

The Intracellular Domain of the Frazzled/DCC Receptor Is a Transcription Factor Required for Commissural Axon Guidance

Highlights

- γ -secretase cleaves Fra to release an ICD that shuttles between nucleus and cytoplasm
- The Fra ICD is sufficient to regulate *comm* expression and midline axon crossing
- The P3 motif in the Fra ICD encodes a transcriptional activation domain
- Fra's transcriptional activation function is required to regulate *comm* expression

Authors

Alexandra Neuhaus-Follini, Greg J. Bashaw

Correspondence

gbashaw@mail.med.upenn.edu

In Brief

Neuhaus-Follini and Bashaw demonstrate that the intracellular domain of the Frazzled/DCC receptor functions as a transcriptional activator to regulate midline axon guidance. They propose that signaling from the nucleus may be a common output of axon guidance receptors.



The Intracellular Domain of the Frazzled/DCC Receptor Is a Transcription Factor Required for Commissural Axon Guidance

Alexandra Neuhaus-Follini¹ and Greg J. Bashaw^{1,*}

¹Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

*Correspondence: gbashaw@mail.med.upenn.edu

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SUMMARY

In commissural neurons of *Drosophila*, the conserved Frazzled (Fra)/Deleted in Colorectal Cancer (DCC) receptor promotes midline axon crossing by signaling locally in response to Netrin and by inducing transcription of *commis sureless* (*comm*), an antagonist of Slit-Roundabout midline repulsion, through an unknown mechanism. Here, we show that Fra is cleaved to release its intracellular domain (ICD), which shuttles between the cytoplasm and the nucleus, where it functions as a transcriptional activator. Rescue and gain-of-function experiments demonstrate that the Fra ICD is sufficient to regulate *comm* expression and that both γ -secretase proteolysis of Fra and Fra's function as a transcriptional activator are required for its ability to regulate *comm* in vivo. Our data uncover an unexpected role for the Fra ICD as a transcription factor whose activity regulates the responsiveness of commissural axons at the midline and raise the possibility that nuclear signaling may be a common output of axon guidance receptors.

INTRODUCTION

During the development of the nervous system, chemotropic cues serve as navigational signals for growing axons. These cues signal through axon guidance receptors, which are expressed on axonal growth cones. In the canonical view of axon guidance receptor signaling, ligand binding recruits protein complexes to receptor cytoplasmic domains to locally remodel the growth cone plasma membrane and underlying cytoskeleton. In this way, guidance receptors are thought to transduce gradients of cues into asymmetrical structural changes in growth cones, to steer them toward sources of attractants and away from sources of repellents (reviewed in O'Donnell et al., 2009). The observation that isolated growth cones that have been physically severed from their cell bodies remain capable of responding to guidance cues provides a particularly dramatic demonstration that local signaling is sufficient to execute some chemotropic responses (Campbell and Holt, 2001).

Growing axons must also modulate their responsiveness to guidance cues in order to navigate intermediate targets on the way to their final synaptic partners. One of the best-studied examples of this phenomenon is the growth of commissural axons across the ventral midline of the embryonic CNS in bilaterally symmetric animals (reviewed in Neuhaus-Follini and Bashaw, 2015). Throughout the period of time when commissural axons are crossing the midline, cells at the midline produce a host of chemotropic cues, including both attractants and repellents. In both insects and vertebrates, these include Netrins, which signal attraction through Frazzled (Fra)/Deleted in Colorectal Cancer (DCC) receptors (Serafini et al., 1994; Kennedy et al., 1994; Harris et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Serafini et al., 1996; Fazeli et al., 1997; Brankatschk and Dickson, 2006), and Slits, which signal repulsion through Roundabout (Robo) receptors (Seeger et al., 1993; Holmes et al., 1998; Kidd et al., 1998a, 1999; Brose et al., 1999; Li et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000; Long et al., 2004; Jaworski et al., 2010). As commissural neurons are growing toward the midline, their responsiveness to midline-derived repellents, including Slits, is suppressed. Once these axons have crossed the midline, they become responsive to Slits and other midline repellents, which facilitates midline exit and prevents re-crossing (Seeger et al., 1993; Kidd et al., 1998a, 1998b; Zou et al., 2000; Keleman et al., 2002, 2005; Sabatier et al., 2004; Chen et al., 2008; Nawabi et al., 2010; Parra and Zou, 2010; Charoy et al., 2012; Philipp et al., 2012; Yam et al., 2012).

In *Drosophila*, while commissural axons are crossing the midline, the endosomal protein Commis sureless (Comm) reduces sensitivity to Slit by inhibiting the trafficking of Robo to the growth cone plasma membrane (Keleman et al., 2002, 2005). Expression of *comm* mRNA is tightly spatially and temporally regulated such that commissural neurons transiently express *comm* while their axons are crossing the midline, but not before or after. Ipsilateral neurons, whose axons do not normally cross the midline, rarely express *comm* (Keleman et al., 2002). Previously, we found that in addition to its canonical role in signaling Netrin-dependent outgrowth and/or chemoattraction, Fra has a second way of promoting midline axon crossing: independent of Netrins, Fra induces *comm* mRNA expression in commissural neurons (Yang et al., 2009). However, the mechanism(s) by which Fra regulates gene expression remain unknown.

The intracellular domains (ICDs) of Fra and its orthologs contain three small, conserved sequence motifs—P1, P2, and

P3 (Kolodziej et al., 1996)—which have been implicated in a variety of protein-protein interactions and receptor signaling outputs. We were particularly intrigued by a pair of in vitro reports that the ICDs of Fra's vertebrate orthologs, DCC and Neogenin (Neo), are capable of translocating to the nucleus and functioning as transcriptional activators in reporter assays following γ -secretase-dependent receptor proteolysis (Taniguchi et al., 2003; Goldschneider et al., 2008). However, whether the ICDs of these receptors function as transcription factors in vivo and what, if any, is the biological significance of their transcriptional outputs is unknown.

