EXTENDED PDF FORMAT
SPONSORED BY
usb
www.usbweb.com

Endogenous Activation Patterns of Cdc42 GTPase Within Drosophila Embryos

Daichi Kamiyama, *et al.*

Science **324**, 1338 (2009);
DOI: 10.1126/science.1170615

The following resources related to this article are available online at www.sciencemag.org (this information is current as of June 5, 2009):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:
http://www.sciencemag.org/cgi/content/full/324/5932/1338

Supporting Online Material can be found at:
http://www.sciencemag.org/cgi/content/full/324/5932/1338/DC1

This article **cites 21 articles**, 5 of which can be accessed for free:
http://www.sciencemag.org/cgi/content/full/324/5932/1338#otherarticles

This article appears in the following **subject collections**:
Development
http://www.sciencemag.org/cgi/collection/development

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:
http://www.sciencemag.org/about/permissions.dtl

Downloaded from www.sciencemag.org on June 5, 2009

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. Copyright 2009 by the American Association for the Advancement of Science; all rights reserved. The title *Science* is a registered trademark of AAAS.

31. D. Littman and A. Rudensky provided reagents for this work, and K. Otipoby prepared recombinant TAT-Cre. We thank J. Hill and K. Leatherbee for technical assistance with microarray data analysis and members of the Rao lab for critical discussion. This work was supported by grants from NIH (to A.R., D.U., and M.W.) and the Juvenile Diabetes Research Foundation (to A.R.). M.S.S. was supported by the Irvington Institute fellowship program of the Cancer Research Institute. D.P.C. was supported by a fellowship from the Portuguese Foundation for Science and Technology. S.K. was supported by an NIH training grant (T32). Further support was provided by a P01 grant to K Rajewsky. M.S.S., A.R., T.K., and M.W. have filed patent applications concerning the use of HF and its derivatives to inhibit T_H17 cell differentiation. Array data can be found at www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15624 with the accession number of GSE15624.

Supporting Online Material
www.sciencemag.org/cgi/content/full/324/5932/1334/DC1
Materials and Methods
Figs. S1 to S14
Tables S1 and S2
References

23 February 2009; accepted 8 April 2009
10.1126/science.1172638

Endogenous Activation Patterns of Cdc42 GTPase Within *Drosophila* Embryos

Daichi Kamiyama and Akira Chiba*

Knowing when and where a given protein is activated within intact animals assists in elucidating its in vivo function. With the use of a genetically encoded A-probe (activation bioprobe), we revealed that Cdc42 guanosine triphosphatase (GTPase) remains inactive within *Drosophila* embryos during the first two-thirds of embryogenesis. Within the central nervous system where Cdc42 activity first becomes up-regulated, individual neurons display patterns restricted to specific subcellular compartments. At both organismal and cellular levels, Cdc42's endogenous activation patterns in the 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 ubiquitously expressed signaling proteins control cellular events through continual regulation of their activities within animals.

Proteins trigger signaling pathways upon activation (*1*). Cdc42 is a member of the monomeric Rho guanosine triphosphatase (GTPase) family expressed ubiquitously in eukaryotes (*2*). It cycles between a guanosine diphosphate (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 overexpression 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 overexpression 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 (*14–16*). However, this visualization approach has 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 by using the fact that, upon activation, Cdc42 reversibly binds to a specific peptide. In the A-probe, Cdc42 and CBD (Cdc42-binding domain) are coexpressed 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 uniquely identified neurons (fig. S1). Use of controls defined the full range of FRET in vivo (fig. S2). Experiments shown used A-probe.1.

