Cell

Transmembrane Receptor DCC Associates with Protein Synthesis Machinery and Regulates Translation

Joseph Tcherkezian,[1,5] Perry A. Brittis,[1,5,*] Franziska Thomas,[2,6] Philippe P. Roux,[3,4] and John G. Flanagan[1,*]

[1]Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
[2]Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA
[3]Institute for Research in Immunology and Cancer
[4]Department of Pathology and Cell Biology
Faculty of Medicine, Université de Montréal, 2900, boulevard Édouard-Montpetit, Montreal QC H3T 1J4, Canada
[5]These authors contributed equally to this work
[6]Present address: Boehringer Ingelheim Pharma GmbH and CoKG, Birkendorfer Strasse 65, 88397 Biberach an der Riss, Germany
*Correspondence: perry_brittis@hms.harvard.edu (P.A.B.), flanagan@hms.harvard.edu (J.G.F.)

SUMMARY

Extracellular signals regulate protein translation in many cell functions. A key advantage of control at the translational level is the opportunity to regulate protein synthesis within specific cellular subregions. However, little is known about mechanisms that may link extracellular cues to translation with spatial precision. Here, we show that a transmembrane receptor, DCC, forms a binding complex containing multiple translation components, including eukaryotic initiation factors, ribosomal large and small subunits, and monosomes. In neuronal axons and dendrites DCC colocalizes in particles with translation machinery, and newly synthesized protein. The extracellular ligand netrin promoted DCC-mediated translation and disassociation of translation components. The functional and physical association of a cell surface receptor with the translation machinery leads to a generalizable model for localization and extracellular regulation of protein synthesis, based on a transmembrane translation regulation complex.

INTRODUCTION

Transmembrane receptors can provide a direct link between extracellular signals and intracellular machinery. It has long been known that receptor cytoplasmic domains can associate with two types of intracellular machinery: the cytoskeleton, and cytoplasmic signaling proteins (Flanagan and Koch, 1978; Rodbell, 1980; Ullrich and Schlessinger, 1990). From there, regulatory information can be conveyed by signaling pathways to regulate functional outputs throughout the cell, including gene expression at both transcriptional and translational levels (Pawson and Nash, 2003).

One key advantage of controlling gene expression at the translational level is that this allows protein synthesis to be spatially localized to specific subregions of the cytoplasm. This localization of protein synthesis is thought to be important in diverse biological functions, such as setting up the primary axes of the *Drosophila* embryo, cell migration and adhesion, axon guidance, and regulation of the synapse (St Johnston, 2005; Sutton and Schuman, 2006; Lin and Holt, 2008; Rodriguez et al., 2008). Moreover, genome-scale analysis in the early *Drosophila* embryo indicates that most messenger RNAs (mRNAs) show localization to diverse and specific subcellular sites, including at the cell membrane, implying that mechanisms to regulate localized translation participate in many, if not most, cell functions (Lécuyer et al., 2007). Regarding mechanisms for translational regulation, a great deal of progress has been made in identifying ribonucleoprotein complexes that mediate mRNA localization and translational control (St Johnston, 2005; Kiebler and Bassell, 2006; Rodriguez et al., 2008), and molecular pathways that transmit signals from cell surface receptors and regulate the translational machinery (Tee and Blenis, 2005; Sonenberg and Hinnebusch, 2007). However, it is still not well understood how extracellular signals may regulate translation with spatial precision.

Translation can be divided into three main stages: initiation, elongation, and termination. The primary site for translational regulation in most systems is the initiation step, and therefore key roles in translational control are played by components involved in initiation (Sonenberg and Hinnebusch, 2007). During initiation, the ribosomal 40S and 60S subunits join the mRNA in a series of steps mediated by eukaryotic initiation factors (eIFs). The small subunit forms the 43S preinitiation complex by recruiting the initiator transfer RNA (tRNA), bound to eIF2, a protein that is an important target of translational control. This complex then recruits mRNAs with a 5′ cap through the action of eIF4E, a cap-binding protein that is a key mediator of multiple pathways of translational control. After scanning to the initiator AUG codon, the small subunit is joined by the large subunit to form the monosome, and elongation can then proceed on polysomes.

In neurons, roles for protein translation have been identified in both dendrites and axons. Developing axons can translate protein, and roles for protein synthesis have been proposed in

axon growth, guidance, and regeneration. Within the axon, there can be further localization of newly synthesized proteins, preferentially within one segment along the length of an axon, or on one side of a growth cone, exposed locally to an extracellular cue (Campbell and Holt, 2001; Zhang et al., 2001; Brittis et al., 2002; Ming et al., 2002; Leung et al., 2006; Yao et al., 2006; Willis et al., 2007). In dendrites, protein synthesis is well established to have roles in synaptic plasticity, including learning and memory. Proteins can be synthesized locally within the dendrite in response to extracellular signals, and localization of translation at or near individual synapses may play a role in plasticity that is synapse specific (Steward and Schuman, 2003; Sutton and Schuman, 2006).

To explain how protein synthesis could occur preferentially in the subcellular region of a neuron receiving an extracellular signal, one model is that transmembrane receptors would operate through signaling pathways that act at a distance, influencing translation with a range extending over part of the cell. We set out here to test an alternative, although not mutually exclusive, model based on the precedent of transmembrane receptor association with other types of machinery inside the cell. In this model, transmembrane receptors that regulate protein synthesis would colocalize with translation machinery in a particle where they would be integrated in a molecular binding complex. To test this second model, we focused on DCC, a well characterized receptor in axon growth and guidance (Keino-Masu et al., 1996; Fazeli et al., 1997) and also found postsynaptically in dendrites (Parent et al., 2005). DCC is one of the receptors for the extracellular factor netrin (Keino-Masu et al., 1996; Fazeli et al., 1997), and netrin is known to promote protein synthesis in axons (Campbell and Holt, 2001).

The results here show a physical complex of DCC with components involved in translation initiation, including eIFs, ribosomal subunits, and monosomes. Furthermore, DCC functionally mediated translational regulation in response to its ligand netrin-1. These studies lead to a generalizable model with transmembrane association of cell surface receptors and the translation machinery contributing to the specificity, efficiency, and spatially precise control of translation, based on a transmembrane complex regulated by extracellular cues.

RESULTS

DCC Colocalizes with Translation Machinery

In initial experiments to explore a potential association of DCC and translation machinery, we tested for colocalization in spinal commissural axon growth cones, where DCC promotes growth and chemoattraction in response to netrin (Keino-Masu et al., 1996; Fazeli et al., 1997). In particular, colocalization would be of interest at filopodial tips, where DCC is known to be located, and which are highly motile structures that explore the environment and respond to extracellular cues (Drees and Gertler, 2008). Immunofluorescence localization showed punctate labeling of both DCC and ribosomal markers, and overlap was seen in a subset of puncta, with a diameter of approximately 0.2 to 1 μm (Figures 1A–1D and Figures S1A–S1C and S1H available online). Notably, colocalization was consistently seen at the tips of filopodia. Quantitation of the overlap within the growth

cone indicated approximately 35% of DCC puncta overlapping with ribosomal puncta and approximately 83% of ribosomal puncta overlapping with DCC puncta. When Pearson's correlation coefficient was calculated for individual DCC puncta that showed detectable ribosome labeling, in >90% of cases the value was between 0.5 and 0.95, confirming true colocalization of puncta rather than fortuitous overlap (see the Experimental Procedures).

Colocalization was also tested in hippocampal neuron dendrites. DCC puncta colocalized with markers of the translational machinery, including at synapses identified by triple labeling with the postsynaptic marker PSD-95 (Figures 1E–1H, Figures S1D–S1G, and data not shown). Approximately 35% of PSD-95 puncta overlapped with DCC puncta, while most but not all DCC puncta overlapped with markers of translational machinery; for example, 89% of DCC puncta overlapped with eIF4E puncta. These results appear to be broadly consistent with electron microscopic studies reporting that polysomes are present in approximately 10%–40% of synaptic spines (Ostroff et al., 2002; Steward and Schuman, 2003).

To complement the overall view of subcellular distribution provided by fluorescence imaging, electron microscopy was used for resolution on a molecular scale. Immunogold labeling showed colocalization of DCC and ribosome markers in axonal growth cones, axon shafts, near the tips of axon filopodia, and in dendritic synaptic spines (Figures 1I–1V). As expected for ultrathin sections (80 nm), labeling density was moderate, and some particles did not show colocalization (Figure S1I), also consistent with the fluorescence results showing colocalization of some but not all puncta. While the immunolocalization procedure does not allow detailed visualization of organelles, counterstaining of the cytoplasm showed that many of the colocalized particles were near the cell surface, while others were in intracellular locations where DCC may be associated with intracellular membranes, perhaps reflecting transport to or from the cell surface. It should be noted, however, that it is not possible here to accurately quantitate the proportion of surface versus intracellular location because of the sectioning geometry, or to determine the precise location of molecules relative to one another or the cell surface, since the gold particles are on a similar scale to the molecules and are tethered via antibodies that can span up to ~30 nm. Addressing colocalization, however, distances among clustered particles were generally 0–30 nm, well within the range for immunogold detection of a direct molecular interaction.

