Dia- and Rok-dependent enrichment of capping proteins in a cortical region

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Summary

Rho signaling with its major targets the formin Dia, Rho kinase (Rok) and non-muscle myosin II control turnover, amount and contractility of actomyosin. Much less investigated has been a potential function for the distribution of F-actin plus and minus ends. In syncytial Drosophila embryos Rho1 signaling is high between actin caps, i.e. the cortical intercap region. Capping protein binds to free plus ends of F-actin to prevent elongation of the filament. Capping protein has served as a marker to visualize the distribution of F-actin plus ends in cells and in vitro. Here, we probed the distribution of plus ends with capping protein in syncytial Drosophila embryos. We found that Capping proteins are specifically enriched in the intercap region similar to Dia and MyoII but distinct from overall F-actin. The intercap enrichment of Capping protein was impaired in dia mutants and embryos, in which Rok and MyoII activation was inhibited. Our observations reveal that Dia and Rok/MyoII control Capping protein enrichment and support a model that Dia and Rok/MyoII control the organization of cortical actin cytoskeleton downstream of Rho1 signaling.
Introduction

Underlying the plasma membrane of cells, a thin layer of cortical F-actin fulfills manifold functions such as playing a central role in cell polarity, providing a scaffold for membrane associated proteins, and ensuring mechanical stiffness, among others. Cortical F-actin contains F-actin regulators, Myosin motor proteins and other cortical proteins. Linker proteins belonging to the ERM protein family mediate attachment of cortical F-actin to the plasma membrane. The F-actin cortex contributes to segregation of proteins and establishment of cortical domains, including polarity proteins that are crucial for formation and maintenance of cell polarity (Honigmann and Pralle, 2016; Schmidt and Grosshans, 2018).

Cortical organization is dynamic during Drosophila development. Following a uniform cortex in preblastoderm stages, the cortex becomes patterned with the arrival of the nuclei at the cortex (blastoderm stages). During interphases, two cortical domains mark the cap region above the nuclei and intercap region in between caps (Karr and Alberts, 1986; Warn et al., 1984). During mitosis, metaphase furrows provide a physical barrier for the individual mitotic spindles. Three distinct cortical domains are observed during this process, namely apical, lateral and basal regions (Mavrakis et al., 2009).

Actin filaments contain an intrinsic polarity with their plus and minus ends or barbed and pointed ends, respectively. The polarity of filaments is reflected by numerous plus end or minus end binding proteins. The formin Diaphanous (Dia) specifically binds as a dimer to plus ends, where it catalyzes the incorporation of G-actin to promote filament elongation (Bogdan et al., 2013; Evangelista et al., 1997; Yan et al., 2013). Similarly Capping protein alpha (Cpa) tightly binds as a heterodimer with Capping protein beta (Cpb) to plus ends of actin filaments (Amândio et al., 2014; Wear et al., 2003). In addition to plus end-binding, the myosin motor protein non-muscle myosin II (MyoII) slides towards the plus ends of actin filaments (Houdusse and Sweeney, 2016) and induces formation of filament asters with plus ends at the center in vitro (Köster et al., 2016; Murrell and Gardel, 2012). Tropomyosin-binding protein Tropomodulin (Tmod) binds to the minus ends of F-actin and has previously been employed as a marker for minus ends (Coravos and Martin, 2016; Fowler et al., 1993; Weber et al., 1994).

Dia and MyoII (via Rho kinase, Rok) are both controlled by Rho signaling. In early embryos, Dia is involved in the formation of pole cells, the metaphase furrow, cellularization furrow and together with Cip4 in the exclusion of lateral markers from the furrow canal (Afshar et al., 2000; Castrillon and Wasserman, 1994; Großhans et al., 2005; Yan et al., 2013).

Unlike acto-myosin in muscle cells, the F-actin cortex is generally assumed to consist of a random meshwork of filaments with uniformly distributed plus ends and plus end binding proteins. In contrast, it is also expected that spatially patterned Rho1 signaling will impinge on the structure, dynamics and organization of the F-actin cortex. These contrasting views have not
been much tested in a physiological context. Here we compare the spatial pattern of Rho1 signaling in early *Drosophila* embryos with the distribution of the heterodimeric capping protein and test the function of two targets of Rho signaling, Dia and MyoII in distribution of Capping proteins.

**Results**

**Rho signaling is locally restricted during syncytial blastoderm development**

Rho signaling controls F-actin in two ways, at least: nucleation and elongation via Dia and contractility via Rok and MyoII (Figure 1A). The cortex of syncytial blastoderm *Drosophila* embryos is structured into two regions during interphase: actin caps and the region between actin caps, namely the intercap region (Figure 1B). Both regions are differentially labeled by cortical markers (Schmidt et al., 2018). For example, MyoII is strongly enriched in the intercap region, whereas Arp2/3 dependent, branched F-actin is highly enriched in the cap region (Stevenson et al., 2002). Using a genetically encoded Rho1 sensor (Munjal et al., 2015), we analyzed the spatiotemporal pattern of Rho signaling in syncytial blastoderm embryos. During interphase, we detected a strongly enriched Rho1 sensor signal in intercaps, but little in the cap region (Figure 1D, Suppl. Figure S1). This finding is consistent with the intercap enrichment of MyoII as previously reported (Royou et al., 2002) and of Dia, assayed by GFP tagging (Figure 1D, Suppl. Figure S2, S3). We labelled Dia by inserting a GFP tag before the stop codon by CRISPR mediated integration at the endogenous dia locus (Suppl. Figure S2A, B). The Dia-GFP construct is fully functional, since we employed a homozygous stock, in which Dia-GFP completely substitutes Dia. It is worth noting that the pattern is rather uniform after Dia immuno staining in fixed embryos (Suppl. Figure S2C). The staining pattern of Dia-GFP depends on formin activity, since the intercap restriction was strongly reduced after treatment with the formin inhibitor SMIFH2, whereas overall F-actin caps were not affected by drug treatment (Suppl. Figure S4A, B).