We expressed our Cdc42 A-probe in all cells of embryos by using a constitutive GAL4 driver (*act'-GAL4*). A-probe FRET remains rare through the first two-thirds of development (Fig. 1A and figs. S3 and S4). At hour 15:00 of embryogenesis (stage 16), the cells at the dorsal midline displayed heightened FRET. By this time, internal tissues such as the trachea and central nervous system (CNS) also exhibited similarly increased FRET. To further examine these internal tissues, we used tissue-specific GAL4 drivers (*btl'-GAL4* and *elav'-GAL4* for, respectively, trachea and CNS). In both tissues, endogenous activities of Cdc42 were suppressed until after the embryos initiated organogenesis (Fig. 1, B and 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 (*17*, *18*). 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, activated 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 and fig. S7). The aCC is one of the first neurons to develop complex cellular morphologies in the CNS (*19–21*). Within the aCC, A-probe displayed highly restricted and reproducible spatiotemporal FRET patterns (Fig. 2B and fig. S8). Before hour 13:00, FRET was rarely detected within the axon, even though it began extension 4 hours earlier. At hour 13:00, FRET became apparent within the axon, peaking at its proximal region from which dendrites were about to emerge. The local elevation of A-probe FRET continued 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 and fig. S11). Despite the specific phenotype displayed by pioneer neurons (Fig. 3A and figs. S12 and S13), the CNS of mutants that lack both maternal and zygotic sup-

Department of Biology and Miami Institute of Molecular Imaging and Computation, University of Miami, Coral Gables, FL 33146, USA.

*To whom correspondence should be addressed. E-mail: akira.chiba@miami.edu

Downloaded from www.sciencemag.org on June 5, 2009

plies of *cdc42* gene (*17*) maintained an overall wild-type morphology at least up to hour 15:00 (fig. S14). There is no evidence that supports these neurons requiring Cdc42 before 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 importance of controlling endogenous activity patterns of Cdc42 within a given cell, we intentionally overrode the endogenous control of Cdc42 activities in the aCC by overexpressing a constitutively activated form of Cdc42 [green fluorescent protein (GFP)::Cdc42^{V12}] (Fig. 3B). When Cdc42 was activated at high amounts and out of normal context from hour 8:00 onward, the aCC (*n* = 56) displayed aberrant migration of the cell body (34%), thickening of the axon (100%), and premature termination of its axonal growth cone before 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 could emerge as early as hour 10:00.

Fig. 1. Cdc42 activation in whole embryo. (**A**) FRET efficiency in whole embryo (mean ± SEM) at hours 5:00 through 15:00 (*n* = 16 to 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 indicates 50 μm. (**B** and **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).

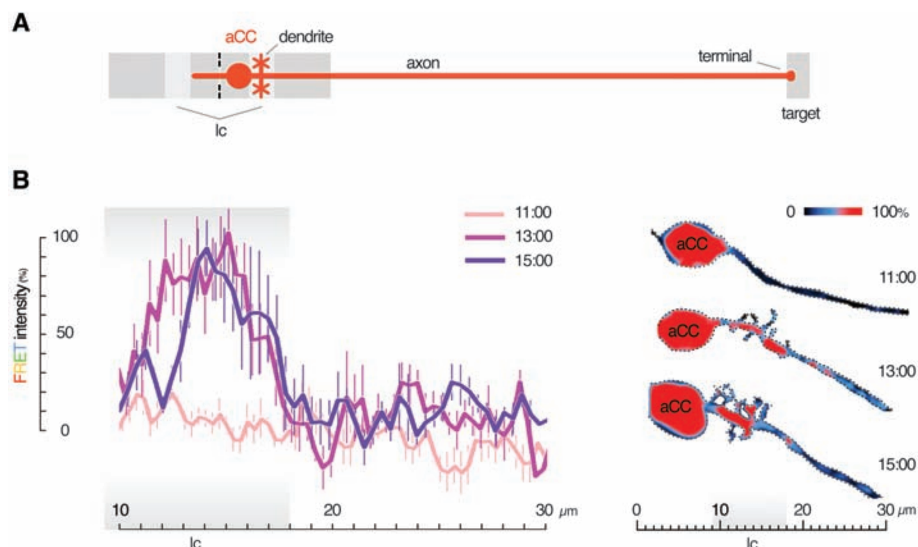

Fig. 2. Cdc42 activation in aCC motoneuron. (**A**) aCC extends its axon laterally to reach the target muscle. The dendrite develops within the longitudinal connective (lc) of the CNS. Anterior is to the top. (**B**) Relative intensity of A-probe FRET (mean ± SEM) along the proximal region of aCC axon 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 analysis of variance ($P < 0.01$). [FRET activities within cell body are mostly nuclear (fig. S1).]