Here, we report that Fra is cleaved by γ -secretase, releasing its ICD, which shuttles between the cytoplasm and the nucleus. This proteolysis is required for Fra's ability to regulate *comm* expression. In rescue and gain-of-function assays in vivo, the Fra ICD is sufficient to induce *comm* expression and midline crossing. In addition, the P3 motif in the Fra ICD encodes a transcriptional activation domain. A point mutant variant of Fra that is specifically deficient for transcriptional activation, but is intact for other P3-dependent functions, cannot regulate *comm* expression in vivo. Moreover, *comm*-regulatory function can be restored to this receptor with a heterologous transcriptional activation domain, providing strong in vivo evidence that Fra's transcriptional activation function is required. Thus, Fra acts in two different cellular compartments to control midline crossing: at the growth cone, Fra regulates local membrane and cytoskeletal dynamics in response to its canonical Netrin ligands and, in the nucleus, Fra functions as a transcription factor to modulate growth cone sensitivity to Slit-Robo repulsion.

RESULTS

Fra Is Cleaved by γ -Secretase

Fra's vertebrate orthologs, DCC and Neogenin (Neo), are substrates for metalloprotease-dependent ectodomain shedding and subsequent γ -secretase-dependent intramembrane proteolysis (Galko and Tessier-Lavigne, 2000; Taniguchi et al., 2003; Parent et al., 2005; Goldschneider et al., 2008; Bai et al., 2011; Okamura et al., 2011), prompting us to examine whether Fra also undergoes proteolytic processing. We pan-neurally expressed C-terminally tagged *UAS-Fra-Myc* with *elav-Gal4* and probed embryo lysates with an antibody against Myc (Figure 1A). We detected an ~200 kDa band corresponding to the full-length receptor, as well as smaller C-terminal fragments of approximately 50 kDa, 35 kDa, and 25 kDa (ICD A, B, and C, respectively). We made a transgenic line that allowed us to express the ICD of Fra, without any extracellular or transmembrane residues, under *Gal4/UAS* control. When we expressed *UAS-Fra ICD-Myc* with *elav-Gal4*, we detected a doublet that corresponds in size to the largest of these C-terminal fragments, as well as the smaller C-terminal species (Figure 1A). To determine whether these C-terminal fragments are specific cleavage products of the Fra cytoplasmic domain, we replaced the Myc epitope with a smaller HA epitope and again examined the sizes of Fra ICD fragments. Consistent with our observations using the Myc-tagged receptor, we detected three C-terminal fragments in lysates from embryos pan-neurally expressing Fra-HA (Figure 1B). All three of these fragments are shifted to lower molec-

ular weights (~45 kDa, 30 kDa, and 20 kDa), commensurate with the decrease in the size of the epitope tag. We also examined lysates from embryos expressing a truncated, C-terminally HA-tagged Fra receptor that is missing its entire cytoplasmic domain (Fra Δ C-HA) and did not detect Fra ICD fragments (Figure 1B). Together these observations indicate that the Fra receptor can be processed to generate distinct C-terminal fragments.

γ -secretase cleaves its substrates in the membrane, releasing their ICDs, which can signal intracellularly in a variety of ways (reviewed in Haapasalo and Kovacs, 2011). The largest C-terminal peptide generated by proteolysis of Fra is approximately the size of the Fra ICD, suggesting that this fragment might be a product of γ -secretase proteolysis. To investigate whether Fra is cleaved by γ -secretase, we examined lysates from embryos in which γ -secretase function was reduced. Presenilin (Psn) is the catalytic subunit of γ -secretase (Wolfe et al., 1999), a multi-protein complex that also includes Aph-1, Nicastrin, and Pen-2 (Yu et al., 2000; Francis et al., 2002; Goutte et al., 2002; Edbauer et al., 2003; Fraering et al., 2004). We analyzed lysates from genetically heterogeneous populations of embryos in which C-terminally epitope-tagged *UAS-Fra* transgene expression was pan-neurally driven by *elav-Gal4* only in *psn* or *aph-1* mutant embryos. To restrict *UAS-Fra* expression to mutant embryos, we used flies in which the *Gal4* and *UAS* elements were recombined onto mutant chromosomes or we used flies in which the chromosomes bearing the mutations were maintained as heterozygotes with balancer chromosomes ubiquitously expressing the *Gal4* repressor Gal80 (see Figures 1C and 1D for details). As γ -secretase components are maternally deposited (Ye et al., 1999; Hu et al., 2002), we analyzed late stage 17 embryos (20–24 hr) in order to minimize the amount of Psn or Aph-1 present. In these embryos, Psn or Aph-1 function is probably strongly reduced, but not absent. In either lysates or immunoprecipitates from *psn*¹² or *aph-1*^{D35} mutant embryos, the abundance of both the Fra ICD and the smaller C-terminal fragments of Fra is reduced (Figures 1E–1H), suggesting that the Fra ICD is a product of γ -secretase proteolysis. In addition, these experiments suggest that even though the smaller fragments are not likely to be directly generated by γ -secretase proteolysis, subsequent processing of the ICD depends on γ -secretase cleavage. DCC and Neo are cleaved by caspases approximately in the middle of their ICDs and this proteolysis is required for the abilities of these receptors to induce apoptosis (Mehlen et al., 1998; Matsunaga et al., 2004). The caspase cleavage site in DCC and Neo is not conserved in Fra, but there are several aspartate residues in the Fra ICD that are candidate caspase cleavage sites.

The Fra ICD Is Sufficient to Induce *comm* Expression

Fra promotes midline crossing of commissural axons both by signaling outgrowth and/or chemoattraction in response to Netrins and by promoting *comm* transcription, independent of Netrins, to inhibit Slit-Robo midline repulsion. We reasoned that if the Fra ICD regulates *comm* by acting as a transcription factor, it should be sufficient to perform the aspects of Fra's function that are due to its regulation of *comm*, but not the aspects that are due to its ability to transduce Netrin signals. To test this idea, we examined the ability of the Fra ICD to rescue *fra* loss-of-function phenotypes. The *eg-Gal4* element is expressed in a