As reported previously (Royou et al., 2002), we detected enriched Rho1 sensor and Dia-GFP signal at the tips of metaphase furrows during mitosis (Figure 1C, E, Suppl. Figure S3) (Großhans et al., 2005; Padash Barmchi et al., 2005; Yan et al., 2013). Furthermore, we detected MyoII at the retracting metaphase furrow (Figure 1E, right panel) (He et al., 2016). In summary, we revealed that Rho signaling and two targets are restricted to the intercap region during interphase. However, we did not test if these proteins colocalize within the region of the basal tip.
Cpa is enriched at the intercap region in a *dia* dependent manner

Having identified a restriction of Rho signaling and two of its targets to the intercap region, we analyzed markers for the organization of cortical F-actin. We found that the plus-end binding proteins Cpa and Cpb as well as the minus end binding protein Tmod were enriched at intercaps.

Cpa immunostaining revealed a strong enrichment outside of actin caps in intercaps during syncytial blastoderm interphases distinct from overall F-actin distribution (Figure 2A). A similar distribution was detected by immunostaining against Cpb (Suppl. Figure S4C). The distinct distribution pattern of Cpa and overall F-actin became also obvious in axial sectioning of frontal views (Figure 2B). Staining of actin caps was prominent in the upper layers, whereas Cpa staining was more prominent in lower sections beside the caps, indicating an enrichment within the intercap regions. Beside a diffuse staining, we detected prominent puncta of a wide range of intensities, especially at basal positions, which may represent Cpa clusters (Figure 2B, red arrows). The prominence and appearance of the puncta depended on fixation conditions (Suppl. Figure S4D-F).

We confirmed the intercap enrichment by live imaging with a C-terminally tagged Cpa at the endogenous locus (Figure 2C, Suppl. Figure S4G, S5). In frontal views, we observed an enrichment of the CpaGFP puncta in regions of basal sections outside of actin caps beyond a diffuse staining of caps and intercaps overlapping with the F-actin label Moesin-GFP. The puncta appeared more prominent than in fixed embryos (Figure 2C, red arrows). The tagged Cpa proteins were functional. Homozygous stocks containing only Cpa molecules at comparable expression levels, were viable and fertile (Suppl. Figure S4H, I). The GFP/Cherry tagging did not affect subcellular distribution, since the staining pattern of fixed wild type and CpaGFP embryos was comparable (Suppl. Figure S4C). The Cpa puncta were mobile with a distribution of their velocities in the range of a few μm/min (Figure 2D, E), which is in the range of cytoskeleton dependent movements (Theriot and Mitchison, 1991).

We also detected an intercap enrichment of the minus end marker Tmod during interphase on top of an overall cortical labeling (Figure 2F, Suppl. Figure S6). We assayed Tmod distribution in living embryos with a functional GFP fusion protein, expressed from the endogenous locus (Coravos and Martin, 2016). Consistent with the typical length of F-actin, we detected both plus and minus ends markers within the intercap region. In the following we focused on the plus end marker Cpa.

Given that both Dia and Cpa bind to plus ends of actin filaments and both are enriched in intercaps, we directly compared their distribution in living embryos coexpressing Dia-GFP and Cpa-mCherry (Figure 2G). We did not detect an obvious overlap of the prominent Cpa-mCherry and Dia-GFP puncta, suggesting that Cpa and Dia interact with distinct populations of F-actin. However, we do not exclude some degree of co-localization given the weak uniform cortical labelling of both proteins.
We next tested whether intercap enrichment of Cpa depended on 

dia
in fixed and live embryos (Figure 3). We generated 
dia
mutant embryos from 
dia
SY5
germline clones, which is a missense mutation within the N-terminal Rho
binding domain (Yan et al., 2013), and contains very little Dia protein (Suppl.
Figure S2 C, D). Strikingly, Cpa was almost uniformly distributed at the cortex
in 
dia
mutants, including actin caps as compared to the sharp lines
representing the intercap regions (Figure 3A). In order to compare wild type
and mutant staining patterns in quantitative terms, we measured the
distribution of the Cpa signal by line profiles in comparison to F-actin (Figure
3B). We quantified fluorescence intensities across cap-intercap and cap-cap
edges and normalized and aligned the curves to their maxima with 0 µm
representing the cap-cap or cap-intercap border. We mirrored the data at the
maxima to receive a curve with decreasing fluorescence intensity to which
we fitted an exponential function. The resulting exponential decay constant \( \kappa \)
describes the decrease of fluorescence intensity over the distance away from
the maxima either towards cap center or towards the cap outside. The
average exponential decay constant in wild type caps was significantly higher
than in 
dia
caps (Figure 3C), which indicates a broader distribution in 
dia
mutants. The F-actin signal has the maximum also at the edge between caps
and intercap regions because of axial distortion of the focal volume and
curved morphology of the caps in interphase 13. As clearly visible by the loss
of the sharp lines of Cpa staining in 
dia
mutants, we measured a significant
difference for the Cpa distribution between wild type and 
dia
mutants
whereas F-actin distribution was statistically not significantly different (Figure
3B, C).

