Turning by asymmetric actin

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Axon guidance requires local protein synthesis at the growth cone. Two new studies show that guidance cues induce asymmetric targeting and translation of β-actin *(Actb)* mRNA. Such asymmetry may be the mechanism that underlies growth cone turning.

The brain is wired up during development by active axon pathfinding in response to environmental guidance cues. The neuronal growth cone, the leading tip of the growing process, makes steering decisions through rearrangement of the cytoskeleton[1], often at a significant distance from the cell body. The autonomy of axons in guidance has been demonstrated in a classic experiment showing that retinal axons are capable of finding their targets in the developing *Xenopus* embryos after being cut off from the cell body[2]. Protein synthesis, a process previously believed to be limited to the cell body, occurs locally in the axons[3] and is required for both acute[4] and long-term adaptive[5] growth cone responses to guidance cues. Since these initial discoveries, there has been a tremendous interest in the identity of newly translated proteins and their role in growth cone guidance. In this issue, two studies by Leung and colleagues[6] and Yao and colleagues[7] show that guidance cues induce asymmetric targeting and translation of β-actin mRNA in the growth cone. This finding is particularly interesting because polymerization of actin filaments provides major driving forces for growth cone motility. Thus, asymmetric actin synthesis may actually underlie growth cone turning.

Localized protein translation allows a cell to fine-tune gene expression in both space and time. Although the existence of local protein synthesis in dendrites and synapses has been well established, the discovery that rapid axonal protein synthesis is required for acute growth cone responses (collapse and turning) to some guidance cues (such as netrin-1, brain-derived neurotrophic factor (BDNF), Sema3A and Slit2) came as a surprise[8–10]. Early studies of cultured *Xenopus* spinal neurons established that these neurons respond to a large number of guidance cues, which can be classified into two groups[11]. The signaling of Group I cues, including netrin-1, BDNF and myelin-associated glycoprotein, requires extracellular Ca^{2+} and is sensitive to modulation by cyclic AMP/protein kinase A (cAMP/PKA) activity[11,12]. Using focal laser–induced photolysis (FLIP) of caged Ca^{2+}, an approach previously developed in their laboratory, Yao and colleagues now show that local Ca^{2+} elevation–induced growth cone attraction and repulsion are both abolished by protein-synthesis inhibitors[7]. This exciting finding suggests a general requirement of new protein synthesis underlying signaling of Ca^{2+}-dependent (Group I) guidance cues.

Regulated local protein synthesis is achieved by two sequential processes—transport of mRNA to specific locations, followed by initiation of mRNA translation upon stimulation. Although several axonal proteins are known to be locally translated and important for growth cone collapse and repulsion[8–10], these two studies newly identify β-actin as another axonally translated protein, and go a step further: they depict nicely how pathways regulating β-actin mRNA transport and translation act in concert to induce the asymmetry that is required for acute growth cone turning[6,7]. Zipcode binding protein 1 (ZBP1) regulates transport of β-actin mRNA through binding to a conserved 54-nucleotide element in the 3′-untranslated region (UTR) of the β-actin mRNA known as the 'zipcode' (ref. 13). Both studies by Leung *et al.* and Yao *et al.* used *Xenopus* neuronal cultures and provide substantial evidence that Vg1RBP, a *Xenopus* homolog of ZBP1, interacts with β-actin mRNA within neuronal growth cones. Using

