The cyclin-dependent kinase Cdk5 controls multiple aspects of axon patterning *in vivo*

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Cyclin-dependent kinase 5 (Cdk5) is one of a subfamily of Cdks involved in the control of cell differentiation and morphology rather than cell division. Specifically, Cdk5 and its activating subunit, p35, have been implicated in growth cone motility during axon extension. Both Cdk5 and p35 are expressed in post-mitotic neurons and are localized to growth cones [1–4]. The Cdk5–p35 complex interacts with the Rac GTPase, a protein required for growth cone motility [5]. Studies using cultured neurons have suggested that Cdk5 activity controls the efficiency of neurite extension [3,4]. Mutant mice lacking p35 exhibit subtle axon-guidance defects [6], but these mice have severe defects in neuronal migration [6–8], making it difficult to define precisely the role of the Cdk5–p35 complex *in vivo*. Here, we examined Cdk5 function in axon patterning in the *Drosophila* embryo. Although our data support the idea that Cdk5–p35 is involved in axonogenesis, they do not support the view that Cdk5 simply promotes growth cone motility. Instead, we found that disrupting Cdk5 function caused widespread errors in axon patterning.

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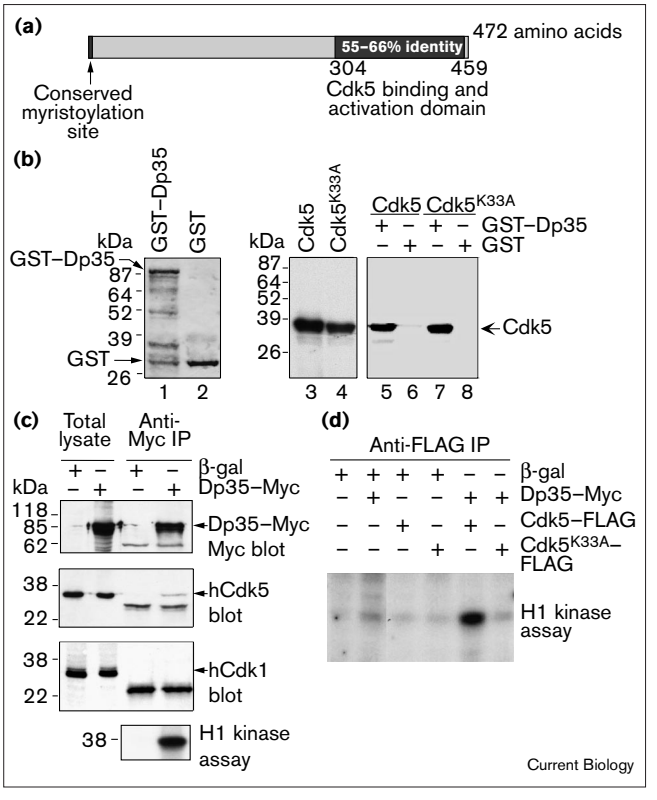
Results and discussion

Cdk5, like all Cdks, must associate with a regulatory subunit to become active [9]. Thus, while Cdk5 is expressed ubiquitously during development [10,11], the spatial and temporal expression of its regulatory subunit limits where and when Cdk5 can be active. We first examined whether a Cdk5 regulatory subunit is present at a time and place consistent with a role in axon outgrowth in the *Drosophila* embryo.

A fly Cdk5 regulatory subunit (Dp35; GenBank accession number AF231134) was identified from the *Drosophila* expressed sequence tag (EST) database using DNA sequence of clones obtained by degenerate PCR followed by cDNA and genomic library screens (Figure 1a and Supplementary material). Dp35 encodes a ~52 kDa protein with 31–40% overall identity to other p35 family members, and 55–66% identity in its carboxy-terminal half, where the Cdk5-binding and activation domains reside [12]. Dp35 also has the amino-terminal myristoylation motif, which is conserved in p35 family members [13], indicating that the cDNA is full length.