We also confirmed the dependence of the accumulation of basal CpaGFP
puncta on 
dia
by live imaging during interphase 13. In wild type embryos, we
could detect a basal accumulation over time in axial slices covering the
intercap region in contrast to 
dia
embryos (Figure 4A). We quantified the
basal accumulation of CpaGFP puncta along the apical-basal axis over time
by counting the number of puncta in the field of view in four embryos per
genotype and normalized to the most apical region to compare the density of
puncta. The normalized density was elevated in wild type intercap regions
compared to 
dia
 intercaps (Figure 4B). To evaluate if the CpaGFP puncta
were overall diminished in dia embryos, we computed orthogonal views from
axial stacks resulting in maximum intensity projections displaying the apical-
basal distribution of CpaGFP (Figure 4C). In wild type embryos we could
detect a basal accumulation of the CpaGFP puncta over time, whereas the
CpaGFP puncta in 
dia
embryos remained distributed along the whole apical-
basal axis (Figure 4D).

**Cpa enrichment depends on Rok activity**

As Rho signaling controls both Dia and MyoII in parallel, we hypothesized
that MyoII may also control Cpa enrichment at intercaps. We indirectly
inhibited MyoII by injecting the Rho kinase inhibitor Y-27632 (Suppl. Figure
S4A) (Ishizaki et al., 2000). We cannot exclude, that other targets of Rok beside MyoII contribute to the observed phenotypes. We detected a loss of Cpa enrichment after inhibitor injection (Figure 5A) reminiscent to the phenotype in dia mutants. Quantification of line profiles revealed a significant difference for Cpa but not F-actin after Rok inhibition (Figure 5B, C). Dia and MyoII function in parallel according to the standard model of Rho signaling (Piekny et al., 2005). We tested for a mutual dependence by analyzing MyoII distribution in dia mutants and Dia-GFP in Rok-inhibited embryos (Figure 5, Suppl. Figure S4A). The overall MyoII levels and restriction to the intercap region appeared comparable between wild type and dia mutants. Yet, the staining in the submicron scale appeared grainier, what may be caused by a partially disrupted and less continuous actomyosin network in the intercap regions (Figure 5D, E) (Jiang and Harris, 2019). We did not observe a change in the pattern of Dia-GFP following Rok inhibition (Suppl. Figure S4A, B). We conclude that Cpa depends on both targets of Rho signaling: Rok/MyoII activity and Dia.

**Cpa distribution in embryos lacking caps**

The intercap enrichment of Cpa is likely an indirect consequence of the function of Dia and Rok/MyoII on actomyosin amount, turnover, organization or activity. The changed Cpa enrichment in dia mutants may be a consequence of altered F-actin dynamics and amounts. To address this option, we quantified cortical F-actin in wild type and dia mutants by measuring overall fluorescence intensity of 4 frames per embryo in three embryos per genotype. Wild type and dia embryos were stained and imaged within the same batch and distinguished by the expression of MyoII-GFP by dia embryos. The frames were imaged in an axial position that captured the actin caps. We did not detect a difference in staining with Phalloidin (Figure 6A, B). dia mutants have rudimentary metaphase furrows and have been reported to have slightly smaller caps (Cao et al., 2010).

We did also not detect changes in the turnover of F-actin in dia embryos. F-actin turnover was measured by FRAP (Fluorescence recovery after photobleaching) with Utrophin-GFP which stably binds to F-actin allowing to assay the exchange kinetics (Burkel et al., 2007) and serving as a good proxy for F-actin dynamics by FRAP. In both the uniform cortex of preblastoderm embryos and the actin caps of blastoderm embryos (Figure 6C, D), we did not detect any obvious difference between wild type and dia embryos. However, we could detect a higher variability in syncytial blastoderm dia mutants compared to the fluorescence recovery in wild type embryos. Taken together, our data indicate that total F-actin amount and dynamics at the cortex is not obviously changed in preblastoderm and syncytial blastoderm dia mutants.
The cortex of syncytial embryos is set by actin caps, which are controlled by Rac signaling and Arp2/3 dependent F-actin (Stevenson et al., 2002). We tested how the pattern of Cpa depended on the actin caps by analyzing embryos lacking actin caps. Rac signaling in the cap region is controlled by a complex of ELMO and Sponge, which constitute an unconventional guanyl nucleotide exchange factor (Postner et al., 1992; Stevenson et al., 2002; Zallen et al., 2002; Zhang et al., 2018). Embryos from ELMO germline clones, similar to embryos from sponge females, display a largely uniform actin cortex without actin caps (Postner et al., 1992; Schmidt et al., 2018; Winkler et al., 2015). In fixed embryos from ELMO germline clones, we detected a largely uniform distribution of Cpa staining comparable to the distribution of F-actin (Figure 6E). We could detect Cpa puncta in ELMO mutants, although they seemed smaller in size and did not display a specific localization compared to the clear intercap localization in wild type. We conclude that Cpa is enriched at intercaps by an exclusion from the cap region.