DCC Coprecipitates with Translation Machinery

We next tested whether DCC and translational machinery might show physical association. Initially, to provide an unbiased sampling of candidate interactors, we transfected full-length DCC or vector control into 293 cells, and then DCC was immunoprecipitated and coprecipitated proteins were identified by tandem mass spectrometry. As shown in Figure 2A, translational components were among the coprecipitated proteins, including translation initiation factors (polypeptides of eIFs 1, 2, 3, 4, and 5) and proteins of the ribosomal small subunit (S10, S13, and S24) and large subunit (L5, L10a, L13, L23, L28, and L38). Most of the proteins from this list were then tested by western blot of transfected 293 cells, confirming specific coprecipitation in
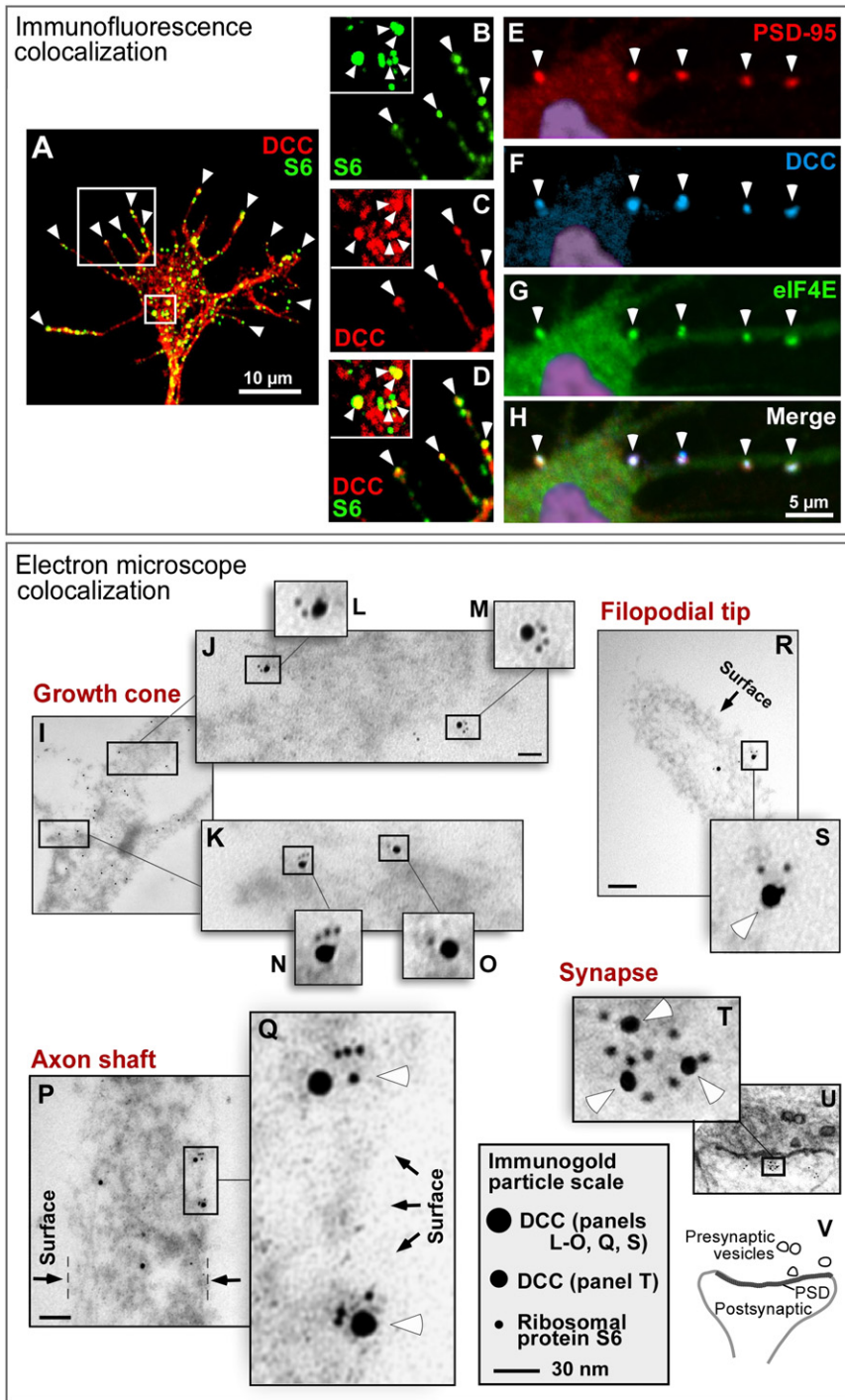
Figure 1. DCC Colocalizes with Translational Components

(A–H) Immunofluorescence.

(A–D) Cultured commissural axons. DCC (red) and ribosomal protein S6 (green) appeared in puncta within the growth cone, with a subset of puncta overlapping (yellow). (B)–(D) are enlargements of boxes from (A), showing filopodial tips, with a central growth cone area in the inset. Arrowheads, examples of colocalized puncta, notably at filopodial tips.

(E–H) Cultured hippocampal dendrites. Labeling for DCC (blue), eIF4E (green), and the postsynaptic marker PSD-95 was seen in puncta at synapses (arrowheads). Nuclear DAPI staining is shown in purple.

(I–V) Electron microscopy, showing double immunogold labeling for endogenous DCC (15 nm gold particles in I–S; 10 nm particles in T–V) and ribosomal protein S6 (5 nm particles). Some variation in particle size is caused by silver enhancement.

(I–S) Cultured commissural axons. (I)–(O) show distal part of growth cone near the base of the filopodia, (P) and (Q) show axon shaft, oriented vertically, and (R) shows filopodium, with distal end toward the upper left.

(T–V) Cultured hippocampal neuron synapse, diagrammed in (V). White arrowheads, examples of clustered particles; black arrows, outer boundary of the cell. Boxes show enlargement of clustered particles.

Scale bars represent 70 nm in (J), (R), and (P). See also Figure S1.

synthesized there, and indicating that these associations are mediated by the DCC cytoplasmic domain.

DCC Cosediments with the Ribosome

To investigate the interaction further, we performed sucrose gradient velocity sedimentation of ribosomes. DCC was found to cosediment with both small and large ribosomal subunits, as well as monosomes, while little or no obvious DCC signal was seen in the polysome fraction under these conditions (Figure 3A and Figure S3). In addition to 293 cells, a comparable distribution was obtained with native embryonic spinal cord (Figure 3B). On a per-subunit basis, the association of DCC with the large and small subunits appeared approximately comparable (Figure 3A). Sedimentation profiles of the key translational regulators eIF4E and eIF2α are also shown for comparison (Figure 3A), with eIF2α cosedimenting with subunits, monosomes and polysomes (Ramaiah et al., 1992), while eIF4E cosedimented primarily with the 40S fraction as in previous studies (Kedersha et al., 2002; Napoli et al., 2008). The lack of prominent association of DCC with polysomes provided further confirmation that the association