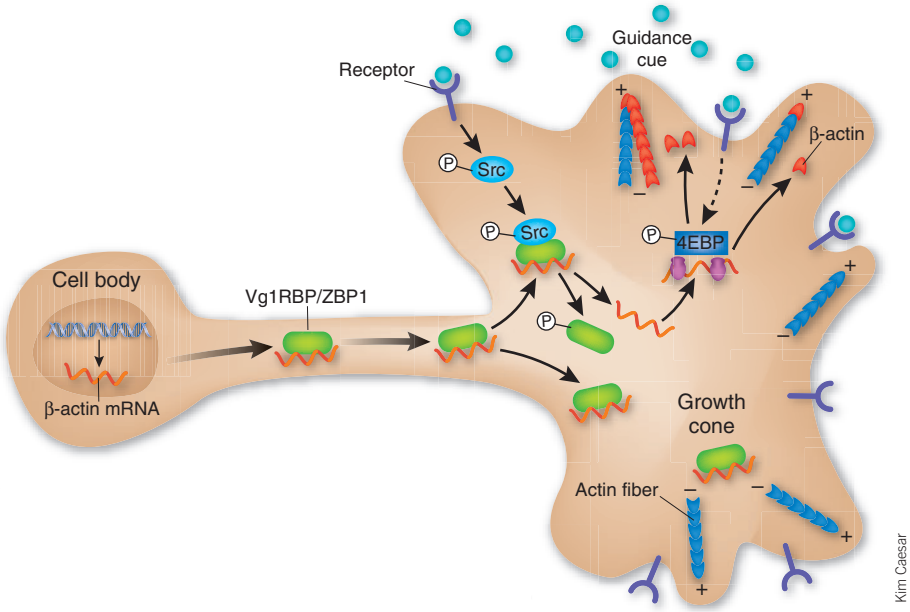

Figure 1 A model for guidance cue–induced asymmetric transport and translation of β-actin mRNA that initiates growth cone turning. Translocation of β-actin mRNA is controlled by ZBP1. ZBP1 associates with the β-actin mRNA in the nucleus and transports it into neuronal growth cones while blocking its translation initiation. A gradient of guidance cues activates yet unknown pathways to promote the asymmetric transport of granules containing ZBP1/β-actin mRNA complex into the growth cone periphery. Translation of β-actin mRNA is regulated by phosphorylation events. Phosphorylation of 4EBP promotes cap-dependent translation initiation, whereas Src kinase phosphorylates ZBP1 to release its block of translation initiation. Together, these cellular events lead to asymmetric distribution of newly synthesized β-actin and the preferential incorporation of this nascent β-actin into the cytoskeleton at one side of the growth cone.

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Vg1RBP-eGFP as a reporter in live cell imaging, Leung and colleagues further show that ZBP1-containing vesicles are actively transported from the growth cone center domain into filopodia of retinal neurons upon netrin-1 stimulation, potentially carrying β-actin mRNA with them. Indeed netrin-1–induced translocation of β-actin mRNA into filopodia was confirmed by fluorescent *in situ* hybridization (FISH) analysis. Furthermore, these authors observed an asymmetrical transport of Vg1RBP granules into filopodia at the near site of the gradient.

Yao and colleagues took a functional approach to examine the role of ZBP1 in growth cone guidance[7]. They found that a cocktail of antisense oligonucleotides to *Xenopus* β-actin 3′ UTR zipcode, but not the control oligonucleotide, attenuated BDNF-induced transport and increase of β-actin mRNA levels in spinal neuron growth cones. Furthermore, they observed a significant increase in the near/far ratio of β-actin mRNA/ZBP1 complex in the presence of a BDNF or Ca^{2+} gradient. More importantly, antisense oligonucleotide treatment blocked both growth cone attraction and repulsion but not neurite extension. These results provide the first functional evidence that ZBP1 is required for growth cone turning.

The above results establish that guidance cues induce active and asymmetric transport of β-actin mRNA, but is its translation regulated by guidance cues as well? Leung and colleagues[6] observed a rapid increase of total β-actin protein within growth cones (by ~30%) upon global application of netrin-1. Such an increase was blocked by a protein synthesis inhibitor, or functional blocking antibodies to the netrin-1 receptor DCC, or an antisense morpholino directed against β-actin mRNA. To directly examine the regulation of β-actin mRNA translation, the authors developed an elegant reporter construct in which the 3′ UTR of β-actin was linked to the coding sequence of Kaede, a green fluorescent protein that can be effectively and irreversibly converted to a highly stable red form upon brief ultraviolet illumination. Thus, analysis of green and red fluorescence signals after photoconversion provides a quantitative measure of both newly synthesized (green) and pre-existing (red) proteins. Imaging studies showed that netrin-1 increased Kaede-green signal in axons separated from the soma. These experiments firmly establish that netrin-1 stimulates local translation of β-actin mRNA.

Growth cone turning requires a break in symmetry. Both groups show that chemoattractants lead to an asymmetric elevation of β-actin within the growth cone[6,7]. Using a morpholino oligonucleotide directed against β-actin mRNA to block its translation, Leung and colleagues showed that the morpholino prevented the asymmetric actin distribution in response to netrin-1 and, more importantly, netrin-1–induced growth cone attraction. Yao and colleagues showed that asymmetric elevation of β-actin protein on the side facing the gradient required ZBP1 function and extracellular Ca^{2+}. Together, results from both groups support the idea that asymmetric localization of β-actin is essential for attractive turning responses.