Discussion
We have revealed that Cpa distribution is surprisingly uneven and divergent from overall F-actin in syncytial blastoderm embryos. Cpa is enriched in areas of Rho1 signaling in a manner depending on the Rho1 targets Dia and Rok. Although cortical organization in syncytial embryos is special, we speculate that Cpa is also differentially distributed within the cell cortex of other cell types as well as in species other than Drosophila. All components, i.e. RhoGTPase, Dia, Rok, MyoII and the Cpa-Cpb complex have been highly conserved through evolution.

Cpa is enriched in regions with Rho signaling
On top of an expected uniform and diffuse staining largely matching global F-actin distribution, we detected an enrichment of Cpa at the intercaps. A corresponding restriction to the intercap region is observed for Rho1 signaling and two of its targets, Dia and Rok/MyoII. Beside levels, turnover and contractility of actomyosin, we wondered whether organization of cortical actin would depend on Dia and Rok/MyoII. To assay the organization of cortical actin we employed the plus end binding proteins Cpa and Cpb. Cpa has been used previously to assay the organization of F-actin in cells of the mesoderm anlage, for example (Coravos and Martin, 2016). We detected an enrichment of Cpa staining in the intercap region of fixed and live embryos, despite the low total F-actin levels compared to the actin caps. Cpa staining is visible in prominent puncta beyond a diffuse staining. The puncta are more obvious in live than fixed embryos, which may be due to sensitivity to fixation conditions of the underlying structure. Given their dynamics and mobility, the puncta may represent sites of interaction between astral microtubules and the actin cortex. Other speculative options for the puncta are the ends of...
actin bundles or centers of asters. Further experiments, especially involving nanoscopic analysis may provide insight into the nature and interaction of the Cpa clusters.

Taking a simple-minded view with Cpa and Tmod as markers of filament ends, the observed enrichment can be interpreted as a preferential accumulation of F-actin plus and minus ends in the intercap region. Our stainings and measurements indicate that the ratio of filament ends versus total amount of F-actin is strongly increased in the intercap region. However, the interpretation and underlying causes may be more differentiated and complicated. One explanation is that filament ends are not equally accessible to the Cpa/Cpb complex and Tmod. For example, the cap region largely contains Arp2/3 dependent branched F-actin, which by structure contain fewer minus ends. The plus ends within the caps may have a reduced preference for Cpa binding or may be subject to competition from other, branched actin specific regulators. Alternatively, the filaments may be much shorter in the intercap than cap region. In vitro studies showed that Myosin-based contractility may lead to severing of actin filaments (Murrell and Gardel, 2012). A consequence of the Rho1 signaling and MyoII activation in the intercap region may be increased contractility and filament severing. Shorter filaments may also arise from dedicated severing proteins like cofilin or from increased actin turnover. Despite these complications, the observed enrichment of markers for filament ends indicates a specific feature of cortical actin organization at the intercap region involving plus ends.

**Dia and MyoII regulate cortical actin organization**

Having identified an intercap enrichment, we characterized the underlying mechanism. We revealed a novel common function of Dia and MyoII for the enrichment of capping proteins in intercaps. Surprisingly, we found that both targets of Rho signaling, Dia and Rok/MyoII, are required in an apparently parallel fashion.

Binding both to plus ends, Cpa and Dia are expected to show an interesting interaction. While some previous reports indicate a mutual exclusion at the plus ends (Bartolini et al., 2012; Kovar et al., 2005), other reports suggest them to be cross regulators that can both simultaneously bind to the plus end and accelerate dissociation of one of the proteins (Shekhar et al., 2015). Concerning the function, it is conceivable that, on the one hand, Dia acts directly on binding and thus orienting plus ends, without a contribution by Cpa. On the other hand, Cpa and Dia may directly cooperate in orienting actin filaments. We did not detect an overlap of Cpa-mCherry clusters and Dia-GFP in our time lapse recordings, whereas the weaker diffuse Cpa signal is overlapping with Dia-GFP clusters.
Similar to Dia’s relation to capping proteins, we discovered a corresponding function of Rok and possibly also of its major target MyoII. A role of MyoII in organization of actin filaments in vitro is well established. Reconstituted F-actin systems lead to asters with MyoII and F-actin plus ends in their center, as demonstrated with Cpa as a plus-end label (Köster et al., 2016; Wollrab et al., 2019). Such an orientation is consistent with the movement of MyoII molecules towards the plus ends of the actin filaments by acting as a crosslinker and sliding actin filaments until reaching their plus ends (Houdusse and Sweeney, 2016). By revealing a common function for both MyoII and Dia in enrichment of Capping protein, the question of their functional relationship arises. We did not detect a mutual dependence of Dia and Rok/MyoII localization. Although we observed a slightly changed pattern, MyoII clusters were still present in dia mutants, suggesting that Dia and MyoII act in parallel in a non-redundant manner. The parallel control and the biochemical activities of Dia and MyoII suggest that they do not directly recruit Cpa to the intercap region but likely act indirectly via the structure of F-actin.

According to in vitro studies as mentions above (Köster et al., 2016; Wollrab et al., 2019), we propose a speculative model where Dia molecules, activated by Rho signaling, serve as fixpoints for a fraction of actin filaments and orient the filaments within the cortex. In parallel, MyoII motor proteins are activated by Rho signaling via Rok within the same regions. Thus, both branches of Rho signaling lead to an accumulation of plus ends within the signaling region. A corresponding model concerning Dia has been proposed for the apical cortical F-actin during apical constriction during mesoderm invagination, where Dia contributes to orientation of cortical F-actin towards the adherens junctions (Coravos and Martin, 2016).

What may be the function of enriched Capping protein?

