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Endogenous activation patterns of Cdc42 GTPase within *Drosophila* embryos

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Abstract

Knowing when and where a given protein is activated within intact animals assists in elucidating its *in vivo* function. Using genetically encoded A-probe (activation bioprobe), we reveal that Cdc42 GTPase remains inactive within *Drosophila* embryos during the first two-thirds of embryogenesis. Within the central nervous system where Cdc42 activity first becomes upregulated, individual neurons display patterns restricted to specific subcellular compartments. At both the organismal and cellular levels, Cdc42's endogenous activation patterns in wild type allow predictions of where loss-of-function phenotypes will emerge in *cdc42/cdc42* mutants. Genetic tests support the importance of suppressing endogenous Cdc42 activities until needed. Thus, bioprobe-assisted analysis uncovers how a ubiquitously expressed signaling protein controls normal cellular events through continual regulation of its activity within animals.

Proteins trigger signaling pathways upon activation (1). Cdc42 is a member of monomeric Rho GTPase family expressed ubiquitously in eukaryotes (2). It cycles between a GDP-bound 'inactive' and GTP-bound 'active' state. As with other family members, Cdc42 has been studied in diverse contexts such as cytoskeletal dynamics, membrane trafficking and gene regulation (3-6) through over-expression of its wild type, constitutively activated and/or other mutant forms (7-12). Such approaches demonstrate profound potentials of the Rho GTPases as versatile signaling proteins. For example, Cdc42 over-expression disrupts the establishment and maintenance of polarity and mobility of various cells in early embryos. In late embryos, the complex morphology of neurons can be drastically altered after expressing mutant Cdc42 proteins. Yet, a biochemical analysis suggests that only a fraction of Rho GTPases may be activated at any given time (13). Advances in genetically-encoded fluorescent proteins and FRET (Forster resonance energy transfer) detection allow the design of molecular bioprobes that reveal activities of specific endogenous molecules (14,15). However, this visualization approach is yet to be implemented *in vivo*.

To examine the activation patterns of Cdc42 within cells and tissues of intact organisms, we generated genetically-encoded molecular bioprobes that can be used in *Drosophila*. We designed an A-probe (activation bioprobe) for Cdc42 utilizing the fact that upon activation Cdc42 reversibly binds to a specific peptide. In the A-probe, Cdc42 and CBD (Cdc42-binding domain) are co-expressed as a single polypeptide. An advantage of this A-probe (A-probe.1) is its FRET donor-to-acceptor ratio stays one-to-one and, thus, allows for quantification of FRET efficiency. A second design (A-probe.2) consisted of Cdc42 and CBD as separate molecules. When introduced *in vivo*, both A-probes revealed similar FRET patterns within

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uniquely identified neurons (fig. S1). Use of controls defined the full range of FRET *in vivo* (fig. S2). Experiments shown below used A-probe.1.

We expressed our Cdc42 A-probe in all cells of embryos using a constitutive GAL4 driver (*act¹-GAL4*). A-probe FRET remains rare through the first two thirds of development (Fig. 1A, fig. S3-4). At hour 15:00 of embryogenesis (stage 16), the cells at the dorsal midline display heightened FRET. By this time, internal tissues such as the trachea and CNS also exhibit similarly increased FRET. To further examine these internal tissues, we used tissue-specific GAL4 drivers (*btl¹-GAL4* and *elav¹-GAL4* for, respectively, trachea and CNS). In both tissues, endogenous activities of Cdc42 are suppressed until after they have initiated organogenesis (Fig. 1B-C). In *Drosophila*, genetic deletion of Cdc42 results in 100% lethality. However, embryos lacking both maternal and zygotic *cdc42* gene functions still undergo normal development for much of embryogenesis. Previous work showed defects in dorsal closure, abnormal tracheal morphogenesis, and incomplete CNS maturation in *cdc42/cdc42* loss-of-function mutants (16,17). Phenotypes that manifest late during embryogenesis were puzzling because all cells maintain endogenous Cdc42 expression and, furthermore, are capable of responding to artificial activation of Cdc42 even in early stages of embryogenesis. However, a simple scenario is possible: the endogenous activation patterns of Cdc42 proteins, and not their mere presence in wild type, allow predictions of when and where loss-of-function phenotypes would emerge within *cdc42/cdc42* mutants (Fig. 1D).

Although Cdc42 proteins are ubiquitously present throughout the cytoplasm (fig. S5), their activation patterns remain uncharacterized within individual cells *in vivo*. The A-probe FRET patterns indicate that neurons, especially within their axons and/or dendrites in longitudinal connectives, activate Cdc42 prominently by hour 15:00 (fig. S6). Therefore, we expressed A-probe specifically in the aCC (anterior corner cell) motoneuron at different stages of its development (Fig. 2A, fig. S7). The aCC is one of the first neurons to develop complex cellular morphologies in the CNS (18-20). Within the aCC, A-probe displays highly restricted and reproducible spatiotemporal FRET patterns (Fig. 2B, fig. S8). Prior to hour 13:00, FRET is rarely detected within the axon, even though it began extension 4 hours earlier. At hour 13:00, FRET becomes apparent within the axon, peaking at its proximal region from which dendrites are about to emerge. The local elevation of A-probe FRET continues as the neuron progressively attains dendritic complexity (fig. S9). Therefore, within an individual neuron, Cdc42's first activation coincides temporarily with the onset of dendrogenesis and, furthermore, its activities are spatially restricted to the compartment from which dendrites emerge. Experiments with A-probe.2 produced similar results (fig. S10).