What are the potential mechanisms that regulate β-actin mRNA translation locally at the growth cone? ZBP1 associates with the β-actin mRNA and prevents its premature translation by blocking translation initiation[14]. Such inhibition is released by the protein kinase Src, which phosphorylates a key tyrosine residue in ZBP1 that is required for binding to RNA (ref. 14). Indeed, a gradient of BDNF led to an asymmetric distribution of phospho-Src (active form) within the growth cone[6]. Another level of regulation lies in the cap-dependent translation initiation for β-actin mRNA. Netrin-1 stimulates rapid phosphorylation of the translation initiation factor eIF-4E binding protein 1 (4EBP)[4], a marker for cap-dependent translation initiation. Leung and colleagues now show that netrin-1 gradient induces asymmetric phosphorylation of 4EBP. Together, these results suggest that guidance cue–induced phosphorylation events may underlie translation initiation of β-actin mRNA.

Whereas both studies agree on the asymmetric localization of β-actin in attractive turning, they differ on growth cone repulsion. Leung and colleagues examined repulsion in response to gradients of netrin-1 in the presence of laminin and Sema3A (not a Group I cue)[6] and observed a slight bias but not significant reverse asymmetry of β-actin protein. Furthermore, morpholino against β-actin did not seem to affect repulsion to either Sema3A or netrin-1. The authors argue that β-actin translation is particularly important for directional guidance toward a positive cue. On the other hand, Yao and colleagues observed reversed asymmetry of both phospho-Src and β-actin levels in response to BDNF under repulsive conditions[7]. Importantly, the cocktail of antisense oligonucleotides against ZBP1 blocked both BDNF- and Ca^{2+} gradient–induced repulsion, suggesting an essential role for ZBP1 in repulsion as well. Furthermore, these authors observed a significant reduction of β-actin protein with BDNF under repulsive conditions. Thus, it is possible that differences in the target of manipulation (β-actin versus ZBP1) contributes to the differential findings from the two groups. ZBP1 may regulate transport of other mRNAs in addition to β-actin mRNA. Further studies are needed to firmly determine whether attraction and repulsion differentially depend on β-actin mRNA transport and translation.

The following model can be drawn from these new findings (**Fig. 1**). Once β-actin mRNA is made in the nucleus, it becomes associated with ZBP1, which transports β-actin mRNA while preventing translation initiation. A gradient of guidance cues activates an unknown pathway to promote the asymmetric transport of granules containing ZBP1/β-actin mRNA complex into the peripheral region of the growth cone. The same gradient also asymmetrically activates Src kinase and 4EBP, promoting translation initiation of β-actin mRNA. The preferential incorporation of nascent β-actin into the cytoskeleton at one side may provide the initial driving force for growth cone steering.

These *in vitro* studies provide substantial evidence for the involvement of asymmetric β-actin mRNA transport and translation in growth cone guidance and also raise many interesting questions. First, is asymmetric β-actin translation instructive or permissive in growth cone guidance? New tools need to be developed to examine whether asymmetry of β-actin mRNA translation within the growth cone is sufficient to induce turning. It is not clear why new β-actin is needed at all, given the large pool of β-actin present in the growth cone. Nascent β-actin may be more effective in the initiation of actin polymerization, and new methodology using tetracysteine tags to differentiate new and old proteins[15] may allow direct testing of this hypothesis. Second, the signaling pathways that regulate transport, stability and rapid translation of specific mRNAs before and after stimulation of guidance cues remain poorly characterized. Third, we know little about the role of local protein synthesis in axonal pathfinding *in vivo*. For example, does midline axon guidance of commissural interneurons, a classic example of netrin-1–regulated Ca^{2+}-dependent guidance[12], require local protein synthesis? And finally, as many inhibitory factors in the adult brain signal through Ca^{2+}-dependent pathways to regulate axonal behaviors[11], could modulation of ZBP1 function and axonal β-actin protein synthesis help to promote axonal regeneration after injury?

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Hooking up new synapses

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Synapse formation requires adhesive interactions between pre- and postsynaptic membranes. A new study reports that netrin-G2 ligand (NGL-2) interactions with netrin-G2 induces excitatory synapses, expanding the range of known synapse-inducing signals.

For most of us, the heyday of synapse formation is over. After all, this process occurs most intensely in the human central nervous system during late prenatal and early postnatal development. Nevertheless, synapse formation vies for attention from both neurobiologists and cell biologists. Neurobiologists are interested in synaptogenesis simply because it is required to set up nervous systems. The cell biological challenge stems from the need to coordinate synapse formation in time and space between pre- and postsynaptic neurons. Only this close coordination allows two neurons to form the asymmetric cell-cell junctions that are synapses. A study by Seho Kim and colleagues[1], in this issue, provides fresh molecular insights into the repertoire of interactions between two neurons as they form a new synapse. The authors demonstrate that NGL-2 is a postsynaptic cell-surface protein that instructs the formation of excitatory synapses.