every case tested (Figure 2B and Figure S2). In addition, coprecipitation of selected proteins was confirmed with embryonic spinal cord, providing a native developmental context where the proteins are expressed at endogenous levels (Figure 3B). Importantly, however, no coprecipitation was seen with a DCC protein lacking the cytoplasmic domain (DCC-Δcyto; Figure 3C), indicating that DCC is not associated with the translation machinery merely because the DCC polypeptide is itself being
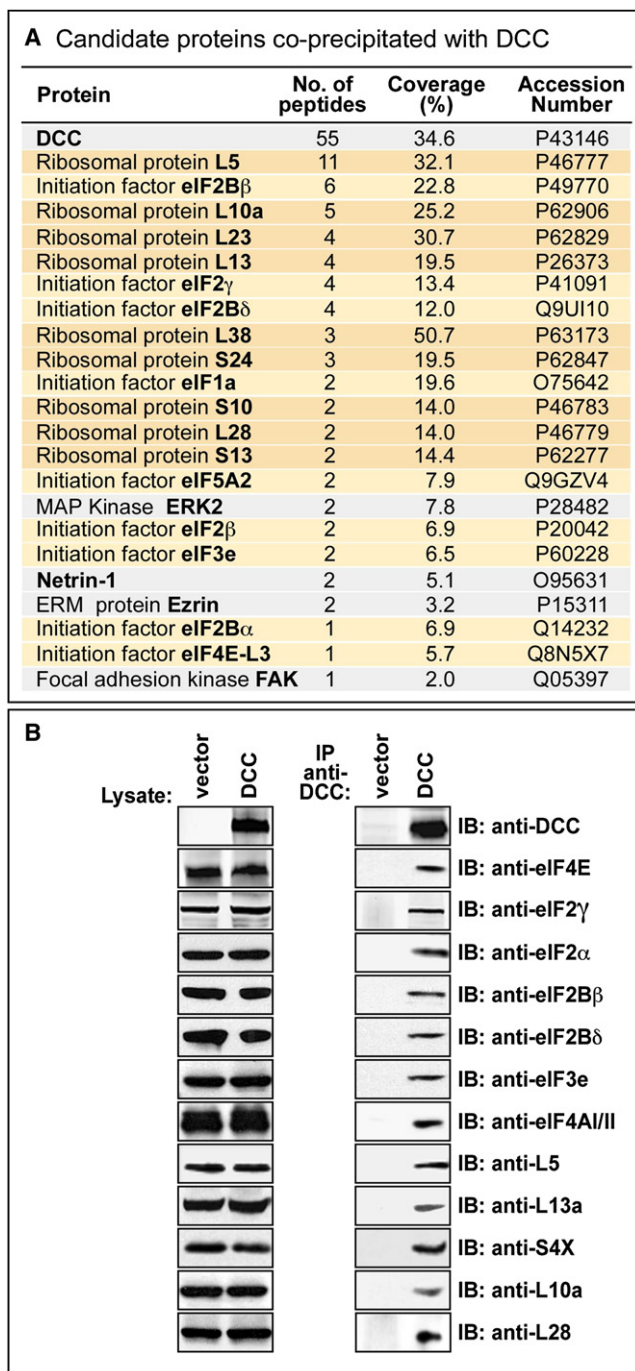
A Candidate proteins co-precipitated with DCC

Protein	No. of peptides	Coverage (%)	Accession Number
DCC	55	34.6	P43146
Ribosomal protein L5	11	32.1	P46777
Initiation factor eIF2Bβ	6	22.8	P49770
Ribosomal protein L10a	5	25.2	P62906
Ribosomal protein L23	4	30.7	P62829
Ribosomal protein L13	4	19.5	P26373
Initiation factor eIF2γ	4	13.4	P41091
Initiation factor eIF2Bδ	4	12.0	Q9UI10
Ribosomal protein L38	3	50.7	P63173
Ribosomal protein S24	3	19.5	P62847
Initiation factor eIF1a	2	19.6	O75642
Ribosomal protein S10	2	14.0	P46783
Ribosomal protein L28	2	14.0	P46779
Ribosomal protein S13	2	14.4	P62277
Initiation factor eIF5A2	2	7.9	Q9GZV4
MAP Kinase ERK2	2	7.8	P28482
Initiation factor eIF2β	2	6.9	P20042
Initiation factor eIF3e	2	6.5	P60228
Netrin-1	2	5.1	O95631
ERM protein Ezrin	2	3.2	P15311
Initiation factor eIF2Bα	1	6.9	Q14232
Initiation factor eIF4E-L3	1	5.7	Q8N5X7
Focal adhesion kinase FAK	1	2.0	Q05397

Figure 2. Tandem Mass Spectrometry Screen Identifying Translational Components Coprecipitated with DCC

(A) Tandem mass spectrometry identification of proteins after immunoprecipitation with anti-DCC antibody. Proteins listed showed specific coprecipitation from 293-net cells transfected with DCC versus vector control. Ribosomal proteins are highlighted in dark orange and initiation factors in light orange. Grey indicates proteins previously shown to interact directly with DCC (although other known interaction partners such as Nck, Src, and Fyn were not seen here, presumably reflecting detection limits in this screen). Accession numbers: UniProtKB/Swiss-Prot database.

with ribosomes is not simply attributable to the DCC polypeptide itself being translated there. The results, however, appeared to be very consistent with a role in translational regulation, since the initiation stage involves recruitment of 40S and 60S subunits to form the 80S ribosome, and is the major site of translational control in most systems.

DCC and Its Cytoplasmic Domain Functionally Affect Translation

Netrin-1 promotion of axon growth and chemoattraction can be mediated not only through DCC, but also two other receptors, neogenin and DSCAM. To assess whether DCC can mediate effects of netrin-1 on translation, we transfected DCC into 293 cells, which did not contain detectable levels of endogenous netrin receptors. Translation was assessed with a dual-luciferase reporter system for cap-dependent translation with a cap-independent cistron as internal control (Figure S4A) (Krüger et al., 2001). The results indeed showed a DCC-dependent effect of netrin-1 on translation in this system (Figure 4A and Figures S4B–S4F). Likewise, an increase in total cell protein synthesis was seen by radiolabeled amino acid incorporation (Figure S4G), although as in previous studies (for example, Pfeifer et al., 2008) the signal-to-background ratio was lower than for reporter translation. The effect of netrin-1 was also tested on the association of DCC with translational components, showing that it promoted dissociation (Figure 4B and Figure S4H). (Note, however, that an association was still detectable in 293-net cells, consistent with the results in Figure 2.) Finding that netrin promoted dissociation of DCC from translational machinery appeared to be very consistent with our earlier experiments which had shown an association of DCC with components involved in translation initiation, but not with polysomes (Figures 2 and 3). These results all appear to be consistent with a model in which full-length DCC associates with translation initiation machinery, and signaling by DCC within this complex promotes formation of actively elongating polysomes that are no longer associated with DCC.

Since our earlier results had indicated that the DCC cytoplasmic domain was required for association with translation machinery, we assessed the role of this domain in translation. The DCC-Δcyto mutant had no significant effect in mediating the effect of netrin-1, showing a requirement for the cytoplasmic domain in regulating translation (Figure 4A).

As an additional test, a truncated receptor was made containing the DCC cytoplasmic domain only (DCCcyto). Expression of a truncated cell surface receptor is a common strategy that can provide information about binding and functional properties of a specific receptor domain. Functional effects of truncated receptors are often interfering rather than activating, since they may retain the ability to bind and occupy some signaling components, while lacking other domains required for productive signaling. For DCCcyto in particular, functional effects on translation, if any, were expected to be inhibitory, since the DCC

(B) Most of the candidate translational components from the mass spectrometry screen were tested by western blot and in every case tested were confirmed to show coimmunoprecipitation with DCC.
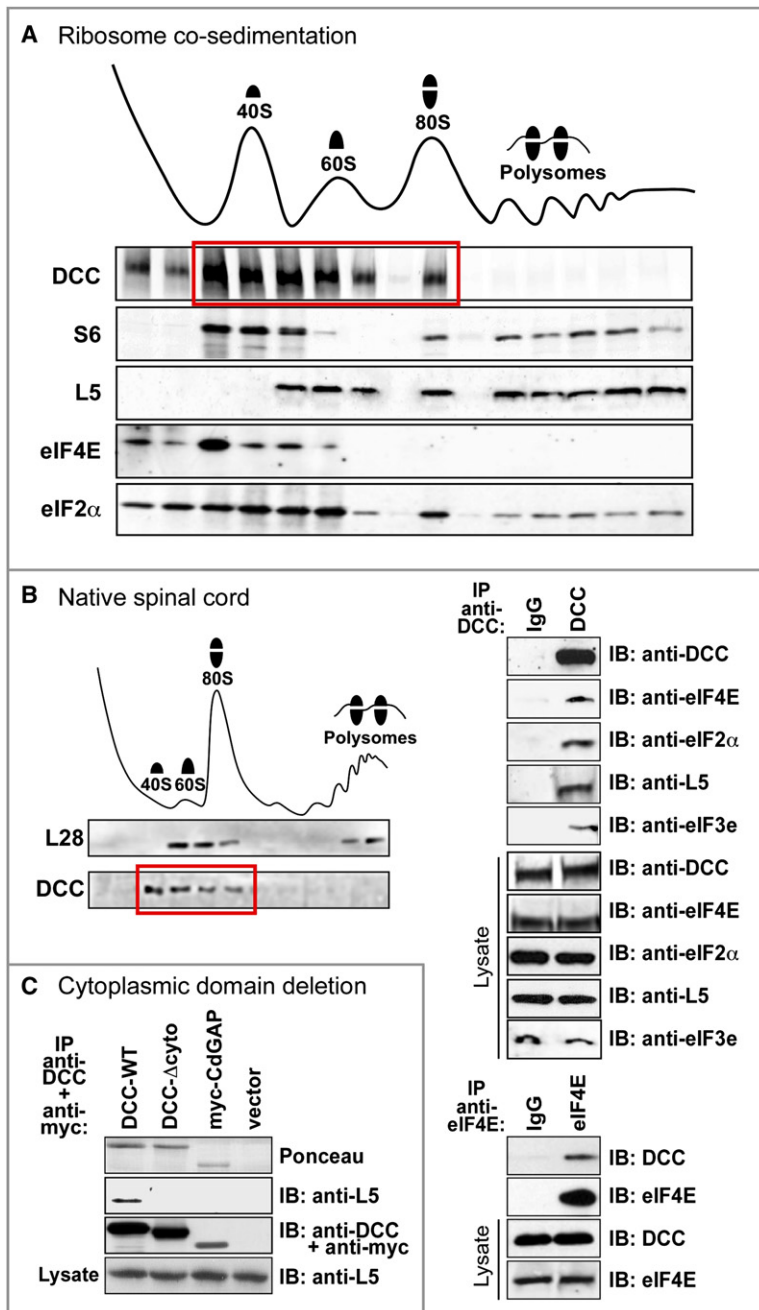See also Figure S2.