Downloaded from www.sciencemag.org on June 5, 2009

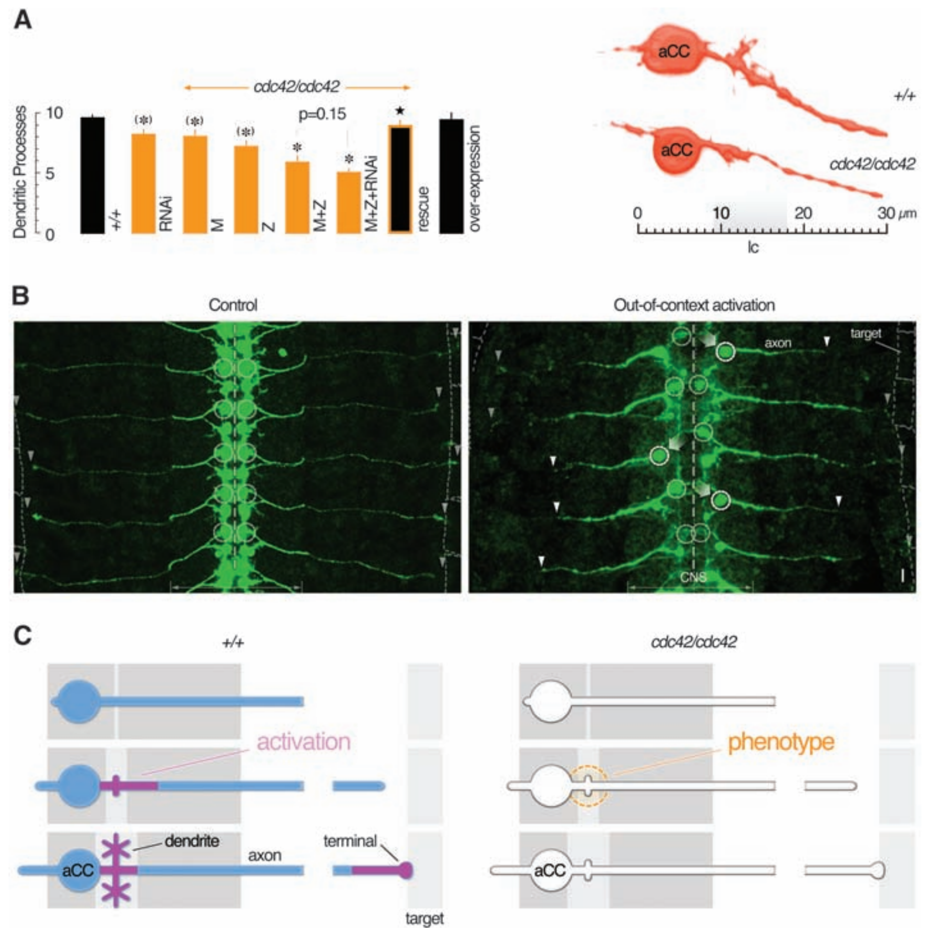

Fig. 3. Genetic tests in aCC motoneuron. (**A**) The number of dendritic processes at 15:00 in wild type (+/+, n = 21), cell-specific RNA interference (RNAi) (*eve'-GAL4/UAS-cdc42^{RNAi}*, n = 13), maternal loss of function (maternally *cdc42^{4}/cdc42^{6}*, n = 9) (*M*), zygotic loss of function (zygotically *cdc42^{4}/Y*, n = 13) (*Z*), maternal and zygotic loss of function (maternally *cdc42^{4}/cdc42^{6}* and zygotically *cdc42^{4} ^{or} ^{6}/Y*, n = 12) (*M+Z*), cell-specific RNAi plus maternal and zygotic loss of function (maternally *cdc42^{4}/cdc42^{6}* and zygotically *cdc42^{4} ^{or} ^{6}/Y;eve'-GAL4/UAS-cdc42^{RNAi}*, n = 14), cell-specific rescue (maternally *cdc42^{4}/cdc42^{6}* and zygotically *cdc42^{4} ^{or} ^{6}/Y;eve'-GAL4/UAS-cdc42*, n = 12), and cell-specific overexpression 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 (*22*). (**B**) Out-of-context activation of Cdc42 in aCC (GFP in green) in the CNS (with pan-neuronal horseradish peroxidase 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).