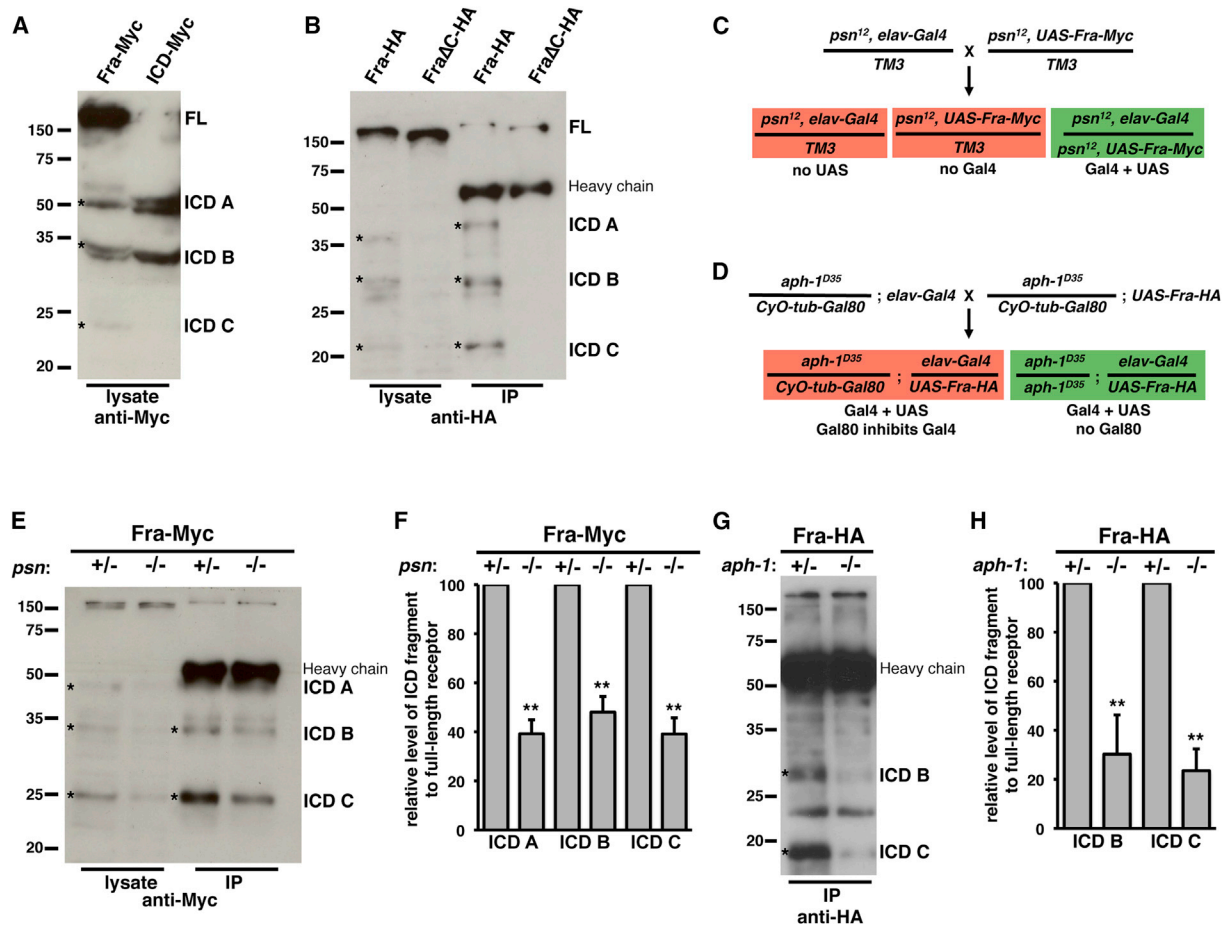


Figure 1. Fra Is Cleaved by γ -Secretase

(A) Protein extracts were made from embryos pan-neurally expressing either a full-length Fra receptor with a C-terminal 6 \times Myc tag (first lane) or an equivalently tagged Fra ICD (second lane). Proteins were resolved by SDS-PAGE and western blots were performed with anti-Myc antibody. We detect full-length receptor at approximately 200 kDa (FL) and several C-terminal fragments, including species at approximately 50 kDa, 35 kDa, and 25 kDa (ICD A, ICD B, and ICD C; indicated by asterisks).

(B) Protein extracts were made from embryos pan-neurally expressing either a full-length Fra receptor with a C-terminal 3 \times HA tag (first lane) or a Fra receptor missing its cytoplasmic domain (second lane) and HA-tagged proteins were immunoprecipitated from these extracts with anti-HA antibody (third and fourth lanes). Proteins were resolved by SDS-PAGE and western blots were performed with anti-HA antibody. The 3 \times HA tag is smaller than the 6 \times Myc tag and, accordingly, ICD A, ICD B, and ICD C are shifted to smaller sizes of approximately 45 kDa, 30 kDa, and 20 kDa in both total protein extracts and immunoprecipitates (first and third lanes, indicated by asterisks). These species are not detected in extracts or immunoprecipitates from embryos expressing Fra Δ C (second and fourth lanes). The position of the IgG heavy chain in the lanes that contain immunoprecipitates is indicated.

(C) Schematic of strategy used to express UAS-Fra-Myc with *elav-Gal4* specifically in *psn* mutants.

(D) Schematic of strategy used to express UAS-Fra-HA with *elav-Gal4* specifically in *aph-1* mutants.

(E) Protein extracts from embryos pan-neurally expressing Fra-Myc in *psn¹²* mutants were resolved by SDS-PAGE and western blots were performed with anti-Myc antibody. All three C-terminal fragments (indicated by asterisks) are reduced in abundance relative to full-length receptor in the total lysates (compare first and second lanes) and the two smaller fragments are reduced in abundance in immunoprecipitates (compare third and fourth lanes). ICD A is obscured in immunoprecipitates by the IgG heavy chain.

(F) Quantification of Fra ICD fragments in total lysates relative to full-length receptor in *psn¹²/+* compared to *psn¹²/psn¹²*. Data were analyzed by Student's t test. **p < 0.005. Error bars indicate SD. Data are from six independent experiments.

(G) Protein extracts from embryos pan-neurally expressing Fra-HA in *aph-1^{D35}* mutants were made and HA-tagged proteins were immunoprecipitated with anti-HA antibody. Proteins were resolved by SDS-PAGE and western blots were performed with anti-HA antibody. The two smaller fragments (indicated by asterisks) are reduced in abundance in immunoprecipitates. The largest fragment is obscured by the IgG heavy chain.