Figure 1

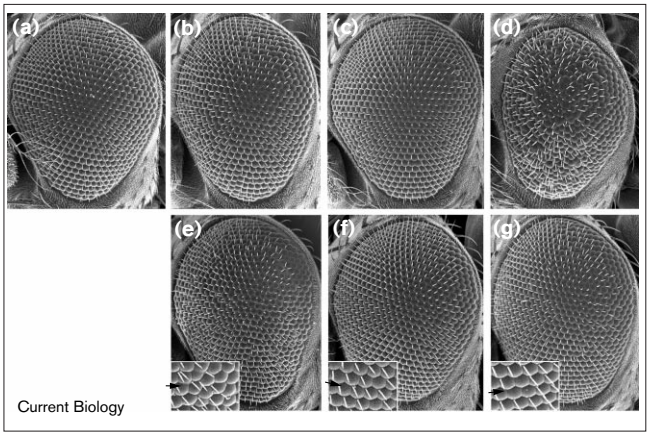


Dp35 binds and activates Cdk5. **(a)** Diagrammatic representation of Dp35. The numbers indicate amino-acid positions. **(b)** GST pull-down assay. Lanes 1 and 2, bacterially expressed GST–Dp35, or GST alone; lanes 3 and 4, *in vitro* translated [^{35}S]methionine-labeled Cdk5 or Cdk5^{K33A}; lanes 5–8, Cdk5 or Cdk5^{K33A} precipitated after incubation with GST–Dp35 or GST beads. All molecular weights are indicated in kDa. **(c)** Human 293T cells transiently expressing UAS–Dp35–Myc or UAS–β-galactosidase (β-gal) and GAL4–VP16 were lysed and subjected to immunoprecipitation (IP) with anti-Myc antibody. Samples were analyzed by western blotting using antibodies to Myc, human Cdk5 (hCdk5), or human Cdk1 (hCdk1) and assayed for histone H1 kinase activity. **(d)** Human 293T cells transiently expressing GAL4–VP16 plus combinations of UAS–Dp35–Myc, UAS–Cdk5–FLAG, UAS–Cdk5^{K33A}–FLAG and UAS–β-galactosidase were lysed, subjected to immunoprecipitation with anti-FLAG antibodies, and kinase activity analyzed as in (c).

We performed three tests, which together confirmed that Dp35 and Cdk5 associate and cooperate functionally *in vitro* and *in vivo*. First, we found that Dp35 binds Cdk5. In an *in vitro* glutathione-S-transferase (GST) pull-down assay, fly Cdk5 associated with GST–Dp35 but not with GST alone, as did a mutant Cdk5 (Cdk5^{K33A}) that is predicted to be kinase inactive (Figure 1b) [3]. Moreover, Myc-epitope-tagged Dp35 (Dp35–Myc) expressed in human 293T cells associated with endogenous human Cdk5 *in vivo*, but not with Cdks 1, 2, 4 or 6 (Figure 1c and Supplementary material). Second, we found that Dp35 activates Cdk5. Anti-Myc antibody immunoprecipitates from human 293T cells containing Dp35–Myc and human Cdk5 phosphorylated histone H1 (Figure 1c). Furthermore, anti-FLAG antibody immunoprecipitates from human 293T cells containing fly FLAG-epitope-tagged Cdk5 (Cdk5–FLAG) exhibited H1 kinase activity in the presence, but not in the absence, of Dp35 (Figure 1d and data not shown). In contrast, Cdk5^{K33A}–FLAG was catalytically inactive, although it did associate with Dp35 (Figure 1b,d and data not shown). Third, we found that Dp35 interacts genetically with Cdk5 in the *Drosophila* eye. Expression of one copy of Cdk5–FLAG in all cells of the eye using the GMR–GAL4 driver had no effect on eye surface morphology, whereas one copy of Dp35–Myc caused a slight rough eye phenotype, presumably by activation of endogenous, low-level Cdk5 (Figure 2b,c). In contrast, coexpression of Cdk5–FLAG with Dp35–Myc severely disrupted eye morphology (Figure 2d). This synthetic phenotype requires Cdk5 kinase activity because coexpression with two copies of Cdk5^{K33A}–FLAG suppressed rather than enhanced the mild rough eye phenotype observed with two copies of Dp35–Myc (Figure 2e–g). Thus, like their mammalian orthologs [3], fly Cdk5 is activated by Dp35, whereas Cdk5^{K33A} appears to act as a dominant-negative mutant by titrating Dp35 and will be referred to below as Cdk5dn.