A function for the intercap enrichment of Cpa is not immediately obvious and we can only speculate about conceivable functions. The most obvious morphological function of Dia and to a lesser degree MyoII is in the formation and elongation of the metaphase furrow during nuclear divisions. dia mutants display short metaphase furrows resulting in perturbed spindle positioning and multinuclear cells (Afshar et al., 2000). A corresponding function of Rok/MyoII activity is less pronounced, possibly because of compensation by Anillin and Septin (Royou et al., 2004; Zhang et al., 2018). It is conceivable that the growth of the metaphase furrow is mediated by oriented actin filaments leading to growth and buckling of the caps (Zhang et al., 2018).

During interphases, Dia is involved in the dynamics and lateral repositioning of the nuclei after mitosis (Kanesaki et al., 2011; Lv et al., 2020). The yoyo movement of nuclei is impaired in dia mutants, in that the return movement is reduced or missing. In general, the actin cortex is assumed to provide mechanical stability to the cell membrane. The viscoelastic properties of the embryonic cortex and membrane have not been assessed concerning a
function of Dia or MyoII. Suitable biophysical measurements will be required to test for such a function.

Lastly, the enrichment of capping proteins may be involved in segregation of cortical markers, although we have not yet detected an impaired segregation of markers for caps and intercaps. Despite this it is possible to speculate that the orientation of actin filaments is used as a template for the more elaborate cortical pattern in the following developmental step, i.e. cellularization.

Taken together, we revealed a novel function of Dia and Rok/MyoII in enrichment of capping proteins and of F-actin plus ends if interpreting Cpa as a plus end probe. Further studies will be necessary to determine if an uneven, anisotropic distribution of capping proteins and F-actin plus ends could be a generic feature of the cell cortex found also in other developmental stages and systems.

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Author contributions
L.L and A.S. conducted the experiments and analyzed the data. Z.L. analyzed turnover of cortical F-actin. S.Y. initially analyzed dia mutant embryos. J.G. supervised the study and acquired funding. A.S. and J.G. wrote and revised the manuscript.

Declaration of interests
The authors declare no competing interests.
Material and Methods
Fly stocks and handling
Fly stocks used were *Ced-12/ELMO* (Drosophila Stock Center, Bloomington), CpaGFP, Cpa-mCherry and Dia-GFP (this work), *dia* 
(Yan et al., 2013), Histone2Av-GFP (Clarkson and Saint, 1999), Moesin-RFP (Großhans lab, w; P[w+, sqh-moesin-RFP]), MyoIIxGFP (Pinheiro et al., 2017), Rho1 sensor (Ubi-Anillin-RBD-EGFP) (Munjal et al., 2015), Tmod-GFP (Drosophila Stock Center, Bloomington), and Utrophin-GFP (Rauzi et al., 2010).

Histone2Av-GFP was used for scoring wild type embryos in stainings with mixed genotypes. *w* 
1118 was used as wild type control. All fly stocks were obtained from the Drosophila Stock Center, Bloomington (Whitworth, 2019), if not stated differently. Genetic markers, annotations and their references are described in Flybase.org (Gramates et al., 2017).

All crosses and cages were kept at 25°C. Germline clones were produced and selected by the ovo/Flipase technique, as described previously (Chou et al., 1993).

Targeted insertions, CpaGFP, Cpa-Cherry, Dia-GFP were generated by CRISPR/Cas9 (InDroso Functional Genomics, France, WellGenetics company, Taiwan). The coding sequence of eGFP or mCherry with a 5’-terminal linker sequence was inserted into the 3’ region of the target gene.