(H) Quantification of Fra ICD fragments relative to full-length receptor in *aph-1^{D35}/+* compared to *aph-1^{D35}/aph-1^{D35}*. Data were analyzed by Student's t test. **p < 0.005. Error bars indicate SD. Data are from four independent experiments.

subset of neurons in the embryo, including three commissural EW interneurons per abdominal hemisegment (Dittrich et al., 1997). We used *eg-Gal4* to drive the expression of UAS-Tau-

Myc-GFP, a marker that labels the axons and cell bodies of the EW neurons and facilitates quantitative evaluation of axonal trajectories. We combined this labeling with fluorescent in situ

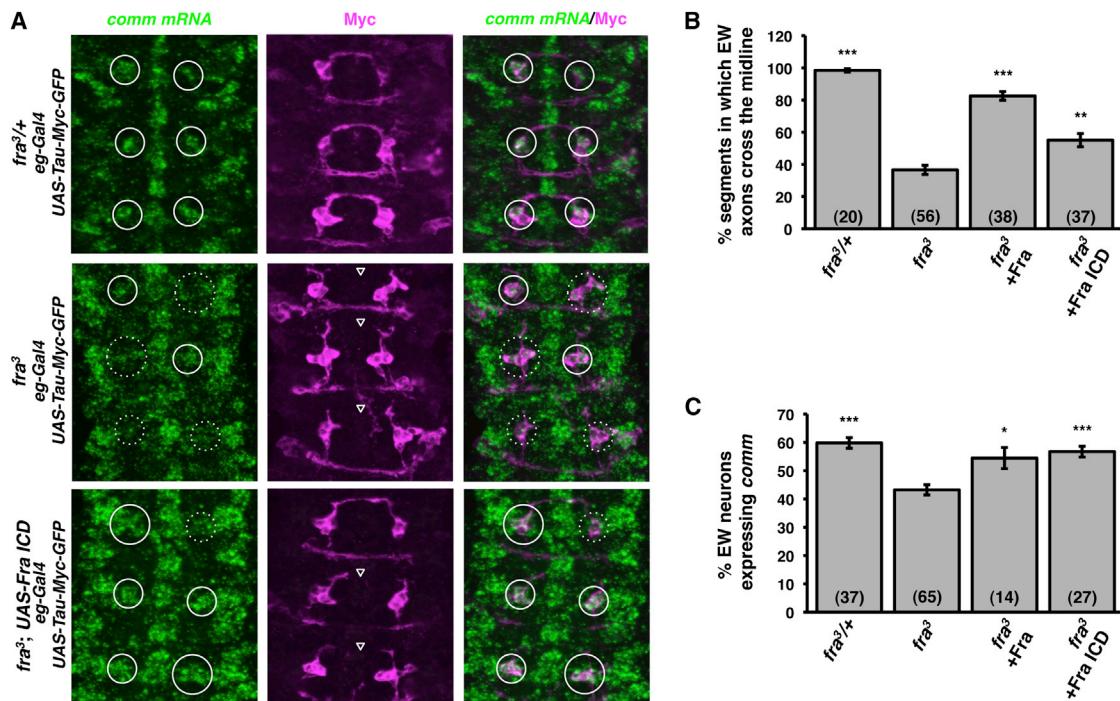


Figure 2. The Fra ICD Is Sufficient to Fully Rescue *comm* Expression and Partially Rescue Midline Crossing Defects in Commissural Neurons of *fra* Mutants

(A) Fluorescent in situ hybridization for *comm* mRNA (green) in stage 14 embryos. Anterior is up. The cell bodies and axons of EW neurons are labeled with *eg-Gal4* driving expression of *UAS-Tau-Myc-GFP*. Anti-Myc immunostaining is shown in magenta. White circles indicate the positions of EW neuron cell bodies. Solid circles indicate EW neurons that express *comm* and dotted circles indicate EW neurons that do not express *comm*. Open arrowheads indicate segments in which EW axons fail to cross the midline.

(B) Quantification of EW axon crossing in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's *t* test. ****p* < 0.0001, compared to *fra³* mutants. ***p* = 0.0002, compared to *fra³* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored. Note that the data for *fra³* heterozygotes and mutants are also shown in Figures 6A and S3.

(C) Quantification of *comm* expression in EW neurons in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's *t* test. ****p* < 0.0001, compared to *fra³* mutants. **p* < 0.01, compared to *fra* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored. Note that the data for *fra³* heterozygotes and mutants are also shown in Figures 6B and S2.

hybridization, using a probe that recognizes *comm* mRNA, so that we could score *comm* expression in each individual EW neuron. In embryos that are wild-type for *fra* or heterozygous for *fra³*, axons of the EW neurons have reached the midline by stage 14, the time when these neurons express maximal amounts of *comm* mRNA (Figures 2A–2C; Keleman et al., 2002; Yang et al., 2009). In *fra³* mutants, these neurons often fail to express *comm* and their axons fail to cross the midline at the appropriate time (Figures 2A–2C; Yang et al., 2009). These midline crossing and *comm* expression defects can be rescued by expression of a full-length *UAS-Fra* transgene with *eg-Gal4* (Figures 2B and 2C; Yang et al., 2009). In addition, expression of *UAS-Fra ICD* with *eg-Gal4* partially rescues midline crossing defects and fully rescues *comm* expression in the EW neurons of *fra³* mutants (Figures 2A–2C).

We also examined the Fra ICD's ability to regulate *comm* expression in a subset of ipsilateral neurons, using a similar approach. The *ap-Gal4* element is expressed in three ipsilateral interneurons per abdominal hemisegment (the ap neurons; O'Keefe et al., 1998), which stochastically express *comm* at stages 16–17 (Figures 3A–3C; Keleman et al., 2002; Yang

et al., 2009). Expression of either full-length *UAS-Fra* or *UAS-Fra ICD* with *ap-Gal4* induces ectopic midline crossing of ap axons and ectopic expression of *comm* in the dorsal ap neuron (Figures 3A–3C; Yang et al., 2009). Together, these rescue and gain-of-function genetic data support the idea that the Fra ICD is sufficient to carry out the transcriptional regulatory component of Fra's activity, but not the local, Netrin-dependent component.