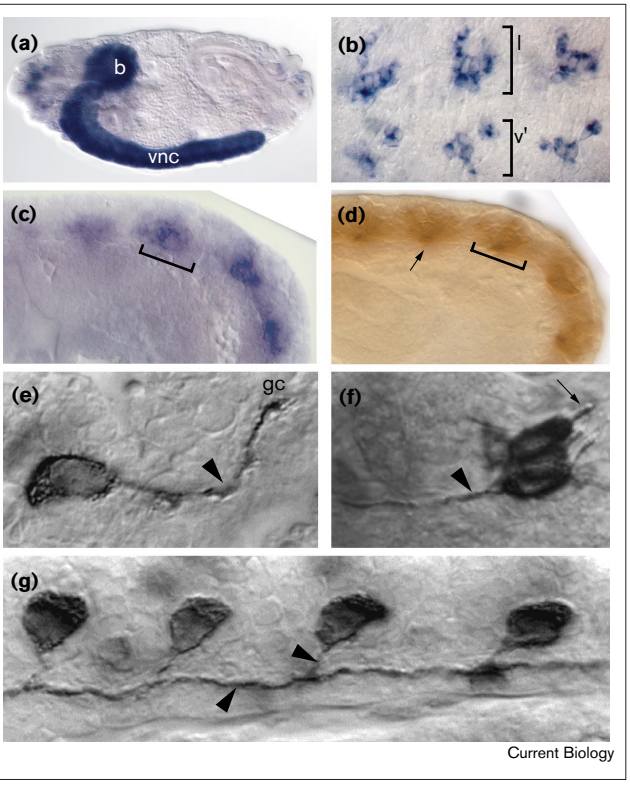
Next, we found that Dp35 is expressed in neurons at a time and place consistent with a role in axon development *in vivo*. Dp35 is expressed exclusively in the nervous system, with strong expression in the brain, ventral nerve cord and post-mitotic neurons of the peripheral nervous

Figure 2



Cdk5 and Dp35 interact genetically. Scanning electron micrograph of adult eyes expressing combinations of Cdk5, Cdk5^{K33A} , and Dp35 with the eye-specific driver GMR–GAL4. **(a)** GMR–GAL4 alone. **(b)** One copy of UAS–Dp35–Myc. **(c)** One copy of UAS–Cdk5–FLAG. **(d)** One copy each of UAS–Dp35–Myc and UAS–Cdk5–FLAG. Note the severe disorganization of eye morphology. **(e)** Two copies of UAS–Dp35–Myc. Note the irregular rows of facets (arrow in the inset). **(f)** Two copies of UAS–Cdk5^{K33A}–FLAG. **(g)** Two copies each of UAS–Dp35–Myc and UAS–Cdk5^{K33A}–FLAG. The regular eye structure here contrasts with the irregular morphology in (d,e).

Figure 3



Dp35 is expressed in neurons and localizes to growth cones. **(a–c)** *In situ* hybridization with a Dp35 probe. (a) Stage 17 embryo; b, brain; vnc, ventral nerve cord. Expression was also observed in anterior and posterior sensillae. Left, anterior; top, dorsal. (b) Lateral view of lateral (l) and ventral′ (v′) clusters of sensory neurons in three abdominal hemisegments. (c) CNS, stage 12.4 embryo. **(d)** Immunostaining of a stage 12.4 embryo with antibody against horseradish peroxidase (HRP) to reveal CNS neurons, neuroblasts and ganglion mother cells. Dp35 expression and HRP immunoreactivity were localized to the same regions of the CNS, but Dp35 was restricted to the dorsal surface of the CNS, where the first neurons are beginning to extend axons (posterior commissure; the dark dot indicated by the arrow). **(e–g)** Embryonic neurons expressing UAS–Dp35–Myc under the control of 15J2–GAL4 and detected with anti-Myc antibodies. (e) A dMP2 neuron, stage 12 CNS. (f) Chordotonal neurons, stage 16 PNS. (g) dMP2 neurons from four hemisegments, stage 14 CNS. Arrowheads, axons; arrow, dendrites; gc, growth cone.