Figure 3. DCC Receptor Physically Associates with Translation Machinery

(A) Ribosome sedimentation from netrin-expressing 293-net cells transfected with full-length DCC. DCC cosedimented in 40S, 60S, and 80S fractions (red box) but not prominently in the polysome fraction. Control proteins were distributed as expected from previous studies: S6 in 40S, 80S, and polysomal fractions; L5 in 60S, 80S, and polysomal fractions; eIF4E primarily in the 40S fraction; and eIF2α in all four fractions.

(B) DCC association with translation machinery, with all proteins expressed at endogenous levels in developing spinal cord. Left: DCC cosedimented in 40S, 60S, and 80S fractions. Other markers such as eIF4E distributed as expected in spinal cord (not shown). Top right: translational components coimmunoprecipitated with DCC. Bottom right: DCC coimmunoprecipitated with eIF4E.

(C) DCC-Δcyto mutant lacking a cytoplasmic domain did not co-precipitate ribosomal marker L5. myc-tagged mCdGAP was a negative control.

See also Figure S3.

under these conditions. Cosedimentation of the DCC$_{cyto}$ polypeptide in these experiments was seen with ribosomal 40S, 60S, and 80S fractions (Figure 4D). Thus, DCC$_{cyto}$, like full-length DCC, associated physically with machinery involved in translation initiation, and produced functional effects on translation, although in the case of DCC$_{cyto}$ these effects were inhibitory not promoting, consistent with this mutant lacking an extracellular domain. These results provided further support for an overall model in which the DCC cytoplasmic domain interacts both physically and functionally with the translation initiation machinery.

Involvement of the DCC P1 Motif and Ribosomal Protein L5

The DCC intracellular domain contains three motifs, P1, P2, and P3, which are conserved from *C. elegans* to mammals, and are therefore likely to mediate downstream functions (Figure 5A) (Garbe and Bashaw, 2004; Round and Stein, 2007). When DCC mutants were tested in the reporter assay for cellular translation, P3 deletion caused some reduction of translation (DCC-ΔP3 mutant; Figure 5B). This is expected, since P3 enhances DCC dimerization, which is believed to mediate signal transduction across the membrane, and it also binds known signaling proteins (Round and Stein, 2007) that might in principle modulate translation. A more drastic effect was seen upon deletion of the P1 motif (DCC-ΔP1 mutant), which completely blocked the ability of DCC to promote translation (Figure 5B). Correspondingly, the P1 deletion reduced the association of DCC with translational components close to background levels (Figure S5A).

Among the translational components that coprecipitated with DCC in the mass spectrometry analysis, the most prominent
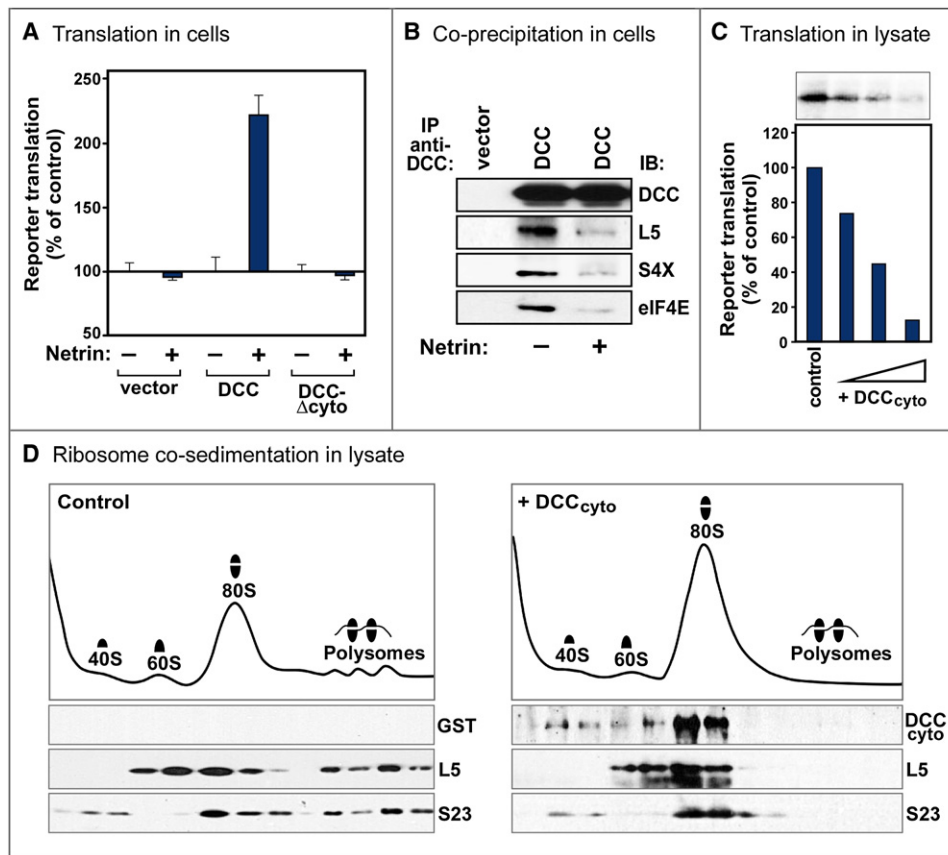
extracellular domain is required for downstream signaling (Round and Stein, 2007). When DCC$_{cyto}$ was tested by transfecting it into 293 cells, this caused cell death (data not shown). While this could be consistent with translation inhibition, it did not provide a good system to study translation further. When DCC$_{cyto}$ was instead tested in a cell-free system, the reticulocyte lysate, it was indeed found to inhibit translation, as indicated both by reduced reporter translation, and reduced polysome levels (Figures 4C and 4D). The reduction in polysomes was accompanied by an increase in the 80S fraction, indicating that a late step after 80S monosome formation became rate limiting

Figure 4. Effects of DCC and Netrin on Translation

(A) Full-length DCC mediated netrin promotion of translation. 293 cells were transfected with DCC constructs, together with a reporter plasmid for cap-dependent translation (Figure S4A). Reporter translation was normalized to a cap-independent internal control and is compared in netrin-expressing 293-net cells (+) versus 100% in control cells (–). DCC lacking a cytoplasmic domain (DCC-Δcyto) showed no effect on translation. n = 3 experiments; error bars represent the standard error of the mean (SEM). Without netrin, full-length DCC promoted translation slightly in some experiments, although to a much lesser degree than with netrin (data not shown).

(B) Netrin reduced the association of DCC with translation components. Western analysis showed reduced coprecipitation of markers of the ribosomal large subunit (L5), small subunit (S4X), and initiation factor eIF4E with exogenous DCC in netrin-expressing 293-net cells (+) compared with control 293 cells (–). An association above background remained, however, in the presence of netrin (see Figure S4H).

(C) DCC cytoplasmic domain (DCC_cyto) inhibited translation. Purified recombinant DCC_cyto (100 ng, 200 ng, and 500 ng) or GST control protein was added to a rabbit reticulocyte lysate translation system.

(D) Ribosome sedimentation profiles of reticulocyte lysates after incubation with purified recombinant DCC_cyto (right) or GST control (left). DCC_cyto cosedimented with 40S, 60S, and 80S fractions. DCC_cyto decreased the polysome fraction and increased the 80S fraction, implying that a step after 80S ribosome assembly was inhibited and became rate limiting in this assay, although earlier steps may also be affected. Ribosomal proteins distributed as expected: S23 in 40S, 80S, and polysome fractions; and L5 in 60S, 80S, and polysome fractions.