γ-Secretase Proteolysis of Fra Is Required for Fra to Regulate *comm* Expression

We used this gain-of-function assay to test whether Fra's ability to regulate *comm* expression depends on its proteolysis by γ-secretase. When we analyzed embryos in which *UAS-Fra* was misexpressed with *ap-Gal4* in *psn* mutants, we found that Fra's ability to induce *comm* expression is reduced in two different *psn* mutant backgrounds (Figure 3C), suggesting that γ-secretase proteolysis of Fra is required for Fra's ability to regulate *comm*. Fra-induced ectopic midline crossing is not suppressed in *psn* mutants (data not shown), but interpretation of midline crossing phenotypes in these experiments is

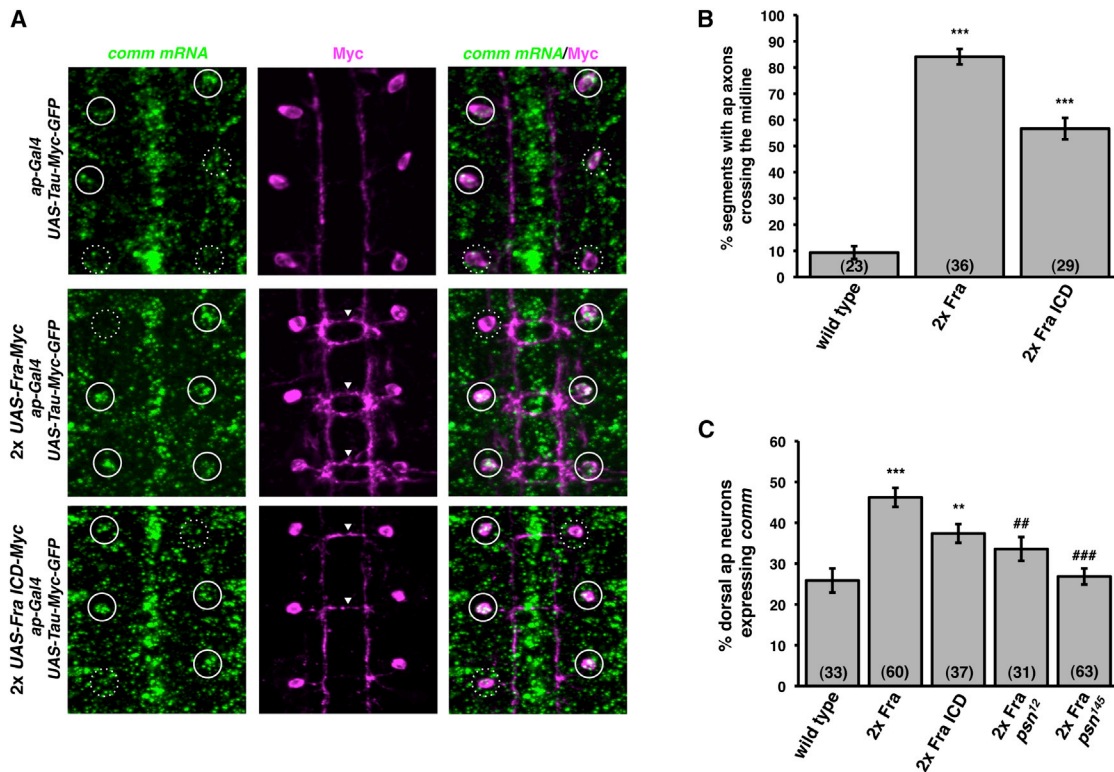


Figure 3. γ -Secretase Proteolysis of Fra Is Required for Fra's Ability to Regulate *comm* Expression

(A) Fluorescent in situ hybridization for *comm* mRNA (green) in stage 17 embryos. Anterior is up. The cell bodies and axons of ap neurons are labeled with *ap-Gal4* driving expression of *UAS-Tau-Myc-GFP*. Anti-Myc immunostaining is shown in magenta. White circles indicate the positions of dorsal apterous neuron cell bodies. Solid circles indicate ap neurons that express *comm* and dotted circles indicate ap neurons that do not express *comm*. Arrowheads indicate segments in which ap axons ectopically cross the midline.

(B) Quantification of ap axon crossing in stage 16–17 embryos. Data were analyzed by ANOVA, followed by Student's *t* test. ****p* < 0.0001, compared to wild-type embryos. Error bars indicate SEM. Number in parentheses indicates number of embryos scored. Note that the data for wild-type embryos are also shown in Figure 5D.

(C) Quantification of *comm* expression in dorsal ap neurons in stage 16–17 embryos. Data were analyzed by ANOVA, followed by Student's *t* test. ****p* < 0.0001, compared to wild-type embryos. ***p* < 0.005, compared to wild-type embryos. ###*p* < 0.0001, compared to wild-type embryos expressing two copies of Fra. ##*p* < 0.002, compared to wild-type embryos expressing two copies of Fra. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

confounded by several factors, including reports that proteolysis of DCC can antagonize canonical Netrin-DCC signaling (Galko and Tessier-Lavigne, 2000; Bai et al., 2011); the observation that Robo activity, which plays a key role in preventing the ap neurons from crossing the midline, is regulated by metalloprotease-dependent ectodomain shedding (Coleman et al., 2010), an event which is typically followed by γ -secretase proteolysis; and the likelihood that ectopic crossing events induced by full-length Fra are primarily a consequence of Netrin-dependent attraction (Figure 5D; O'Donnell and Bashaw, 2013).

The Fra ICD Shuttles between the Cytoplasm and the Nucleus

If Fra regulates *comm* expression by functioning as a transcription factor, its ICD should be localized in nuclei. We initially investigated the subcellular localization of the Fra ICD in *Drosophila* S2R+ cells expressing a C-terminally epitope-tagged Fra ICD. In these experiments, we labeled nuclei by staining cells with an antibody against nuclear lamin, a component of the nuclear

envelope. Under control conditions, the Fra ICD appears to be excluded from the nucleus. However, when nuclear export is blocked, either pharmacologically, with Leptomycin B, an inhibitor of chromosome region maintenance 1 (CRM1)-dependent nuclear export, or genetically, by deleting the P3 motif, which encodes Fra's nuclear export signal (NES), the Fra ICD accumulates in the nucleus (Figure 4A), suggesting that the Fra ICD normally shuttles between the nucleus and the cytoplasm. To examine the subcellular localization of the Fra ICD in vivo, we expressed *UAS-Fra ICD-Myc* with the restricted drivers *eg-Gal4* and *ap-Gal4*, which allows for single-cell resolution of nuclear localization. We observed some cells in which the Fra ICD is enriched in the nucleus and others in which the Fra ICD is mostly cytoplasmic (Figures 4B and 4C). When we expressed *UAS-Fra ICD Δ P3-Myc* with either *eg-Gal4* or *ap-Gal4*, we detected its expression in the nucleus in every cell we examined, suggesting that the Fra ICD shuttles between the nucleus and cytoplasm in vivo and indicating that the NES we mapped in vitro appears to have the same activity in vivo (Figure 4D and data not shown).