system (PNS) (Figure 3a,b). The earliest Dp35 expression was observed at stage 12 in clusters of cells in the central nervous system (CNS) where the neurons that extend the first CNS axons reside (Figure 3c,d). Dp35 expression was not observed in neuroblasts, suggesting that fly Dp35–Cdk5, like mammalian p35–Cdk5, is not involved in cell division [2]. We examined the subcellular localization of Dp35–Myc expressed in a small subset of neurons using the 15J2–GAL4 driver. Consistent with the localization of mammalian p35 [3,4], Dp35–Myc protein expressed in CNS dMP2 neurons was present in the neuron cell body and axon, including the growth cone (Figure 3e,g). Dp35–Myc, expressed in PNS lateral chordotonal neurons, was present in neuron cell bodies, dendrites and axons (Figure 3f and data not shown).

Finally, we tested the functional consequences of altering neuronal Cdk5 activity *in vivo*. We expressed Cdk5, Cdk5dn and/or Dp35 in all post-mitotic neurons using the elav–GAL4 driver, and then examined motor nerves innervating lateral and dorsal body wall muscles of stage 16–17 embryos.

Altering neuronal Cdk5 activity led to two important observations. First, either increasing or decreasing Cdk5 activity produced errors in axon patterning. Second, there was no simple correlation between Cdk5 activity and axon/dendrite length. We activated endogenous neuronal Cdk5 by expressing one copy of Dp35, which caused subtle nerve defects in 32–40% of the embryos (Figure 4j). The majority of defects occurred in the transverse nerve (TN), with a few defects observed in segmental nerve a (SNa) and the intersegmental nerve (ISN; see Supplementary material for distribution of defects). In contrast to observations in cultured neurons [3,4], we found overextended nerves, stalled nerves, and nerves with errors in pathfinding and target recognition (Figure 4a–i). Two copies of Dp35 increased the percentage of embryos with defects (~61%; Figure 4j), suggesting that the effects of Dp35 are dose dependent. The severity of the phenotype was greatly enhanced by coexpressing Cdk5 with Dp35 (Figure 4k); 80% of the embryos exhibited nerve defects that were more severe and widespread than when Dp35 alone was expressed (Figure 4a–i and see Supplementary material). Nevertheless, the spectrum of phenotypes observed (overextended, stalled and misrouted nerves) was similar to that seen when Dp35 alone was expressed.

We decreased neuronal Cdk5 activity by expressing Cdk5dn [3]. One copy of Cdk5dn had no effect on axon patterning, but two copies caused 32–35% of the embryos