They are a result of the activation of Cdc42 protein, and not its mere presence, because overexpression of wild-type Cdc42 (GFP::Cdc42) at similar amounts produces no changes in the aCC (Fig. 3B). Therefore, precise spatiotemporal control of Cdc42 activities is critical to achieve the normal development of neurons.

With use of our in vivo bioprobe imaging technology, 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.

References and Notes

1. S. E. Egan, R. A. Weinberg, *Nature* **365**, 781 (1993).
2. S. Etienne-Manneville, A. Hall, *Nature* **420**, 629 (2002).
3. L. Luo, *Nat. Rev. Neurosci.* **1**, 173 (2000).
4. J. S. da Silva, C. G. Dotti, *Nat. Rev. Neurosci.* **3**, 694 (2002).
5. M. Fukata, M. Nakagawa, K. Kaibuchi, *Curr. Opin. Cell Biol.* **15**, 590 (2003).
6. E. E. Govek, S. E. Newey, L. Van Aelst, *Genes Dev.* **19**, 1 (2005).
7. L. Luo, Y. J. Liao, L. Y. Jan, Y. N. Jan, *Genes Dev.* **8**, 1787 (1994).
8. R. Threadgill, K. Bobb, A. Ghosh, *Neuron* **19**, 625 (1997).
9. M. L. Ruchhoeft, S. Ohnuma, L. McNeill, C. E. Holt, W. A. Harris, *J. Neurosci.* **19**, 8454 (1999).
10. Z. Li, L. Van Aelst, H. T. Cline, *Nat. Neurosci.* **3**, 217 (2000).
11. X. B. Yuan *et al.*, *Nat. Cell Biol.* **5**, 38 (2003).
12. L. Van Aelst, H. T. Cline, *Curr. Opin. Neurobiol.* **14**, 297 (2004).
13. M. A. Del Pozo *et al.*, *Nat. Cell Biol.* **4**, 232 (2002).
14. P. Nalbant, L. Hodgson, V. Kraynov, A. Toutchkine, K. M. Hahn, *Science* **305**, 1615 (2004).
15. T. Nakamura, K. Aoki, M. Matsuda, *Brain Res. Mol. Brain Res.* **139**, 277 (2005).
16. O. Pertz, L. Hodgson, R. L. Klemke, K. M. Hahn, *Nature* **440**, 1069 (2006).
17. J. L. Genova, S. Jong, J. T. Camp, R. G. Fehon, *Dev. Biol.* **221**, 181 (2000).
18. A. Lundström *et al.*, *Genes Dev.* **18**, 2161 (2004).
19. J. B. Thomas, M. J. Bastiani, M. Bate, C. S. Goodman, *Nature* **310**, 203 (1984).
20. M. Landgraf, V. Jeffrey, M. Fujioka, J. B. Jaynes, M. Bate, *PLoS Biol.* **1**, E41 (2003).
21. M. P. Furrer, S. Kim, B. Wolf, A. Chiba, *Nat. Neurosci.* **6**, 223 (2003).
22. Materials and methods are available as supporting material on *Science* Online.

23. We thank M. Hayden, K. Chittanon-Buranachai, R. Clegg, S. Rogers, S. Siechen, and I. Vasenkova for advice; S. Hsu, O. Alpcan, M. Furrer, S. Deng, and R. Kamiyama for technical assistance; Bloomington *Drosophila* Stock Center and Developmental Studies Hybridoma Bank for reagents; and E. Giniger, J. Baker, and M. Kim for comments on manuscript. Supported by grants from NIH/National Institute of Neurological Disorders and Stroke and NIH/National Institute of Mental Health (A.C.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/324/5932/1338/DC1
Materials and Methods
Figs. S1 to S15
References

7 January 2009; accepted 20 April 2009
10.1126/science.1170615

Downloaded from www.sciencemag.org on June 5, 2009