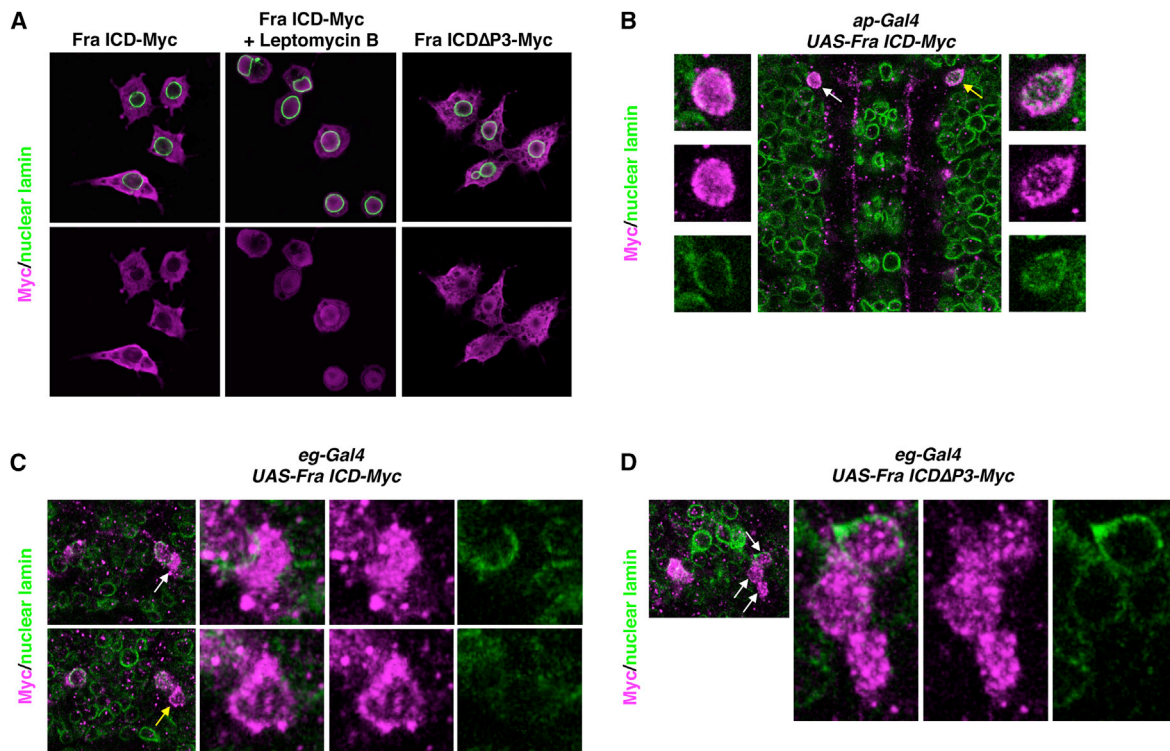


Figure 4. The Fra ICD Shuttles between the Cytoplasm and the Nucleus

(A) S2R+ cells were transfected with the indicated Myc-tagged constructs and treated with Leptomycin B or vehicle, as indicated. Cells were immunostained with antibodies against Myc (magenta) and nuclear lamin (green). For each condition, a single optical plane is shown.

(B) Stage 16 embryo in which *ap-Gal4* is driving expression of *UAS-Fra ICD-Myc*. Anterior is up. A single optical plane is shown. The embryo is stained with antibodies against Myc (magenta) and nuclear lamin (green). White arrow indicates a cell in which the Fra ICD is enriched in the nucleus (enlarged in the panels on the left). Yellow arrow indicates a cell in which the Fra ICD is largely excluded from the nucleus (enlarged in the panels on the right).

(C) One segment of a stage 14 embryo in which *eg-Gal4* is driving expression of *UAS-Fra ICD-Myc*. Anterior is up. Two different single optical planes are shown. The embryo is stained with antibodies against Myc (magenta) and nuclear lamin (green). In the top row, the white arrow indicates a cell in which the Fra ICD is enriched in the nucleus (enlarged in the panels on the right). In the bottom row, the yellow arrow indicates a cell in which the Fra ICD is largely excluded from the nucleus (enlarged in the panels on the right).

(D) One segment of a stage 14 embryo in which *eg-Gal4* is driving expression of *UAS-Fra ICDΔP3-Myc*. Anterior is up. A single optical plane is shown. The embryo is stained with antibodies against Myc (magenta) and nuclear lamin (green). White arrows indicate three cells in which the Fra ICD is enriched in the nucleus (enlarged in the panels on the right).

See also Figure S1.

When we expressed full-length Fra in S2R+ cells or in embryos, we could not detect its C terminus in the nucleus, even when nuclear export was blocked (data not shown), suggesting that the amount of nuclear ICD generated from full-length receptor is too low at any given time for us to detect. Our inability to detect the C terminus of full-length Fra in nuclei is reminiscent of reports that the C terminus of full-length Notch cannot be detected in the nucleus by conventional immunostaining (Fehon et al., 1991; Lieber et al., 1993; Rebay et al., 1993), despite the finding that nuclear localization of the Notch ICD is necessary for its function (Struhl and Adachi, 1998).

We attempted to make a variant of the Fra ICD that lacks the ability to enter the nucleus, in order to test whether nuclear localization of the Fra ICD is required for its ability to regulate *comm*. We made serial deletions across the entire Fra ICD and tested the localization of these variants in S2R+ cells. Using this assay, we did not identify a sequence that is required for

nuclear localization (Figure S1A). We also used a reporter assay in yeast to test which sequences within the Fra ICD are sufficient to confer nuclear localization. We used a strain of yeast in which a *lexAop* insertion upstream of the *ADE2* gene disrupts endogenous *ADE2* expression, causing the cells to accumulate a red pigment. In this strain, *ADE2* is under the control of *lexAop*, so the expression of a transcriptional activator with a LexA DBD causes the yeast to turn white (Figure S1B). We fused a series of sequences spanning the entire Fra ICD to a transcription factor consisting of a DNA-binding domain from the bacterial transcription factor LexA (mutLexA DBD) and an activation domain from the yeast transcription factor Gal4 (Gal4 AD). The mutLexA DBD that we used in these experiments has mutations that abolish its intrinsic ability to enter the nucleus (Rhee et al., 2000; Marshall et al., 2007) and the Gal4 AD does not localize to the nucleus (Silver et al., 1988). We expressed these fusion proteins in *ADE2* reporter yeast and