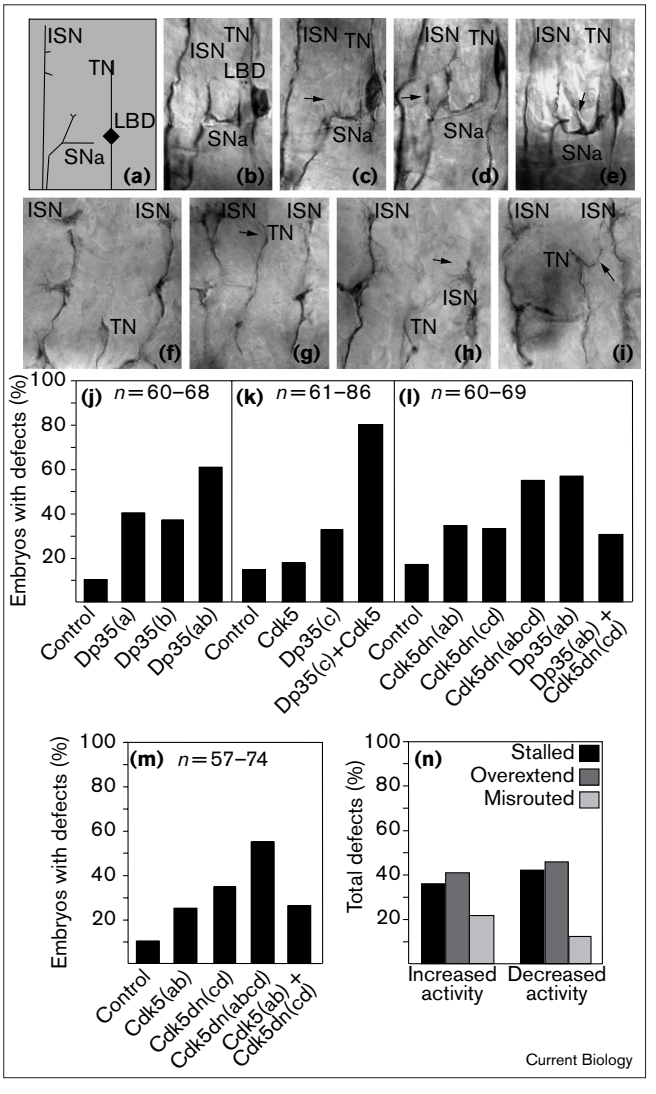


Figure 4

Altering Cdk5 function causes axon patterning defects. **(a)** Diagram of an abdominal hemisegment depicting the three nerves examined in this study. LBD, lateral bipolar dendritic neuron. **(b–i)** Peripheral motor nerves of stage 17 (b,f) wild-type embryos, or embryos expressing (c,d,g–i) Cdk5–FLAG with Dp35–Myc, or (e) Cdk5dn–FLAG alone, visualized with anti-fasciclin 2 antibody. Arrowheads denote defective nerves. (b) Wild-type SNa. (c) SNa lacking the dorsal branch. (d,e) SNas with ectopic muscle contacts. (f) Wild-type dorsal terminations of ISN and TN. (g) An overextended TN. (h) A stalled ISN. (i) A misrouted TN that has extended towards the ISN in the next segment. **(j–m)** Percentage of embryos exhibiting one or more nerve defects when neuronal Cdk5 activity was altered by expression of the indicated proteins. Control embryos expressed elav–GAL4 alone. Letters in parentheses indicate independent UAS construct insertions into the genome; each letter represents one insertion. Each bar represents an average of two independent experiments; the results of each experimental pair never differed by more than 14%, with an average difference of less than 10%. For example, in four independent trials with Cdk5dn, 31, 32, 33 and 33% of the embryos exhibited defects. **(n)** Percentage of stalled, overextended, or misrouted nerves in response to increased or decreased Cdk5 activity. Increased activity includes defects seen with expression of Dp35 or Dp35 plus Cdk5; decreased activity refers to defects seen with expression of Cdk5dn. Expression of UAS–Cdk5, UAS–Cdk5dn, and UAS–Dp35 did not alter neuron cell fate as assayed by staining with anti-eve antibody and monoclonal antibody 22C10 (data not shown).

to exhibit subtle nerve defects (Figure 4l and data not shown). The majority of defects occurred in the TN, with some defects in SNa and the ISN (see Supplementary material). In addition, we observed a similar spectrum of phenotypes as that seen with increased Cdk5 activity (Figure 4n). With four copies of Cdk5dn, the percentage of embryos with defects increased to 56%, indicating that the effects of Cdk5dn, like those of Dp35, are dose dependent (Figure 4j,l). Cdk5dn is likely to act, in part, by titrating endogenous Dp35, because coexpression of two copies of Cdk5dn with two copies of Dp35 suppressed the axonal defects produced by two copies of Dp35 alone (31% versus 57%; Figure 4l). The phenotypic suppression observed with Cdk5dn/Dp35 coexpression contrasts with the phenotypic enhancement caused by Cdk5/Dp35 coexpression and indicates that the Cdk5/Dp35-induced defects require kinase activity. Additionally, the phenotypes of Cdk5 and Cdk5dn appear to be mutually suppressive because, while the effects of either Cdk5 or Cdk5dn are dose dependent, coexpression of two copies of Cdk5 with two copies of Cdk5dn caused a smaller percentage of embryos to exhibit defects than did four copies of Cdk5dn (26% versus 56%; Figure 4m).