See also Figure S4.

was ribosomal protein L5, with 11 peptides (Figure 2A). L5 also appeared to be interesting because it is thought to be located at or near the ribosomal P site, and L5 mutations can affect peptidyl-tRNA binding (Fabijanski and Pellegrini, 1981; Meskauskas and Dinman, 2001; Spahn et al., 2001; Takahashi et al., 2005). Purified recombinant L5 was therefore tested for binding to purified recombinant DCC cytoplasmic domain, and the results showed direct binding (Figure 5C). While this direct association of DCC with a ribosomal protein is striking, it should be emphasized that this is unlikely to be the only interaction of DCC relevant to translation (see the Discussion).

A common strategy to test the biological function of protein associations is to mutate the interaction site. We were therefore interested to test whether the association of DCC with L5 could be mapped to a specific site within the DCC primary sequence. When nested deletions were examined by removal of successively larger regions from the C terminus (Figure 5A), a region containing the P1 motif was found to be sufficient for L5 binding (Figure 5E). The P1 motif was found also to be necessary for L5 binding, as shown by testing the DCC-ΔP1 mutant (Figure 5D). It may be worth noting that although the P1 motif can also interact with coreceptors in the UNC5 family, they mediate repellent
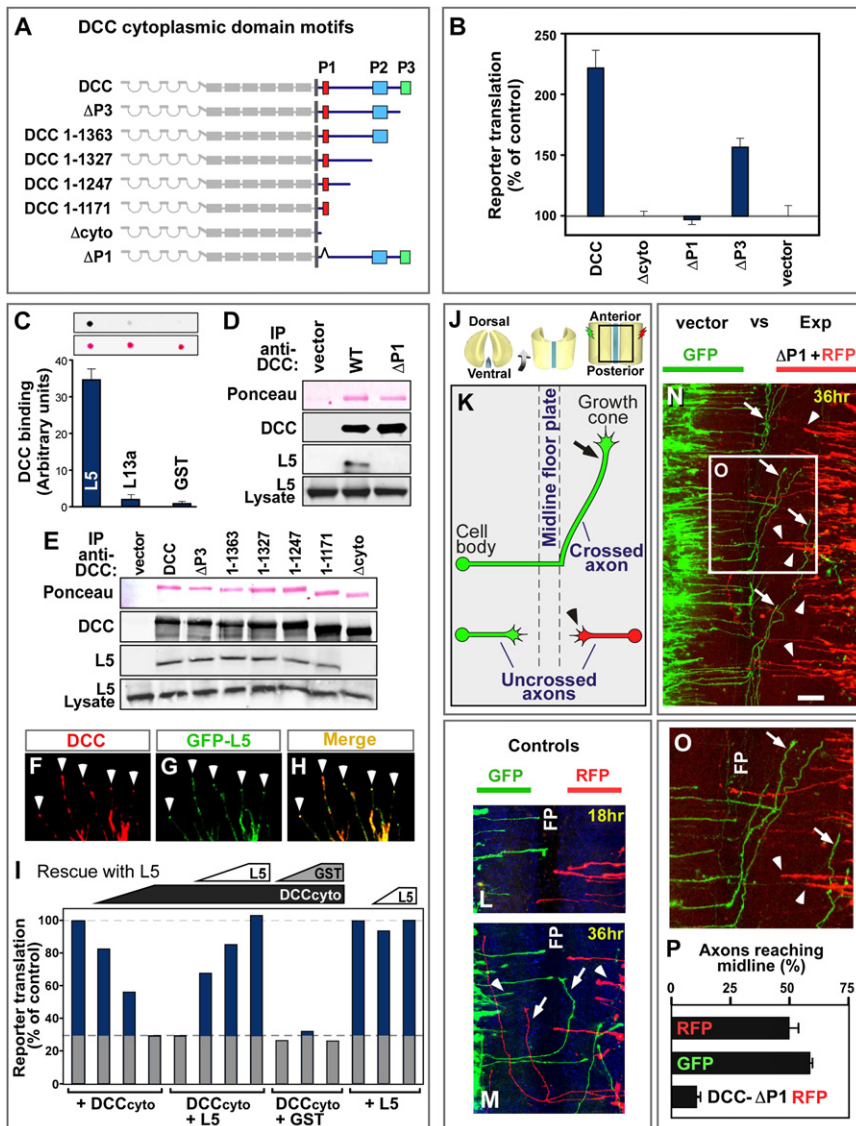
Figure 5. Involvement of the DCC P1 Domain and Ribosomal Protein L5

(A) DCC cytoplasmic domain contains three conserved motifs: P1, P2, and P3. A nested series of deletions were made, and an internal deletion of the P1 motif.

(B) DCC promotion of translation was prevented by P1 deletion (DCC-ΔP1), and partially reduced by P3 deletion (DCC-ΔP3). The assay was as in Figure 4A, with cap-dependent reporter translation normalized to a cap-independent control. n = 3 experiments; error bars represent the SEM.

(C–I) Physical and functional interactions with ribosomal protein L5.

(C) Far-western dot blot quantitation of direct binding of purified recombinant DCC cytoplasmic domain to purified recombinant L5. Ribosomal protein L13a and GST provide controls. Ponceau stain shows similar loading. n = 4 experiments; error bars represent the SEM.

(D and E) Western analysis of endogenous L5 coimmunoprecipitation with DCC, showing L5 associated with all mutants in the nested deletion series except DCC-Δcyto, and not with DCC-ΔP1.

(F–H) Immunofluorescence of DCC (red) and GFP-L5 (green) cotransfected into 293 cells, showing colocalization in neurite-like extensions, notably at the tips (arrowheads).

(I) Functional interaction between ribosomal protein L5 and DCC_cyto. The inhibitory effect of DCC_cyto in a reticulocyte lysate (see Figure 4C) was rescued in a dose-dependent manner by recombinant L5 (100 ng, 200 ng, and 500 ng). GST control at the same concentrations did not rescue. L5 alone (200 ng and 500 ng) had no noticeable effect. Column 5 is a duplicate of column 4 to clarify control value.

(J–P) Effect of DCC-ΔP1 as a dominant inhibitory mutant on commissural axon pathfinding in developing spinal cord. Chick spinal cords at E3.5 (HH 23) were electroporated and analyzed 36 hr later, compared with controls electroporated on the contralateral side of the same spinal cord.

(J) Illustration of spinal cord open book preparations, with imaged area in box.

(K) Diagram of axon trajectories seen in (N): axons grow toward the midline floor plate (arrowheads), cross it, then turn and grow anteriorly (arrow).

(L and M) Control plasmids expressing GFP and RFP; equivalent axon numbers, growth rates, and patterns were seen for both tracers after 18 or 36 hr.

(N and O) Axons electroporated with DCC-ΔP1 (right side, red) compared to GFP control (left side, green) showed greater numbers remaining at locations midway toward the floorplate (red; examples indicated by arrowheads), and fewer had reached or crossed the floor plate compared with control (green; examples indicated by arrows). (O) shows an enlargement of the boxed area.

(P) Quantitation of axons reaching the midline after electroporation with control or DCC-ΔP1 mutant construct. A total of 530 axons were analyzed in seven experiments; error bars represent the SEM.

The scale bar in (N) represents 100 μm. FP, floor plate. See also Figure S5.

actions of netrin, whereas commissural neurons are attracted, and they are not expressed in commissural neurons or the cell lines used in this study (Round and Stein, 2007) (unpublished data). The experiments here show that the P1 motif, which we had already found to be required to promote translation, was also the sequence necessary and sufficient for association of DCC with ribosomal protein L5.

DCC and L5 were also examined for overlapping subcellular expression. Since none of the L5 antibodies tested gave a signal in immunocytochemistry, a GFP-L5 fusion protein was coexpressed with DCC in 293 cells, where DCC promotes neurite-like extensions. Colocalization was seen in the neurite-like extensions, notably at their tips (Figures 5F–5H). Similarly, GFP-L5 expressed in commissural neurons colocalized with DCC at filopodial tips in axon growth cones (Figure S5B).

Having found that the P1 motif of DCC is required both for regulation of translation and for association with L5, we addressed the other side of the interaction by testing whether

perturbations of L5 might affect actions of the DCC cytoplasmic domain. Disruption of L5 within cells is problematic because of the key roles of L5 in ribosome biogenesis and function. Therefore, we used the cell-free reticulocyte system that we had already shown to be inhibited by DCC$_{cyto}$. A functional interaction between L5 and DCC$_{cyto}$ was tested by the logic of dominant interference: if L5 provides a bridge between DCC and the ribosome, then addition of sufficient L5 should functionally occupy both its binding partners and therefore block the effects of the DCC cytoplasmic domain on translation. Accordingly, L5 addition was found to cause a dose-dependent and highly effective reversal of the DCC$_{cyto}$ effect on translation (Figure 5I). These experiments demonstrate a functional interaction between the DCC cytoplasmic tail and L5 in translation.

