text{RhoA}_{\text{cortex}}, \text{ROCK1}_{\text{cortex}}, \text{NMIIA}_{\text{cortex}}, \text{p190B}_{\text{cortex}}\) and \(\text{Rnd3}_{\text{cortex}}\)

### Supplementary Note Table 4. Steady state cortical concentrations of the different species in the 3 dimensional model

<table>
<thead>
<tr>
<th>Expression</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\frac{[\text{ROCK1}<em>{\text{cortex}}]</em>{\text{s.s.}}}{[\text{ROCK1}<em>{\text{cyt}}]</em>{\text{s.s.}}} = \left(\theta_{\text{ROCK1}}^{\text{ROCK1}}\right)<em>{\text{s.s.}} + \left(\theta</em>{\text{NMIIA}}^{\text{ROCK1}}\right)<em>{\text{s.s.}} \cdot K_a^{\text{ROCK1}</em>{\text{cortex}}})</td>
<td>(Eq.30)</td>
</tr>
<tr>
<td>(\frac{[\text{Rnd3}<em>{\text{cortex}}]</em>{\text{s.s.}}}{[\text{Rnd3}<em>{\text{cyt}}]</em>{\text{s.s.}}} = \left(1 - \left(\theta_{\text{ROCK1}}^{\text{Rnd3}}\right)<em>{\text{s.s.}}\right) \cdot K_a^{\text{Rnd3}</em>{\text{cortex}}})</td>
<td>(Eq.31)</td>
</tr>
<tr>
<td>(\frac{[\text{p190B}<em>{\text{cortex}}]</em>{\text{s.s.}}}{[\text{p190B}<em>{\text{cyt}}]</em>{\text{s.s.}}} = \left(\theta_{\text{Rnd3}}^{\text{p190B}}\right)<em>{\text{s.s.}} \cdot K_a^{\text{p190B}</em>{\text{cortex}}})</td>
<td>(Eq.32)</td>
</tr>
<tr>
<td>(\frac{[\text{RhoA}<em>{\text{cortex}}]</em>{\text{s.s.}}}{[\text{RhoA}<em>{\text{cyt}}]</em>{\text{s.s.}}} = \left(\theta_{\text{ROGEP}}^{\text{RhoA}}\right)<em>{\text{s.s.}} \cdot \left(1 - \left(\theta</em>{\text{p190B}}^{\text{RhoA}}\right)<em>{\text{s.s.}}\right) \cdot K_a^{\text{RhoA}</em>{\text{cortex}}})</td>
<td>(Eq.33)</td>
</tr>
</tbody>
</table>
Using the above set of equations, it is possible to define relationships at which the different adsorption/desorption reactions proceed efficiently (assuming cytosolic concentrations of species equal to unity):

1) At steady state active RhoA is able to promote the cortical accumulation of ROCK1 only if:

\[
\left( \theta_{\text{RhoGEF}}^{\text{RhoA}} \right)_{s.s.} \cdot K_{\text{RhoA} \text{cortex}}^{\text{RhoA}} \gg K_{\text{RhoA}}^{\text{ROCK1}}
\]

2) At steady state p190B at the cortex is able to cause RhoA to dissociate from it only if:

\[
K_{\text{p190B cortex}}^{\text{p190B}} \gg K_{\text{p190B}}^{\text{RhoA}}
\]

3) At steady state Rnd3 is able to promote cortical localization of p190B only if:

\[
K_{\text{Rnd3 cortex}}^{\text{Rnd3}} \gg K_{\text{p190B}}^{\text{p190B}}
\]

4) At steady state ROCK1 is able to promote cortical accumulation of NMIIA only if:

\[
K_{\text{ROCK1 cortex}}^{\text{ROCK1}} \gg K_{\text{ROCK1}}^{\text{NMIIA}}
\]

5) At steady state ROCK1 is able to antagonize the cortical localization of Rnd3 only if:

\[
K_{\text{ROCK1 cortex}}^{\text{ROCK1}} \gg K_{\text{ROCK1}}^{\text{Rnd3}}
\]

6) At steady state NMIIA is promote the cortical localization of ROCK only if:

\[
K_{\text{NMIIA cortex}}^{\text{NMIIA}} \gg K_{\text{ROCK1}}^{\text{ROCK1}}
\]
2. Transformation of variables from the one-dimensional stimulation-repression model to three dimensions.

Given that normally the space volume in the cytoplasm is larger than the corresponding one at the membrane, we can then assume that the cytosolic concentrations of the different components remains constant throughout the simulation. This allows the following transformation of variables from the one-dimensional stimulation-repression model to its extension in 3 dimensions (Supplementary Note Table 5).