The gene region of CpaGFP is as followed (EXON, intron, **LINKER**, **UTR**):

```
CCAGTTGCCCATCACCAGGACCAAGATCGACCTTGAGCACAAATCGTCTCGTACAGCATTGGCAAGGAACTGAAGACGCAA
CCACATCCATCGAGCAAGCAGGACCAAGTGGAGCAAAATCGTCTCGTACAGCATTGGCAAAGAACTGAAGACGCAA
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Cpa-mCherry (EXON, **LINKER**, **mccherry**, **LoxP**):

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CGACATTCAAGGCAATGCGTCGCCAGTTGCCCATACACCAGGACCAAGATCGACCTTGAGCACAAATCGTCTCGTACAGCATTGGCAAGGAACTGAAGACGCAA
CCACATCCATCGAGCAAGCAGGACCAAGTGGAGCAAAATCGTCTCGTACAGCATTGGCAAAGAACTGAAGACGCAA
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Dia-GFP (EXON, LINKER, egfp, LoxP):
CGGACGCGTGTCACCAACGGCAACTAATTGACCCGCGAAATGTCTCAACGAGGTTC
TAGGCTCCGCGTCTAGAatggtgagcaagggcgaggagctgttcagccgggtgtgtgtgcccacactcttgtcgacgctga
ccgccacacatgaaagagcagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacactttcttcaagag
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
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cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
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cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
cccagttccgctgagaccaaggacagccagcagctcttctcaagtcgcagccgagaggcttcaggttcagggcgacacacttt
cagacgcaactaagacccgagccaggtgaagttcagtcagggcagacccgagccacactttcttcaagagcttcagccgta
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**Immunostaining and antibodies**

Following primary antibodies were used: mouse anti-α-Tubulin (1:50000 for Western blot, B512, Sigma); rabbit anti-Cpa (1:200, 1:2000 for Western blot; Amândio et al., 2014 and this work); rabbit anti-Cpb (1:200, Amândio et al., 2014), rabbit anti-Dia (1:1000, Großhans et al., 2005), guinea pig anti-Dia (1:5000 for Western Blot, Großhans et al., 2005). F-actin was stained by Phalloidin coupled to Alexa647 (Thermo Fisher). Secondary antibodies for immunostainings were labeled by Alexa 488, 568, 647 (Thermo Fisher). The staining of GFP was done by making use of GFP-booster coupled with Atto488 (1:500, Chromotek). DNA was stained by DAPI (0.2 µg/ml, Thermo Fisher). Secondary antibodies for Western blots were IRDye-800CW and IRDye-680 (1:20000, Li-COR Biotechnology).

The full length Cpa coding sequence was cloned into pGEX-His leading to a GST domain at the N-terminus and a 6x His tag at the C-terminus. The Cpa antibody was raised in rabbit against recombinant GST-Cpa-His6 protein expressed in E. coli and purified under denaturing conditions according standard protocols (Qiagen).

For histological stainings, embryos were fixed by formaldehyde or heat fixation using standard methods described previously (Großhans et al., 2005). After fixation, embryos were stored in methanol at –20°C. Embryos for manual devitellinization were fixed by 8% formaldehyde (F-actin or Cpa staining). Fixed embryos were transferred to PBT (Phosphate buffered saline (PBS)+0.2% Tween20) and washed thrice for 5 min and afterwards blocked for 30–60 min in PBT+5% bovine serum albumin (BSA). Incubation with primary antibodies was done in PBT+0.1% BSA overnight at 4°C or for 2–3 h at room temperature. Afterwards the embryos were washed with PBT thrice for 15 min before incubation with secondary antibodies in PBT for 1–2 h at room temperature followed by three washing steps with PBT for 15 min. DNA staining by DAPI was done for 10 min at room temperature, followed by washing for 15 min. The embryos were mounted in Aquapolymount (Thermo Fisher).
The ROCK inhibitor Y-27632 (Sigma) was injected into embryos expressing CpaGFP during preblastoderm with a concentration of 10 mM. Embryos were fixed after reaching syncytial blastoderm and stained as described after hand-removal of the vitelline membrane.

**Western blot**

Embryo extracts were produced by freezing dechorionated embryos in liquid nitrogen. The frozen embryos were homogenized with a pestle in 2x Lämmli buffer to generate an extract with 1.5 embryos/µl. Afterwards the extract was heated to 95°C for 10 min and loaded onto a 10% SDS-Gel. Plotting onto a nitrocellulose membrane was performed for 1.5 h and the membrane was then blocked for 1 h in PBS+5% milk powder. Incubation with primary antibodies in PBT was done overnight at 4°C followed by 4 times washing with PBT and incubation with the secondary antibodies for 2 h at room temperature in PBT. Afterwards 4 times washing with PBT was performed. The membrane was imaged with an Odyssey CLx infrared imaging system (LI-COR Biosciences) with 16-bit depth. Image processing was done in Adobe Photoshop and Illustrator.

**Imaging and quantifications**

Imaging was performed with a Zeiss LSM 780 confocal microscope equipped with Airyscan or LSM980 AiryScanII. Fixed samples were imaged with an LCI Plan Neofluar 63×/water NA 1.3 objective and live imaging was done with a Plan Neofluar 63×/oil NA 1.4 objective. Embryos for live imaging were handled as described previously (Kanesaki et al., 2011). Embryos expressing the Rho biosensor were live imaged with a frame size of 512x512 pixel (33.7×33.7 µm; 70 nm lateral pixel size) and z-stacks were conducted with a step size of 0.5 µm. Orthogonal views were made with Fiji/ImageJ (Schindelin et al., 2012). Planar XY projections were generated with IMARIS from axial image stacks of CpaGFP (Figure 4C). Wild type or dia germline clone embryos expressing CpaGFP were live imaged with a frame size of 488x488 pixel (32.16x32.16 µm), a z-stack step size of 0.5 µm and a frame rate of 120 s.

F-actin in fixed wild type and dia embryos (Figure 6) was quantified by the fluorescence intensity of 4 frames with a size of 256x256 pixel per embryo. The embryos of both genotypes were stained in the same tube and imaged with the same settings. The genotypes were discriminated by a fluorescent tag, which was expressed by dia embryos. Three embryos per genotype were used for quantification.