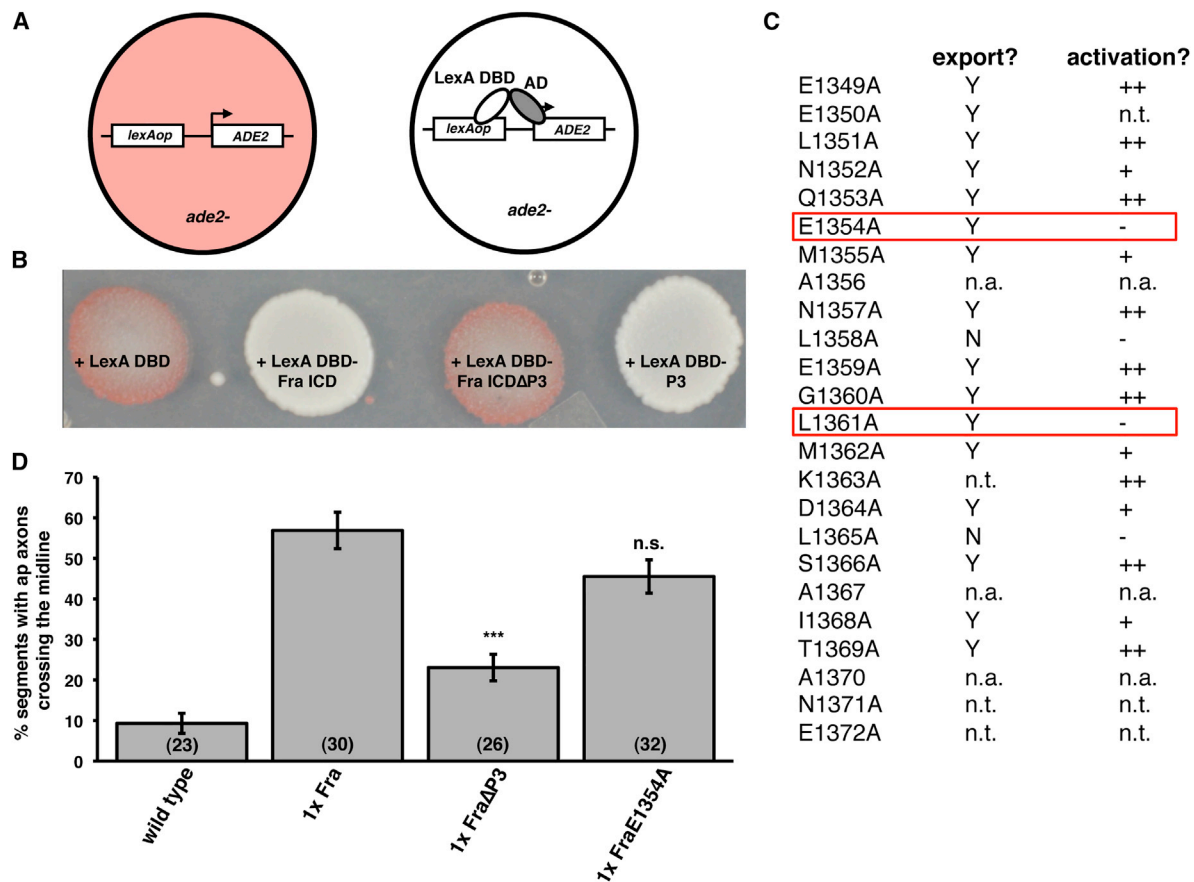


Figure 5. The Fra ICD Encodes a Transcriptional Activation Domain

(A) Schematic of yeast activation assay.

(B) Yeast were transformed with plasmids encoding LexA DBD and the indicated forms of the Fra ICD. Note that P3 is necessary and sufficient for activation.

(C) Summary of an alanine mutagenesis scan to identify point mutations within P3 that specifically disrupt transcriptional activation. Data in the export column indicate whether the mutant ICD was exported from the nucleus in S2R+ cells. Y indicates that the ICD did not accumulate in the nucleus in the absence of Leptomycin B. N indicates that the ICD accumulated in the nucleus in the absence of Leptomycin B. Data in the activation column indicate whether the mutant ICD functioned as transcriptional activator in the yeast assay. ++ indicates that the yeast appeared white; + indicates that the yeast appeared light pink; – indicates that the yeast appeared dark pink. n.t. indicates that the mutant was not tested. n.a. indicates alanine residues within P3. The mutants enclosed in the red boxes appear functional for nuclear export but non-functional for transcriptional activation.

(D) Quantification of ap axon crossing in stage 16–17 embryos. Data were analyzed by ANOVA, followed by Student's t test. *** $p < 0.0001$, compared to embryos expressing Fra. n.s. indicates $p > 0.05$, compared to embryos expressing Fra. Error bars indicate SEM. Number in parentheses indicates number of embryos scored. Note that the data for wild-type embryos are also shown in Figure 3B.

See also Figure S2.

identified three different regions of the Fra ICD that are sufficient to confer nuclear localization (Figure S1C). This redundancy prevented us from generating a Fra ICD variant that is defective for nuclear localization.

The Fra ICD Encodes a Transcriptional Activation Domain

The ICDs of DCC and Neo have been shown to function as transcriptional activators in reporter assays in vitro (Taniguchi et al., 2003; Goldschneider et al., 2008), suggesting a potentially direct mechanism through which Fra could regulate *comm* expression and prompting us to examine whether the Fra ICD shares this property with its vertebrate orthologs. To determine whether the Fra ICD, like its vertebrate orthologs,

contains an activation domain, we returned to the *ADE2* reporter yeast strain. For these experiments, we took advantage of the fact that expression of a transcription factor consisting of a LexA DBD (fused, in this case, to a strong nuclear localization signal [NLS]) and any activation domain drives expression of *ADE2*, causing the yeast to turn white (Figure 5A). Expression of a LexA DBD-Fra ICD fusion produces white yeast, indicating that the Fra ICD can function as a transcriptional activator (Figure 5B). A fusion between a LexA DBD and a Fra ICD lacking the conserved P3 motif (Fra ICDΔP3) fails to drive reporter expression, while a fusion between LexA DBD and P3 functions as a transcriptional activator, indicating that P3 is necessary and sufficient for Fra's transcriptional activation function (Figure 5B).