**Supplementary Note Table 5. Variable transformation between the one-dimensional stimulation-repression model and its extension in 3 dimensions.**

<table>
<thead>
<tr>
<th>Equation</th>
<th>Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a_1 = k_{ROCK1\text{cortex}}^{ROCK1 \text{cortex}} )</td>
<td>(Eq. 35)</td>
</tr>
<tr>
<td>( b_1 = b_6 = k_{ROCK1 \text{cortex}}^{ROCK1 \text{cortex}} \cdot K_{ROCK1 \text{cortex}} \cdot [ROCK1 \text{cyt}] )</td>
<td></td>
</tr>
<tr>
<td>( a_2 = k_{Rnd3 \text{cortex}}^{Rnd3 \text{cortex}} )</td>
<td>(Eq. 36)</td>
</tr>
<tr>
<td>( b_2 = k_{Rnd3 \text{cortex}}^{Rnd3 \text{cortex}} \cdot K_{Rnd3 \text{cortex}} \cdot [Rnd3 \text{cyt}] )</td>
<td></td>
</tr>
<tr>
<td>( a_3 = k_{p190B \text{cortex}}^{p190B \text{cortex}} )</td>
<td>(Eq. 37)</td>
</tr>
<tr>
<td>( b_3 = k_{p190B \text{cortex}}^{p190B \text{cortex}} \cdot K_{p190B \text{cortex}} \cdot [p190B \text{cyt}] )</td>
<td></td>
</tr>
<tr>
<td>( a_4 = k_{RhoA \text{cortex}}^{RhoA \text{cortex}} )</td>
<td>(Eq. 38)</td>
</tr>
<tr>
<td>( b_4 = \theta_{RhoA}^{RhoA} \cdot K_{RhoA \text{cortex}}^{RhoA \text{cortex}} \cdot k_{RhoA \text{cortex}}^{RhoA \text{cortex}} \cdot [RhoA \text{cyt}] )</td>
<td></td>
</tr>
</tbody>
</table>
\[ a_5 = k_{\text{off}}^{\text{NMIIA cortex}} \]
\[ b_5 = k_{\text{off}}^{\text{NMIIA cortex}} \cdot K_a^{\text{NMIIA cortex}} \cdot [\text{NMIIA cyt}] \]  
(Eq. 39)

3. Numerical simulations for the 3 dimensional model.

To extend the model to 3D, we then used the set of equations described in Supplementary Note Table 3 to create a biomodel in Vcell 5.2 \(^10\) where reactions were solved using a fully implicit finite volume with regular grid and variable time step. We used a sphere of 25x25x25 mesh. In order to specifically test the capacity of the NMII-ROCK1 feedback network (Scheme 1) to influence spatial distribution of RhoA, the RhoGEF activity was distributed uniformly throughout the cell cortex in the simulations. Simulations were started with cytosolic concentrations of all components equal to unity in \(\text{uM}\) units and in the cortex as null and data presented correspond to steady state (\(\sim 500\) s).

The initial concentration of species as well as their diffusion coefficients and association, activation and kinetic constants used in this model are shown in Supplementary Note Table 6 and 7. In principle we assumed default values for molecular diffusion of 10 \(\mu\text{m}^3/\text{s}\) for proteins in the cytosol and 0.1 \(\mu\text{m}^2/\text{s}\) at the cell membrane. In the model, we also assumed that the dynamic behavior of p190B at the cortex mirrored that of Rnd3, as p190B can exist at the cortex only when bound to Rnd3 \(^11\); this was implemented by setting a higher diffusion rate on the membrane for Rnd3 than for p190B. In our simulations, we did this by preventing p190B diffusion from the membrane.

In the case of myosin, we used a diffusion constant of 0.01 \(\mu\text{m}^2/\text{s}\), which corresponds to diffusion dynamics of myosin \(\sim 10\) times slower than RhoA and Rnd3. We used this difference in order of magnitude based on FRAP results (Fig. 5d, 7g) and previously reported data on the lateral mobility of Rho GTPases \(^12,13\). Of note, a spatially defined RhoA zone did not form in simulations when the diffusion rate of NMIIA was higher or comparable to that of RhoA.
The $k_{off}$ for RhoA and Rnd3 were chosen based on experimental values previously used in the literature for prenylated GTPases \(^{14}\). When we used lower values of these constants, a RhoA zone did not form, as it was compromised by the molecular diffusion on the membrane.

To modulate the stability of myosin locally, an expression for $k_{off}^{NMIIA_{cortex}}$ was introduced.

$$k_{off}^{NMIIA_{cortex}} = \frac{1}{(1+[NMIIA\text{ stabilizer}])^2} \quad (\text{eq. 40})$$

Here the NMIIA stabilizer represents a species/factor that modulates NMIIA stability at the cortex. In order to define a fixed zone where the myosin is stable, the diffusion constant of the stabilizer was set to 0 although cortical NMIIA was allowed to diffuse. Then, as an initial condition we defined $[\text{NMIIA\_stabilizer}]=3-10$ for regions for higher stability of myosin and $[\text{NMIIA\_stabilizer}]=0$ for regions with unstable myosin.