FRAP experiments were conducted with wild type and dia germline clone embryos expressing Utrophin-GFP (Figure 6C, D). The bleaching and recording were carried out with a Zeiss microscope (Axio Observer) equipped with a spinning disc (40×/NA1.3 oil) and an additional diode laser (473 nm, 100 mW; Rapp OptoElectronic, Hamburg/Germany). Bleaching of Utrophin-
GFP was carried out in a 5x5 µm region within a single actin cap using 60% laser power output for 1 s during the image acquisition. The images were taken for 2 to 3 min with a frame rate of 1 s/frame. To quantify the mobile fraction of F-actin, the fluorescence intensity in the bleaching region was measured in Fiji/Image J manually. The fluorescence intensity was normalized as follows: Normalized intensity: \( I = \frac{(I_t - I_{\text{min}})}{(I_{\text{max}} - I_{\text{min}})} \). Where \( I_t \) is the intensity at time \( t \), \( I_{\text{min}} \) is the intensity immediately post-bleach and \( I_{\text{max}} \) is the intensity pre-bleach.

The formin inhibitor SMIFH2 (Abcam) was dissolved in DMSO with a concentration of 2 mM and injected into syncytial embryos expressing Dia-GFP (Suppl. Figure 4AF). The fluorescence intensity of Dia-GFP in living embryos during interphase 13 with or without SMIFH2 treatment was measured with frames of 28.0x1.67 µm at the intercap region. The fluorescence intensities of Dia-GFP were normalized.

To track the movement of CpaGFP puncta (Figure 2D), living embryos expressing CpaGFP during interphase on the cap region were recorded with a frame size of 488x488 pixel (21.44x21.44 µm), a z-stack step size of 0.25 µm and a frame rate of 2 s.

The dynamics of Cpa puncta in CpaGFP embryos during interphase (Figure 2E) was quantified by measuring the distance that Cpa puncta move in 2s on the cap region with a projection of 0.5 µm in Fiji/Image J manually. In total the movement of 73 Cpa puncta was measured.

The fluorescence intensity of Cpa in fixed embryos (Figure 3) was quantified by measuring fluorescence intensities across cap edges with a line width of 10 pixel. For both genotypes, 3 embryos in interphases 11–13 were used. The fluorescence intensities of each cap edge were normalized to their individual peak and all aligned to 1, which is position 0 µm in the plots. Averages of all measurements in each embryo were plotted in a graph. Each averaged quantification was mirrored at position 0 µm and subjected to exponential fitting (OriginPro 8.5G, GraphPad Prism 6) in a region from position 0–1.5 µm.

The density of CpaGFP puncta (Figure 4B) was determined by quantification of puncta in each z-slice along the apical-basal axis. The numbers of puncta were normalized to 1 in the first slice.

Embryos expressing Dia-GFP or MyoII-GFP (Suppl. Figure 4) were collected and injected with ROCK inhibitor Y-27632. Imaging was performed with a Spinning disc microscope 20 min after injection.
References


**Figure 1 Spatiotemporal pattern of Rho signaling.** (A) Scheme indicating the signal pathway downstream of Rho1, activating Dia that leads to F-actin nucleation and elongation and MyoII activation via ROCK that leads to actomyosin contractility. (B) Scheme of syncytial blastoderm interphase with F-actin rich caps above nuclei and MyoII-rich intercap regions as indicated. (C) Scheme of syncytial blastoderm mitosis with metaphase furrows invaginating towards the interior of the embryo. Rho signaling, Dia and MyoII enriched at the basal tip of the metaphase furrows. (D–E) Living wild type embryos during syncytial blastoderm interphase (D) and mitosis (E) expressing a Rho sensor (Rho binding domain of Anillin) tagged with GFP, Dia-GFP, or MyoII labeled with 3x GFP. Embryos were imaged from the top and apical-basal positions are as indicated within images. Computed sagittal views are shown in bottom panels. Yellow circles indicate the position of interphase nuclei. Scale bars are 10 µm.
Figure 2 Cpa and CpaGFP are enriched at intercap regions. (A, B) Embryos in interphase 13 fixed and stained for Cpa (grey/green), F-actin (grey/red) and DNA (blue). (A) Sagittal section. (B) Frontal sections. Pictured are top views arranged along the apical-basal axis with positions as indicated. Arrows in red point to Cpa puncta. (C) Live embryo expressing CpaGFP (grey/green) and Moesin-RFP (grey/red). Interphase 13. Frontal sections along apical-basal axis are shown as indicated. Arrows in red point to CpaGFP puncta. (D) Track of a single cortical cap CpaGFP puncta represented by colors over time as indicated. (E) Dynamics of CpaGFP puncta. (F) Live embryo expressing Tmod-GFP to mark F-actin minus ends. Displayed are stills along the apical-basal axis as indicated. (G) Images from
live embryos expressing Cpa-mCherry (grey/red) and Dia-GFP (grey/green) during interphase 13. Region marked with dotted square shown in high magnification. Scale bars are 10 µm, Scale bars in D and right panel of F are 5 µm.
Figure 3 Cpa enrichment depends on dia. (A) Wild type and dia embryos in interphase 13 fixed and stained against Cpa (grey/green), F-actin (grey/red) and DNA (blue). Sagittal sections are shown in top panels, frontal sections in bottom panels. Regions marked by dotted square are magnified in panels on the right. The red line indicates the measurement by line profiles. (B) Averaged and normalized intensity profiles of wild type (blue) and dia (red) interphase 13 embryos across cap edge, as indicated in (A). Fluorescence intensities of three embryos per genotype were measured (wild type 98 edges, dia 108 edges in total) across cap-intercap borders normalized to their peak and mirrored at the peak. S.E.M. is indicated by transparent regions. (C) Fitted exponential constants of intensity profiles per embryo for Cpa and F-actin signal were plotted. Mean values and S.E.M. are shown by bars. *** p ≤ 0.001 (p value was determined by two-tailed t-test). Scale bars are 10 µm.