Our data confirm that Cdk5 is involved in axon development *in vivo*, consistent with predictions of Cdk5 function from studies using cultured neurons. Analysis of the phenotypes resulting from Cdk5/Dp35 expression *in vivo* revealed, however, a different picture of Cdk5 function than that observed in cultured neurons. Experiments using cultured neurons suggested a simple correlation between Cdk5 activity and the efficiency of neurite outgrowth [3,4]. In these experiments, it was not possible to examine axon patterning, however. It is clear that Cdk5 and p35 are not absolutely required for axon growth *in vivo* ([7,8] and data not shown). Rather, we found that altering Cdk5 activity degrades the accuracy of axon patterning: either increasing or decreasing activity could promote or retard axon growth, and cause errors in pathfinding and target recognition. Perhaps the main function of Cdk5 is to ensure coordination of the many signaling pathways that are active in the growth cone, akin to the 'checkpoint' function of cell-cycle Cdks [14], and not simply to increase growth cone motility. Such a 'surveillance' mechanism would be consistent with the relatively subtle phenotypes observed when we modulate Cdk5 activity.

It is interesting that increasing or decreasing Cdk5 activity both caused qualitatively similar nerve defects. This is reminiscent of the effects produced when activity of the Rac GTPase is increased or decreased [15]. As p35 interacts with Rac [6], it will be of interest to determine whether the axonal effects of Cdk5/Dp35 *in vivo* are mediated or regulated by Rac. More generally, the ability to modulate Cdk5 activity *in vivo* will permit us to place Cdk5 genetically within the signaling pathways that control growth cone motility and guidance.

Supplementary material

Additional results and methodological details are available at http://current-biology.com/supmat/supmatin.htm.

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References

1. Lew J, Huang Q-Q, Qi Z, Winkfein RJ, Aebersold R, Hunt T, *et al.*: **A brain-specific activator of cyclin-dependent kinase 5**. *Nature* 1994, **371:**423-426.
2. Tsai L-H, Delalle I, Caviness VS, Chae T, Harlow E: **p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5**. *Nature* 1994, **371:**419-423.
3. Nikolic M, Dudek H, Kwon YT, Ramos YFM, Tsai L-H: **The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation**. *Genes Dev* 1996, **10:**816-825.
4. Pigino G, Paglini G, Ulloa L, Avila J, Caceres A: **Analysis of the expression, distribution and function of cyclin dependent kinase 5 (cdk5) in developing cerebellar macroneurons**. *J Cell Sci* 1997, **110:**257-270.
5. Nikolic M, Chou MM, Lu W, Mayer BJ, Tsai L-H: **The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak activity**. *Nature* 1998, **395:**194-198.
6. Kwon YT, Tsai LH, Crandall JE: **Callosal axon guidance defects in p35(−/−) mice.** *J Comp Neurol* 1999, **415:**218-229.
7. Chae T, Kwon YT, Bronson R, Dikkes P, Li E, Tsai L-H: **Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality**. *Neuron* 1997, **18:**29-42.
8. Gilmore EC, Ohshima T, Goffinet AM, Kulkarni AB, Herrup K: **Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex**. *J Neurosci* 1998, **18:**6370-6377.
9. Morgan DO: **Cyclin-dependent kinases: engines, clocks, and microprocessors**. *Annu Rev Cell Dev Biol* 1997, **13:**261-291.
10. Hellmich MR, Kennison JA, Hampton LL, Battey JF: **Cloning and characterization of the *Drosophila melanogaster* CDK5 homolog**. *FEBS Lett* 1994, **356:**317-321.
11. Sauer K, Weigmann K, Sigrist S, Lehner CF: **Novel members of the cdc2-related kinase family in *Drosophila*: cdk4/6, cdk5, PFTAIRE, and PITSLRE kinase**. *Mol Biol Cell* 1996, **7:**1759-1769.
12. Poon RYC, Lew J, Hunter T: **Identification of functional domains in the neuronal Cdk5 activator protein**. *J Biol Chem* 1997, **272:**5703-5708.
13. Patrick GN, Zuckerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai L-H: **Conversion of p35 to p25 deregulates cdk5 activity and promotes neurodegeneration**. *Nature* 1999, **402:**615-622.
14. Lew DJ, Reed SI: **A cell cycle checkpoint monitors cell morphogenesis in budding yeast.** *J Cell Biol* 1995, **129:**739-749.
15. Luo L, Liao YJ, Jan LY, Jan YN: **Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion**. *Genes Dev* 1994, **8:**1787-1802.