As a further test of our models, the effect of the P1 motif was tested on commissural axon behavior. Previous studies have implicated the P1 motif in axon attraction and growth promotion in *C. elegans* (Gitai et al., 2003; Garbe and Bashaw, 2004) and in UNC-5H dependent axon repulsion (Garbe and Bashaw, 2004; Round and Stein, 2007) and also have shown a role for the P3 motif in commissural axon guidance (Finger et al., 2002). Here, to assess P1 functionally in commissural axons, we used DCC-ΔP1 as a dominant interfering construct. The rationale for this type of dominant interfering experiment is that a mutant receptor can block the actions of endogenous wild-type receptor by heterodimerization or by binding unproductively to upstream or downstream components. The DCC-ΔP1 construct was confirmed to have an interfering effect on translation and to be expressed in neuronal growth cones (Figures S5C–S5E). To test commissural axon behavior, we electroporated DCC into chick spinal cord open book explants, using constructs that also expressed a fluorescent protein reporter, and 36 hr later axon trajectory was traced (Figures 5J–5P). Previous work has shown that DCC promotes axon growth and chemoattraction in response to floor plate derived netrin: after *DCC* gene disruption, axons initially grow in a ventral direction, but many fail to reach the floor plate (Fazeli et al., 1997). In control experiments here, full-length DCC, or the use of GFP versus RFP markers, did not impair the ability of axons to reach the floor plate (Figures 5L, 5M, and 5P and data not shown). When DCC-ΔP1 was introduced, however, axons grew out in a ventral direction, but a greatly reduced proportion reached the midline (p < 0.0001; Figures 5N–5P), producing a phenotype comparable to that of *DCC* mutant mice (Fazeli et al., 1997). While the P1 motif might in principle affect other downstream processes in addition to translation, these results in spinal cord appear to be consistent with all of our other data and indicate that P1, a short motif that mediated translational regulation and was necessary and sufficient for associations with ribosomal components also affected DCC function in promoting outgrowth or attraction of spinal commissural axons toward the midline.

Newly Synthesized Protein Colocalizes with DCC

The finding that DCC colocalizes and physically associates with the translation machinery and regulates translation implied that DCC should be capable of regulating protein synthesis in a localized manner within the cell. To investigate this, we used a tracer of newly synthesized protein, azidohomoalanine (AHA), which acts as an amino acid analog incorporated in place of methionine and can be visualized after cell fixation by covalent cycloaddition with a tagged alkyne (Dieterich et al., 2007; Beatty and Tirrell, 2008). AHA labeling was found to overlap with DCC in axons of commissural neurons, notably in puncta at the tips of filopodia (Figures 6A and 6B and Figures S6A and S6B). We also used an independent tracer of newly synthesized protein that operates by a different mechanism, Cy3-puromycin, a fluorescently tagged derivative of the aminoacyl-tRNA analog puromycin (Smith et al., 2005), and obtained comparable results (Figures S6C–S6I). In hippocampal neurons, too, AHA showed punctate labeling that overlapped with DCC puncta (Figure 6C). When the effect of netrin was tested, DCC puncta in commissural axon filopodia showed increased labeling of newly synthesized protein (Figure 6D and Figure S6G). Thus, the accumulation of newly synthesized protein within DCC microdomains was increased by netrin transmembrane signaling.

DISCUSSION

Physical association of cellular components plays a central role in establishing both the spatial organization of the cell and the specificity of cellular pathways. Cell surface receptors and the translation machinery are two of the basic classes of cellular components. The results here lead to a generalizable model in which a transmembrane receptor interacts both extracellularly with signaling cues and intracellularly with the translation machinery to form a transmembrane translation regulation complex.

Interactions of DCC with Protein Synthesis Machinery

The results here showed that DCC associated with eIFs, ribosomal 40S and 60S subunits, and 80S ribosomes, though not prominently with polysomes. Taken together with finding that netrin-1 promoted translation in a DCC dependent manner and reduced the association of DCC with translation components, these results lead to the following model. DCC forms a complex with translation initiation machinery. Signaling by DCC within this complex is enhanced by netrin and promotes the formation of actively elongating polysomes that are no longer associated with DCC (Figure 7A). Signaling by DCC is believed to be mediated by dimerization or higher-order clustering, and, in the absence of netrin, spontaneous dimerization and signaling by DCC may promote some translation, although at a lower level than with netrin. These interactions with the translation machinery are mediated via the DCC cytoplasmic domain, which was found to be necessary and sufficient for interactions with translational components (Figures 7B and 7C).

The association of DCC with translation initiation machinery, but not prominently with the polysome fraction, is reminiscent of the key translational regulator eIF4E, which cosedimented mainly with the ribosomal small subunit and not prominently with polysomes, as confirmed under the conditions here and seen in previous results (Kedersha et al., 2002; Napoli et al., 2008). Why DCC, or eIF4E, would not show more prominent association with the polysome fraction is an interesting question, since polysomes undergo repeated rounds of initiation as
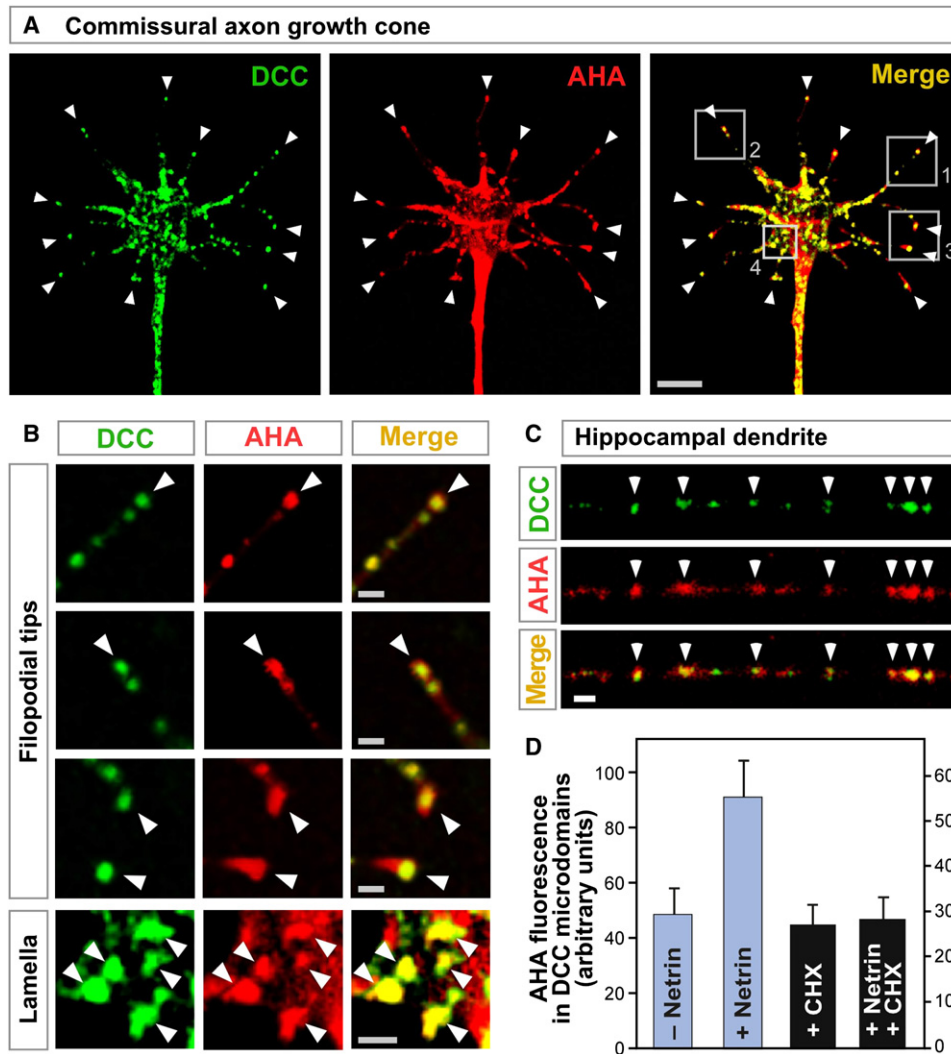
Figure 6. DCC Colocalizes with Newly Synthesized Protein

(A and B) Localization of DCC (green) and AHA-labeled sites of newly synthesized protein (red) in cultured commissural axon growth cone. (B) shows enlargement of boxes in (A). Arrowheads show examples of overlapping puncta (yellow) notably at filopodial tips.