Figure 4 Basal accumulation of CpaGFP puncta depends on dia. (A) Live wild type and dia embryos during interphase 13 expressing CpaGFP. The top panels display a z-section of the actin caps (1 µm) and whereas the bottom panels show a z-section of the intercap region (5 µm) as indicated in schematic representation during early (2 min) and late (8 min) interphase. (B) Apical (cap) to basal (intercap) distribution of CpaGFP clusters within the field of view of 4 imaged embryos per genotype as indicated. The number of clusters was normalized to the first apical slice. Solid lines represent early interphase (2 min), dashed lines late interphase (8 min) in wild type (blue) and dia (red) embryos. (C) To obtain maximum intensity projections in (D) z-stacks of frontal sections were projected as orthogonal views. (D) Orthogonal views of wild type (left) and dia (right) embryos expressing CpaGFP during interphase 13 at indicated timepoints. The position of nuclei is indicated by dashed circles. Scale bars are 10 µm.
Figure 5 MyoII controls Cpa distribution. (A) Fixed embryos in interphase 13 injected with or without Rok inhibitor Y-27632, fixed and stained for F-actin (grey/red) and CpaGFP (grey/green). The lines in red indicates the measurement with line profiles (B). (B) Normalized fluorescence intensity profiles of CpaGFP and F-actin in Y-27632 injected and control embryos. Six embryos were quantified per genotype (56 edges in control, 63 edges in Y-27632 injected embryos in total). Average in solid line, SD as band. (C) Fitted exponential constant from intensity profiles of injected and control embryos. Corresponding averages and SD are shown by bars. ** p ≤ 0.001 (p value was determined by two-tailed t-test). (D) Live wild type and dia embryos expressing MyoII-GFP in interphase 13. Frontal sections of the cap and intercap layers as indicated. (E) Wild type and dia embryos in interphase 13 fixed and stained for F-actin (grey/red) and MyoII-GFP (grey/green). The images are maximum intensity projections. Scale bars 10 µm.
Figure 6 F-actin amount and dynamics in dia mutants. (A) Wild type and dia embryos fixed and stained against F-actin (grey/red) and DNA (blue). (B) Quantification of F-actin signal in (A) as indicated. In total 12 frames of three embryos per genotype were quantified. Corresponding averages and S.E.M. are shown by bars. (C–D) FRAP in pre-blastoderm (C) and syncytial blastoderm (D) embryos expressing Utrophin-GFP. Schematic representation of embryonic stages in frontal sections of Utrophin-GFP are shown on the left sides. Graphs represent the averaged percentage of fluorescence recovery over time in wild type (blue) and dia (red) embryos. For (C) 4 embryos per genotype were tested; for (D) 6 wild type and 4 dia embryos were tested. SD are represented by transparent regions along the curves. Scale bars are 10 µm. (E) Wild type and ELMO germline clone embryos in interphase 13 fixed and stained for F-actin (grey/red), Cpa (grey/green) and DNA/DAPI (grey/blue). Scale bars 10 µm.
Fig. S1. Spatial-temporal localization of Rho sensor during interphase and mitosis. Live imaging of a syncytial blastoderm embryo expressing the Rho sensor tagged with GFP. Axial position and time are as indicated. Time 0 was defined by the localization of the Rho sensor to new furrows. Scale bar 10 µm.
Fig. S2. GFP tagging of Dia. (A) Scheme of the genetic region of dia with eGFP insertion. (B) Western blot with wild type and Dia-GFP embryonic lysates stained for Dia (upper blot) and Tubulin as loading control (lower blot). (C) Wild type and dia embryos in interphase 13 fixed and stained for Dia (grey/green), Dlg (grey/red) and DNA (blue). Sagittal sections (D) Western blot with wild type and dia embryonic lysates stained for Dia (upper blot) and Tubulin as loading control (lower blot). Scale bar 10 µm.
Fig. S3. Dia-GFP is enriched in intercaps and basal tips of metaphase furrow. Images from a movie of a syncytial blastoderm embryo expressing Dia-GFP. Axial position and time are as indicated. Time 0 was defined by the localization of Dia-GFP to new furrows. Scale bar 10 µm.
**Fig. S4.** Injection of inhibitors diminishes fluorescence of Dia-GFP and Sqh-GFP. (A) Embryos expressing indica-ted tagged proteins were injected with the indicated drugs. Images from live embryos during interphase 13. (B) Normali-zed Dia-GFP fluorescence intensity at the intercap domain. (C) Wild type embryo during interphase 13 fixed and stained for F-actin (grey/red), Cpb (grey/green) and DNA (blue). Upper panels shows frontal sections, lower panel sagittal views. (D, E) Histone2Av-GFP and CpaGFP embryos fixed with 4% (D) or 37% (E) formaldehyde and stained for Cpa antibody or GFP antibody as indicated. (F) Embryos expressing indicated tagged proteins were heat-fixed and stained for RFP and Cpa. Arrows in red indicate Cpa punctae. (G) Cpa locus on the second chromosome with eGFP or mCherry insertions as indicated. (H, I) Western blots with wild type and CpaGFP or Cpa-mCherry embryonic lysates probed for Cpa (upper blot) and α-Tubulin as loading control. Scale bars 10 µm.
Fig. S5. Dynamics of Cpa-mCherry during mitosis and interphase. Live syncytial blastoderm embryos expressing Cpa-mCherry from mitosis 12 to interphase 13. Axial position and time as indicated. Time 0 was defined by the expansion of the cap region at the beginning of mitosis. Scale bar 10 μm.
Fig. S6. Dynamics of Tmod-GFP during mitosis and interphase. Live syncytial blasto-derm embryos expressing Tmod-GFP from mitosis 12 to inter-phase 13. Axial position and time as indicated. Time 0 was defined by the expansion of the cap region at the beginning of mitosis. Scale bar 10 µm.
# Table S1. Fly stocks

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# Table S2. Antibodies, stains and inhibitors

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