We hypothesized that removal of Cdc42 from the aCC would induce abnormal development of this neuron only when and where the protein is normally activated. To demonstrate this, we designed single-cell genetic tests that would reveal cell-autonomous functions of Cdc42 within the aCC motoneuron in the CNS, while paying attention to its normally stereotyped dendritic development (Fig. 3A, fig. S11). Despite the specific phenotype displayed by pioneer neurons (Fig. 3A, fig. S12-13), the CNS of mutants that lack both maternal and zygotic supplies of *cdc42* gene (16) maintains an overall wild type morphology at least up to hour 15:00 (fig. S14). There is no evidence that supports these neurons requiring Cdc42 prior to dendrogenesis when the molecule would first become activated. Together, the results not only support the cell-autonomous requirement of endogenous Cdc42 during the initiation of dendrites by a model neuron, but also point to spatiotemporal correlation between the activation of Cdc42 and its role within this morphologically complex cell down to the level of subcellular compartments (Fig. 3C).

To investigate the significance of controlling endogenous activity patterns of Cdc42 within a given cell, we intentionally overrode the endogenous control of Cdc42 activities in the aCC

by over-expressing a constitutively activated form of Cdc42 (GFP::Cdc42^{V12}) (Fig. 3B). When Cdc42 is activated at high levels and out of normal context from hour 8:00 onward, the aCC displays aberrant migration of the cell body (34%, n=56), thickening of the axon (100%), and premature termination of its axonal growth cone prior to reaching the target muscle (77%). These abnormalities are not only qualitatively distinct from the loss-of-function phenotype, but also variable from segment to segment within a given embryo and can emerge as early as hour 10:00. They are a result of the activation of Cdc42 protein, and not its mere presence, because over-expression of wild type Cdc42 (GFP::Cdc42) at similar levels produces no changes in the aCC. Therefore, precise spatiotemporal control of Cdc42 activities is critical to achieve the normal development of neurons.

In conclusion, we described an *in vivo* bioprobe imaging technology. Using this we revealed the surprisingly restricted pattern for endogenous activities of a potent and ubiquitously expressed signaling protein both within individual cells and in whole animals. Genetic experiments further supported the causal link between the molecule's activation and its function *in vivo*. The bioprobe-assisted approach is indispensable in elucidating the function of molecules whose activities receive continual controls within organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cdc42 activation in whole embryo

A. FRET efficiency in whole embryo (mean±SEM) at hours 5:00 through 15:00 (n=16-20). Asterisk indicates $p < 0.01$ by two-tailed t-test against all other time points. The FRET range for Cdc42 activation is based on fig. S2. Anterior is to the left. White dots outline yolk. Scale bar is 50 μ m. **B-C.** Cdc42 activation in trachea (**B**) and CNS (**C**). **D.** Cdc42 is ubiquitous throughout development but remains inactive during early stages (blue areas) and tissue-wide activation (pink areas) occurs at late stages (top). *cdc42/cdc42* embryos develop phenotypes (dashed ovals) at late stages (bottom).

Figure 2. Cdc42 activation in aCC motoneuron

A. aCC extends its *axon* laterally to reach the *target* muscle. The *dendrite* develops within the longitudinal connective (*lc*) of the CNS. Anterior is to the top. **B.** Relative intensity of A-probe FRET (mean $\pm$ SEM) along the proximal region of aCC axon before (11:00), during (13:00) and after (15:00) the onset of dendrogenesis (n=6 each). The patterns at 13:00 and 15:00 differ from that at 11:00 by ANOVA ($p<0.01$). (FRET activities within cell body are mostly nuclear (fig. S1).)

Figure 3. Genetic tests in aCC motoneuron

A. The number of dendritic processes at 15:00 in: wild type (+/+, $n=21$), cell-specific RNAi (*eve'-GAL4/UAS-cdc42^{RNAi}*, $n=13$), maternal loss-of-function (maternally *cdc42⁴/cdc42⁶*, $n=9$) (*M*), zygotic loss-of-function (zygotically *cdc42⁴/Y*, $n=13$) (*Z*), maternal and zygotic loss-of-function (maternally *cdc42⁴/cdc42* and zygotically *cdc42⁴ or ⁶/Y*, $n=12$) (*M+Z*), cell-specific RNAi plus maternal and zygotic loss-of-function (maternally *cdc42⁴/cdc42⁶* and zygotically *cdc42⁴ or ⁶/Y;eve'-GAL4/UAS-cdc42^{RNAi}*, $n=14$), cell-specific rescue (maternally *cdc42⁴/cdc42⁶* and zygotically *cdc42⁴ or ⁶/Y;eve'-GAL4/UAS-cdc42*, $n=12$), cell-specific over-expression of wild type Cdc42 (*eve'-GAL4/UAS-cdc42*, $n=15$). Asterisk and parenthetical asterisk indicate, respectively, $p<0.01$ and $p<0.05$ against wild type, and *star* indicates $p<0.01$

against loss-of-function mutant. See Supplement for details. **B.** Out-of-context activation of Cdc42 in aCC (GFP in *green*) in the CNS (with HRP antibody in *purple*) of wild type embryos (*eve'*-GAL4/*eve'*-GAL4;*uas-gfp::cdc42*^{V12}/*uas-gfp::cdc42*^{V12}) causes changes in migration of the cell body (*white circle*), thickening of axons, and premature termination of axonal growth cone (*arrowhead*). The *control* expresses GFP-tagged wild type Cdc42 (*eve'*-GAL4/*eve'*-GAL4;*uas-gfp::cdc42*/*uas-gfp::cdc42*). **C.** Cdc42 protein is present in entire cytoplasm of aCC. Its activation (*pink area*) occurs at the onset of dendrogenesis (*left*), where the phenotype (*dashed circle*) resulting from its genetic deletion appears (*right*).