Previous work had identified two synaptic adhesion systems that not only hold synapses together, but also instruct their formation. Neuroligins, a small family of postsynaptic membrane proteins[2], were the first molecules shown to induce the assembly of functional presynaptic specializations[3]. They act in synaptogenesis through binding of their presynaptic partners, neurexins[4]. This heterophilic interaction also induces the assembly of postsynaptic protein complexes[5–7]. Another synaptic adhesion system involves the cell adhesion molecule SynCAM 1, a homophilic immunoglobulin (Ig) domain–containing protein belonging to its own small gene family[8]. SynCAM 1 drives the formation of functional presynaptic specializations that release excitatory neurotransmitter and concurrently promotes synaptic transmission[9,10]. Obviously synaptogenesis is too important to be left to only two synaptic adhesion systems, so NGL-2 constitutes the third example of a trans-synaptic interaction mediating synaptogenesis.

NGL-2 may literally hook up new synapses: its extracellular domain contains leucine-rich repeats that form a horseshoe-shaped hook. (ref. 11 and **Fig. 1**) The extracellular sequence of NGL-2 also includes an Ig-like domain, implying that this protein could serve as adhesion molecule. However, the feature that led to the identification of NGL-2 in this new study is its intracellular carboxyl-terminal PDZ (postsynaptic density-95/Discs large/zona occludens-1) domain interaction sequence. The authors found NGL-2 in a yeast two-hybrid screen for proteins interacting with PDZ domains of the adaptor molecule postsynaptic density–95 (PSD-95). They confirmed that this interaction occurs in the brain and that NGL is a postsynaptic membrane protein at excitatory but not inhibitory synapses. Synaptic membrane proteins interacting with scaffolding molecules such as PSD-95 are, of course, generally good candidates for the organization of postsynaptic membrane specializations. However, a more direct motivation probably drove the authors' screening strategy as well: neuroligins bind PSD-95 (ref. 12), and thus other PSD-95–binding synaptic membrane proteins might similarly function in synaptogenesis.

Knockdown of NGL-2 in dissociated hippocampal neurons, which reduces the density of excitatory but not inhibitory postsynaptic specializations, demonstrates its role in synapse formation or stability. Furthermore, NGL-2 is involved in excitatory neurotransmission, as knockdown of the protein's expression reduces the frequency of excitatory miniature potentials (minis) but not of inhibitory minis in dissociated hippocampal neurons. What are the functions of NGL-2 on the cellular level? Here, the authors provide two key results. First, NGL-2 promoted the assembly of postsynaptic scaffolding molecules and receptors. Exogenous clustering of overexpressed NGL-2 in the postsynaptic membrane led to the recruitment not only of PSD-95 and other components of the postsynaptic scaffold, but also of AMPA and NMDA receptors, indicating that functional postsynaptic assemblies had been formed. Consistently, NGL-2 overexpression promoted dendritic spine protrusions. The second key result of the new work is that NGL-2 induced presynaptic assembly through a trans-synaptic interaction. Presentation of NGL-2 to growing axons induced specializations that contained not only clustered markers of excitatory presynaptic sites, but also actively recycling synaptic vesicles. This was demonstrated by uptake of an antibody to the luminal domain of the synaptic vesicle protein synaptotagmin I into these NGL-2–induced specializations. The adhesive interactions of NGL-2 are sufficient to achieve this effect, as its purified extracellular sequence can be presented on beads to neurons in order to induce this presynaptic differentiation. These experiments place NGL-2 into the same group as neuroligins and SynCAM 1, synaptic membrane proteins capable of inducing functional presynaptic terminals.

An open question is which presynaptic protein the NGL-2 hook catches during synaptogenesis. The only known extracellular partner of the related NGL-1 leucine-rich repeats is netrin-G1, a glycosylphosphatidylinositol (GPI)-anchored membrane protein that is also known as laminet-1 (ref. 13). Netrin-G1 shares extensive sequence similarities with netrins, secreted proteins that are critical regulators of axon outgrowth in early development. Consistent with functions in axon guidance, NGL-1 and netrin-Gs promote neurite outgrowth[13]. No other binding partners are known

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