### Supplementary Note Table 6. Dissociation rates, binding affinities and activation constants used for modeling in three dimensions

<table>
<thead>
<tr>
<th>Dissociation rates</th>
<th>$k_{off}^{ROCK_{cortex}}$</th>
<th>0.15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{off}^{Rnd3_{cortex}}$</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>$k_{off}^{p^{190B}_{cortex}}$</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>$k_{off}^{RhoA_{cortex}}$</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>$k_{off}^{NMIIA_{cortex}}$</td>
<td>$\frac{1}{(1+[NMIIA\text{ stabilizer}])^2}$</td>
</tr>
</tbody>
</table>

| Binding affinities | $K_{a}^{p^{190B}_{cortex}}$ | 3 |

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### Supplementary Note Table 7. Initial concentrations and diffusion coefficients of species in the 3-dimensional modeling.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration</th>
<th>Diffusion Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{RhoGEF}_{\text{cortex}}])</td>
<td>1</td>
<td>well mixed</td>
</tr>
<tr>
<td>([\text{NMIIA}_{\text{cyt}}])</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>([\text{NMIIA}_{\text{cortex}}])</td>
<td>0</td>
<td>&quot;(0.1 - (0.09 * ((4.75 &lt; z) &amp;&amp; (z &lt; 5.25))))&quot;</td>
</tr>
<tr>
<td>([\text{NMIIA stabilizer}_{\text{cortex}}])</td>
<td>(10.0 * ((4.75 &lt; z) &amp;&amp; (z &lt; 5.25))) &amp;¹</td>
<td>0</td>
</tr>
<tr>
<td>([\text{p190B}_{\text{cortex}}])</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([\text{p190B}_{\text{cyt}}])</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>([\text{RhoA}_{\text{cyt}}])</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>([\text{RhoA}_{\text{cortex}}])</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>([\text{Rnd3}_{\text{cortex}}])</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>([\text{Rnd3}_{\text{cyt}}])</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>([\text{ROCK1}_{\text{cyt}}])</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>([\text{ROCK1}_{\text{cortex}}])</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

&¹ For simulations where the stability of NMIIA was set homogenous everywhere,
NMIIA_stabilizer was set to 0.

The diffusion constant of NMIIA was set to occur in a different scale when compared to others as RhoA or Rnd3. Our experimental data suggest the order of magnitude of this difference is ~10 (Fig 5d and 7g and refs 12 and 13).
Implications of the model for cortical signaling at the ZA.

The model that we developed explicitly analyses how network interactions described in Scheme 1 can affect the steady-state cortical localization of its constituent components at the ZA. However, several lines of evidence argue that once present at the junctional cortex, a majority of RhoA, ROCK-1, Rnd3 and p190B molecules are active and having biological effects.

Firstly, our use of both the GFP-AHPH location biosensor and a FRET-based activity biosensor identify the ZA as a site where RhoA molecules are active. This is further substantiated by our observation that the steady-state accumulation of RhoA at the ZA requires that it be active, as direct inhibition of RhoA with C3-transferase rapidly and substantially depletes detectable RhoA from the ZA. This implies that differences in the steady-state content of RhoA at junctions will be reflected in differences in the amount of GTP-loaded RhoA at junctions and the amount of inactive (GDP-loaded) RhoA is likely to correspond to a minority of the total junctional RhoA. Secondly, inhibition of ROCK activity with Y-27632 decreases its well-established targets: junctional myosin and pMRLC at the ZA. This implies that a substantial proportion of the junctional ROCK1 population is active and, therefore, significant changes in the steady-state junctional content of ROCK1 can significantly alter ROCK1 signaling at the ZA. We interpret this as arising from the co-recruitment and activation of ROCK1 at the ZA by GTP-RhoA, a well-established ROCK1 activator. This interpretation is supported by our observation that junctional ROCK is reduced when RhoA is inhibited with C3-transferase (Supplementary Fig 4b,c). Third, Rnd3 is known to be constitutively active; indeed control of cortical localization by its phosphorylation is thought to be a major avenue for its biological regulation and is implicit in the model that we developed. This predicts that significant alterations in junctional Rnd3 content will be accompanied by significant changes in junctional Rnd3 signaling. Fourth, several observations suggest that p190B RhoGAP is active when it accumulates at the junction. Thus, junctional recruitment of p190B is accompanied by reduced junctional RhoA signaling when myosin II is inhibited; however, inhibition of RhoA does not occur when p190B is depleted. This implies that the p190B that accumulates at junctions in myosin II-inhibited cells...
is substantively active at this location. Further, expression of a Rnd3 mutant
that cannot interact with p190B (T55V) causes both a reduction in p190B
junctional staining and rescues junctional RhoA localization/activity when
myosin is inhibited, establishing that localization of p190B by Rnd3 is a key
determinant of its action at the ZA (Fig 4 a,b,c). We emphasize that this
analysis considers the signaling of populations of molecules at the ZA, rather
than regulation of their catalytic activity at the molecular level. Overall, these
observations imply that regulation of cortical localization by the feedback
network that we have modeled (Scheme 1) ultimately serves to affect
junctional signaling at the population level.

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