(C) DCC (green) and AHA (red) labeling in cultured hippocampal neuron dendrite. Arrowheads indicate overlap.

(D) Quantitation of AHA labeling in DCC puncta in filopodia. AHA was added for 20 min, then cycloheximide (CHX) for 20 min, then 200 ng/ml netrin-1 for 10 min. Netrin induced an increase in labeling that was blocked by cycloheximide. n = 60 DCC puncta per column; error bars represent the SEM; p < 0.02 for effect of netrin without cycloheximide.

(A)–(C) were in the presence of netrin-1. Scale bars represent 5 μm in (A) and 1 μm in (B) and (C). See also Figure S6.

successive ribosomes are added. One potential explanation is suggested by observations that the initial recruitment of translation machinery to an mRNA involves formation of specialized ribonucleoprotein structures where the mRNA is looped to place the 5′ cap next to the 3′ polyA tail, whereas subsequently added ribosomes join an mRNA loop that is already undergoing active translation and may no longer require all the same components (Amrani et al., 2008). Although eIF4E and DCC shared the property of promoting translation yet not cosedimenting prominently with polysomes, they are very different in other respects. Whereas eIF4E initially recruits mRNA to the small subunit, we found that the truncated DCC$_{cyto}$ fragment affected a late stage

after 80S ribosome assembly. While it is possible that this late step is the primary site of regulation by full-length DCC, additional stages of translation initiation are likely to be regulated by full-length DCC, as discussed further below.

Regarding protein interactions linking DCC to translational control, our studies implicated the conserved cytoplasmic DCC P1 motif and ribosomal protein L5. Previous studies of eukaryotic L5 indicate that it is located at or near the ribosomal P site and showed that mutations in L5 can influence binding of the peptidyl-tRNA to the ribosome (Fabijanski and Pellegrini, 1981; Meskauskas and Dinman, 2001; Spahn et al., 2001; Takahashi et al., 2005). Therefore, one model could be that effects of
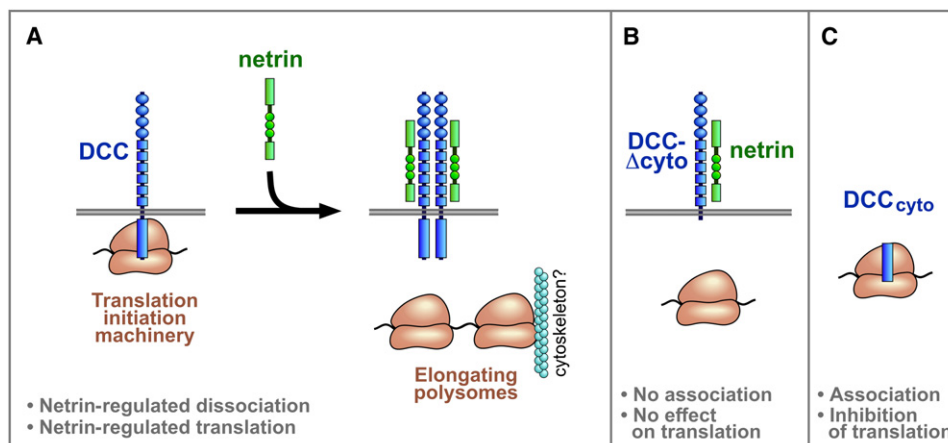
Figure 7. Models for Interaction of DCC with Translational Machinery

(A) In the model illustrated, full-length DCC associates with translation initiation machinery, including 40S, 60S, and 80S ribosomes and eIFs. Signaling by DCC within this complex is enhanced by netrin and promotes formation of actively elongating polysomes that are no longer associated with DCC. Polysomes may nevertheless tend to remain locally within micron scale structures such as a filopodial tip or synaptic spine, and this may be facilitated by their known interaction with the cytoskeleton. Transduction of a signal across the membrane by DCC and other receptors is believed to be mediated by dimerization or higher-order clustering. In the absence of netrin, spontaneous dimerization and signaling by DCC may promote some translation, although at a lower level than with netrin. Signaling by some DCC molecules in the cell would not preclude the coexistence of other DCC molecules that remain in a complex with translation initiation machinery. In addition to DCC and translation initiation machinery, the complex is proposed also to include signal transduction proteins, and DCC may regulate more than one step of initiation.

(B) No prominent association with translation components or functional effect on translation was seen with DCC mutants lacking the cytoplasmic domain (DCC-Δcyto) or lacking the 20 amino acid P1 motif (DCC-ΔP1), showing a requirement of these regions for physical and functional interactions with translation machinery.

(C) The DCC cytoplasmic domain by itself, DCC_{cyto}, cosedimented with 40S, 60S, and 80S ribosomes, and inhibited protein synthesis in a cell-free system, showing that this region is sufficient for physical and functional interactions with translational machinery. DCC_{cyto} is proposed to act here as an interfering mutant, occupying downstream components but lacking an extracellular domain necessary for positive signaling.

Although the diagrams are not to a precise scale, receptor length and ribosome diameter are approximately in proportion: the eukaryotic ribosome is ~20–30 nm across (Spahn et al., 2001), each Ig domain (oval) and FNIII domain (small square) is ~4 nm long, and the intracellular domain of DCC (rectangle) is approximately 25% of the protein sequence, as illustrated here in proportion with the extracellular length to account for potential folding, or in more extended conformation it could be up to 100 nm long.

DCC on translation might be mediated via structural changes in L5. An alternative model would be that L5 serves more passively as a tether helping link DCC to the ribosome, while translational regulation would be mediated via other DCC interactors. The second of these models may be favored by our finding that DCC preferentially affected cap-dependent rather than cap-independent translation, since functional effects transmitted by a general translation component such as L5 might be expected to influence both types of translation equally. In either model, the location of L5 is notable because it could provide a mechanism to position DCC, together with other associated proteins, near the ribosomal P site, where the ribosomal large and small subunits, the initiator tRNA, and the initiator AUG codon all come together during translation initiation.

Another protein that may be involved in linking DCC to translation is Nck-1, which has been found to bind directly to the DCC cytoplasmic domain (Li et al., 2002). In a separate study, Nck-1 was shown to bind directly to eIF2β, and to regulate translation (Kebache et al., 2002). Since eIF2β is part of the 43S preinitiation complex, Nck-1 therefore has the necessary binding specificities to act as a bridge between DCC and the ribosomal small subunit. Thus, together, L5 and Nck-1 can provide a model for DCC to be linked to both the ribosomal large and small subunits.

DCC associates with several other signaling proteins, some of which have been implicated in translational control in other systems (Garbe and Bashaw, 2004; Round and Stein, 2007). Also, eIF4E and its upstream regulator 4E-BP1 are thought to mediate netrin signaling (Campbell and Holt, 2001; Leung et al., 2006), and we find here that eIF4E associates in a complex with DCC. We therefore favor an overall model in which DCC would act as a platform for the assembly of a large multicomponent complex, containing not only ribosomes and eIFs but also multiple signal transduction components, providing more than one pathway structurally and functionally linking DCC to translational regulation within an integrated molecular apparatus.

In relation to the lack of prominent DCC association with polysomes, it is interesting that ribosomes have long been known to associate with the cytoskeleton, and, in contrast to DCC, this cytoskeletal association has been reported to involve few or no monosomes or subunits, but most or all polysomes (Lenk et al., 1977; Hesketh and Pryme, 1991). This suggests a model where DCC forms a complex with translation initiation components, whereas actively translating polysomes would instead become associated with the cytoskeleton. Cytoskeletal attachment of polysomes could provide mechanisms either for directed movement of translating polysomes or to retain them within specific subcellular sites close to the receptor (Figure 7A).

The identification of a complex of a transmembrane receptor with translation machinery opens up many possibilities for functions of this complex in ribonucleoprotein transport, localization, metabolism, and regulation. In this regard, it would be interesting to know whether the DCC-containing particles identified here have any relationship with previously described ribonucleoprotein particles (Kiebler and Bassell, 2006) or mRNA binding proteins (St Johnston, 2005; Kiebler and Bassell, 2006; Lin and Holt, 2008; Rodriguez et al., 2008). Also, while DCC may regulate general translation of all mRNAs that are present locally, an appealing model is that its association with translation components may provide a mechanism to preferentially localize and regulate a specific subset of mRNAs.

Translation in Axons and Dendrites

Our observations on the association of DCC with translational machinery in neurons appear consistent with previous work showing functional roles for local protein translation in chemoattractant responses to netrin (Campbell and Holt, 2001; Ming et al., 2002). Meanwhile, guidance receptors also interact directly with the cytoskeleton, and, accordingly, translation inhibitors do not block all axon morphological responses, as reported for example by Roche et al. (2009) for cues other than netrin, although their study like others confirmed an involvement of translation in chemoattraction. The association of DCC with sites of protein synthesis within micron scale subcellular structures such as the filopodial tip and synaptic spine, as seen here, can provide a mechanism to help regulate translation with spatial precision in processes such as axon guidance and synaptic plasticity.

Other Receptors

While the study here focuses on DCC, the model of receptor association with translation machinery may be more broadly applicable. Many imaging studies have found ribosomes, or newly synthesized protein, in the same vicinity as cell surface receptors, for example in fibroblast lamellipodia, near synapses or other sites of cell-cell contact, and under the surface membrane of axons (Koenig and Martin, 1996; Steward and Schuman, 2003; Rodriguez et al., 2006; Sutton and Schuman, 2006). Although it is not clear whether this localization places ribosomes in direct association with the membrane, it is worth noting that the electron microscopic approaches that are generally used identify polysomes but not subunits or monosomes (Sutton and Schuman, 2006), whereas we found here an association of DCC with subunits and monosomes. Other potentially consistent observations include the presence of a Wnt receptor in particles that sediment with approximately the velocity of a ribosome (Bilic et al., 2007) and the association of focal adhesion proteins with initiation factors and ribosomal proteins (de Hoog et al., 2004). We find that a subset of receptors other than DCC show physical and functional interactions with the translation machinery (unpublished data). While specific molecular mechanisms are likely to vary with different receptors, the concept of a transmembrane translation regulation particle, where cell surface receptors form a physical complex with protein synthesis machinery and regulate translation, could be widespread in cell biology.

General Conclusions

Regulated protein synthesis is implicated in a wide variety of cellular functions controlled by extracellular cues in both normal biology and pathology (St Johnston, 2005; Tee and Blenis, 2005; Sutton and Schuman, 2006; Lécuyer et al., 2007; Lin and Holt, 2008; Rodriguez et al., 2008). Linking a cell surface receptor in a complex together with the translation machinery could have several potential advantages. One may be to enhance the efficiency or speed of regulation by bringing components together in an integrated molecular machine. Another is the potential for each receptor to associate with a unique repertoire of mRNAs, providing an additional layer of specificity in biological regulation. A third advantage may be to allow spatial precision of translation, which may be crucial for cellular functions such as motility, adhesion, guidance, and synaptic plasticity. Association of DCC or other cell surface receptors with translation machinery in a transmembrane translation regulation complex could contribute to varied developmental, physiological, and pathological processes.

EXPERIMENTAL PROCEDURES

Primary Neuron Cultures

Commissural neuron cultures were prepared from E10.5–E11.5 mouse spinal cords by trituration and plated on polylysine; >95% of cells were positive for commissural markers TAG-1 and DCC. Hippocampal neuron cultures from E18–E19 rat brain were cultured for 3 weeks on laminin polylysine.

Coprecipitation, Western Analysis, and Tandem Mass Spectrometry

Cell lysates were prepared in Triton X-100 buffer. For spinal cord lysates, approximately 15 mouse E11.5 cords were homogenized. Immunoprecipitation used protein G-Sepharose beads washed with RIPA buffer. Far-western binding analysis used affinity-purified recombinant glutathione S-transferase (GST) fusion proteins probed with recombinant purified DCC cytoplasmic domain, followed by anti-DCC antibody and detection by chemiluminescence. For tandem mass spectrometry, after gel purification and trypsin digestion, peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). All major peaks in the spectrum were assigned to predicted peptide ions, peptide representation of 138 proteins was present in immunoprecipitates from cells with DCC and not from control cells, and peptides for proteins with two or fewer peptide hits were individually analyzed to confirm tryptic peptide identity. The entire immunoprecipitation and mass spectrometry analysis was performed twice; L5 and other ribosomal proteins and initiation factors were identified in both experimental series, and aggregate peptide counts are shown in Figure 2A.

Sucrose Gradient Velocity Sedimentation of Ribosomes

293-net cells transiently transfected with DCC were treated with cycloheximide, and lysed in 1% Triton X-100. For each spinal cord gradient, 12 cords were homogenized in low-salt buffer with digitonin. Lysates were precleared, centrifuged on a sucrose velocity gradient, and eluted on a fractionator monitored by 254 nm ultraviolet (UV).

Functional Assays of Translation and Newly Synthesized Protein

Cellular cap-dependent translation assays used bicistronic reporter plasmid pRL-5′-IRES-FL (Krüger et al., 2001), which directs cap-dependent translation of Renilla luciferase (RL) and also cap-independent IRES-mediated translation of firefly luciferase (FL), which was used as an internal control. In vitro translation experiments used Retic Lysate IVT Kit (Ambion), with Xef-1 RNA template, adding purified recombinant GST-DCC$_{cyto}$, GST-L5 or GST control. Translated Xef-1 protein was resolved on SDS-PAGE, transferred to nitrocellulose, and quantitated by phosphoimager. For AHA labeling, cells were incubated in methionine-free medium without serum containing 100 µM AHA for the times

indicated and then fixed and permeabilized with Triton X-100 and incubated in Click-iT tetramethylrhodamine (Invitrogen).

Image Analysis

For electron microscopy, cultured neurons were permeabilized in saponin or Triton X-100, glutaraldehyde postfixed, embedded in resin, and 80 nm sectioned. Nonspecific distribution of particles in areas devoid of cells was negligible, and controls showed no gold particle clustering. Fluorescence imaging used an inverted laser confocal at 0.25–2.0 μm per Z series slice. All fluorescence quantitation used original unprocessed image data, with no pixels at zero intensity or saturated. In panels displayed in the figures, for consistent visibility across the intensity range, contrast and brightness were adjusted uniformly within each experimental series. To confirm that individual yellow puncta reflected true colocalization, rather than fortuitous overlap, regions of interest (ROIs) for individual puncta were selected by automatic thresholding in one channel, and then the Pearson's correlation coefficient (r) was measured for the two channels. This tests all pixels within an ROI for correlation over the full range of intensity values: chance overlap corresponds to r = 0; identical patterns produce r = 1, which would suggest an artifact; and colocalization of two molecules in a common structure should produce an intermediate value. For the growth cone as a whole, r values up to 0.8 were obtained. As a control, a circular ROI was selected containing a central part of the growth cone, and one channel was gradually rotated; although the density of yellow pixels remained similar, the r value for correlation of intensities was confirmed to fall to approximately zero. For quantification of numbers of overlapping puncta, ROIs were selected in each channel independently by automatic thresholding to select 0.2–1 μm puncta, and the number of puncta that overlapped was then counted. For quantification of AHA intensity, each ROI for quantitation was selected automatically using the green channel, with a constant threshold above background, to select 0.2–1 μm puncta from twenty different filopodia in twenty different growth cones, chosen at random and blind to the red channel. Mean pixel intensity was then measured within each ROI in the red channel.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2010.04.008.

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Update

Cell

Correction

Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation

Joseph Tcherkezian, Perry A. Brittis,* Franziska Thomas, Philippe P. Roux, and John G. Flanagan*
*Correspondence: perry_brittis@hms.harvard.edu (P.A.B.), flanagan@hms.harvard.edu (J.G.F.)
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Our paper reported that cell surface receptor DCC associates with translation machinery and regulates protein synthesis. During the preparation of Figure 2B and related Figure S2 we inadvertently introduced two duplications. In Figure 2B, the eIF2Bδ IP immunoblot was duplicated and superimposed over the eIF3e IP immunoblot, and in Figure S2 the eIF4E lysate immunoblot was duplicated in place of the S23 lysate immunoblot. Corrected versions are below derived from original experimental data. The error does not affect any results or conclusions described in the paper. We apologize for any confusion this error may have caused.
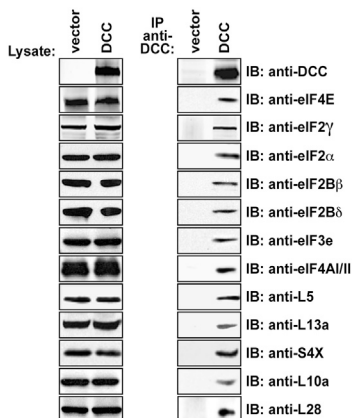

Figure 2. Tandem Mass Spectrometry Screen Identifying Translational Components Coprecipitated with DCC (corrected)
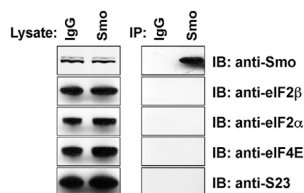

Figure S2. Negative Control Showing that Translational Components Did Not Co-precipitate with All Cell Surface Proteins (corrected)