Fra Regulates Midline Axon Crossing and *comm* Expression by Functioning as a Transcriptional Activator

To determine whether Fra's ability to regulate commissural axon guidance and *comm* expression depends on its function as a transcriptional activator, we examined whether Fra Δ P3, which lacks Fra's activation domain, could rescue *fra* loss-of-function phenotypes. Expression of *UAS-Fra Δ P3* with *eg-Gal4* fails to rescue *comm* expression in the EW neurons of *fra*³ mutants (Figure S2). To more directly test whether this lack of rescue is a consequence of the loss of Fra's activation domain or reflects other defects in the receptor, we performed a domain replacement experiment using the VP16 AD. Expression of *UAS-Fra Δ P3-VP16AD* with *eg-Gal4* does not rescue *comm* expression in *fra*³ mutants (Figure S2).

This result could either mean that Fra's function as a transcriptional activator is not required for its ability to regulate *comm* or that P3 has an additional function in Fra's *comm*-regulatory pathway besides its function as an activation domain. To distinguish between these possibilities, we attempted to make mutations in Fra that specifically abrogate its transcriptional function, while leaving P3, which forms an alpha helix (Hirano et al., 2011; Wei et al., 2011), structurally intact. We performed an alanine mutagenesis scan across P3 and determined whether each point mutant had a functional activation domain and NES (Figure 5C). We used the presence of a functional NES as a proxy for the structural integrity of P3, as leucine-rich NESs, such as the one in P3, are alpha helices and this structure, rather than primary sequence, is the basis of their recognition by the nuclear export karyopherin CRM1 (Dong et al., 2009). Therefore, we reasoned that mutant ICDs that lacked functional activation domains but retained functional NESs were good candidates to have specific deficits in transcriptional activation without deficits in other P3-dependent functions. To determine whether a point mutant ICD had a functional activation domain, we fused it to LexA DBD and expressed it in *ADE2* reporter yeast. To determine whether a point mutant ICD had a functional NES, we fused it to a C-terminal epitope tag and examined its localization in S2R+ cells. Using this approach, we identified two point mutants, L1351A and E1354A, that are deficient for transcriptional activation but are normally exported from the nucleus (Figure 5C) and we selected E1354A for further study. When we misexpressed *UAS-FraE1354A* with *ap-Gal4*, we found that it induces ectopic midline crossing almost as effectively as wild-type Fra, suggesting that this mutant is able to carry out canonical Netrin signaling (Figure 5D). In contrast, expression of *UAS-Fra Δ P3* with *ap-Gal4* causes a much weaker ectopic crossing phenotype (Figure 5D).

Having defined specific mutations that disrupt transcriptional activation without disrupting other P3-dependent activities of the receptor, we next tested whether FraE1354A is able to rescue Fra's midline guidance and transcriptional regulatory activities. Expression of *UAS-FraE1354A* with *eg-Gal4* fails to rescue the loss of *comm* expression in EW neurons of *fra*³ mutants, strongly suggesting that Fra's transcriptional activation function is required for this activity (Figure 6B). We were surprised to find that FraE1354A provides no rescue of midline crossing (Figure 6A), even though this receptor is probably intact

for Netrin-dependent signal transduction (Figure 5D). In fact, we found that expression of FraE1354A antagonizes midline crossing in embryos heterozygous for *fra*³ (Figure S3), suggesting that FraE1354A acts as a dominant negative with respect to midline crossing. To rigorously test whether FraE1354A's inability to rescue midline crossing and *comm* expression stems from the disruption of Fra's activation domain, we generated a *UAS-FraE1354A* transgene with the VP16 AD fused to its C terminus and evaluated its ability to rescue midline crossing and *comm* expression in *fra*³ mutants. Strikingly, we found that addition of a heterologous VP16 AD to the FraE1354A receptor restores its ability to rescue both midline crossing and *comm* expression, providing compelling *in vivo* evidence that Fra's function as a transcriptional activator is required for its ability to promote midline crossing and regulate *comm* (Figures 6A and 6B).

DISCUSSION

In this study, we identify the Fra ICD as a transcription factor that regulates the expression of *comm*, a key modulator of axonal responsiveness at the midline. γ -secretase proteolysis of Fra releases its ICD, which is capable of nuclear translocation and is sufficient to promote midline crossing and regulate *comm* expression in rescue and gain-of-function assays *in vivo*. The conserved P3 motif within the Fra ICD functions as a transcriptional activation domain and this activity is required for Fra's regulation of *comm* expression. Thus, in addition to its canonical role signaling locally to regulate growth cone dynamics, Fra functions as a transcription factor to regulate axonal responsiveness at the midline.

Regulation of Fra's Function as a Transcription Factor

comm is expressed in commissural neurons with exquisite temporal specificity (Keleman et al., 2002). How might the transcriptional activity of the Fra ICD be regulated to contribute to *comm*'s expression pattern? γ -secretase proteolysis is typically the second cleavage event in a proteolytic cascade, preceded by ectodomain shedding. Indeed, pharmacological experiments suggest that DCC's ectodomain is shed as a result of metalloprotease cleavage and that this proteolytic event is required for subsequent γ -secretase-dependent processing (Galko and Tessier-Lavigne, 2000; Bai et al., 2011). Metalloprotease-dependent ectodomain shedding is often ligand-dependent, while subsequent γ -secretase processing depends on the shape of the membrane-tethered metalloprotease cleavage product. For example, metalloprotease-dependent shedding of the Notch ectodomain is stimulated by the binding of Notch ligands (Brou et al., 2000; Mumm et al., 2000), and the subsequent γ -secretase cleavage of the membrane-tethered ICD is constitutive (Struhl and Adachi, 2000). As Fra regulates *comm* independent of Netrins (Yang et al., 2009), Fra ectodomain shedding may occur in response to the binding of a different ligand. Alternative ligands for DCC have been identified, including the vertebrate-specific proteins Draxin (Ahmed et al., 2011) and Cerebellin 4 (Haddick et al., 2014). In addition, the secreted protein MADD-4 physically associates with the *C. elegans* ortholog of Fra/DCC, UNC-40, and guides sensory neurons and muscle

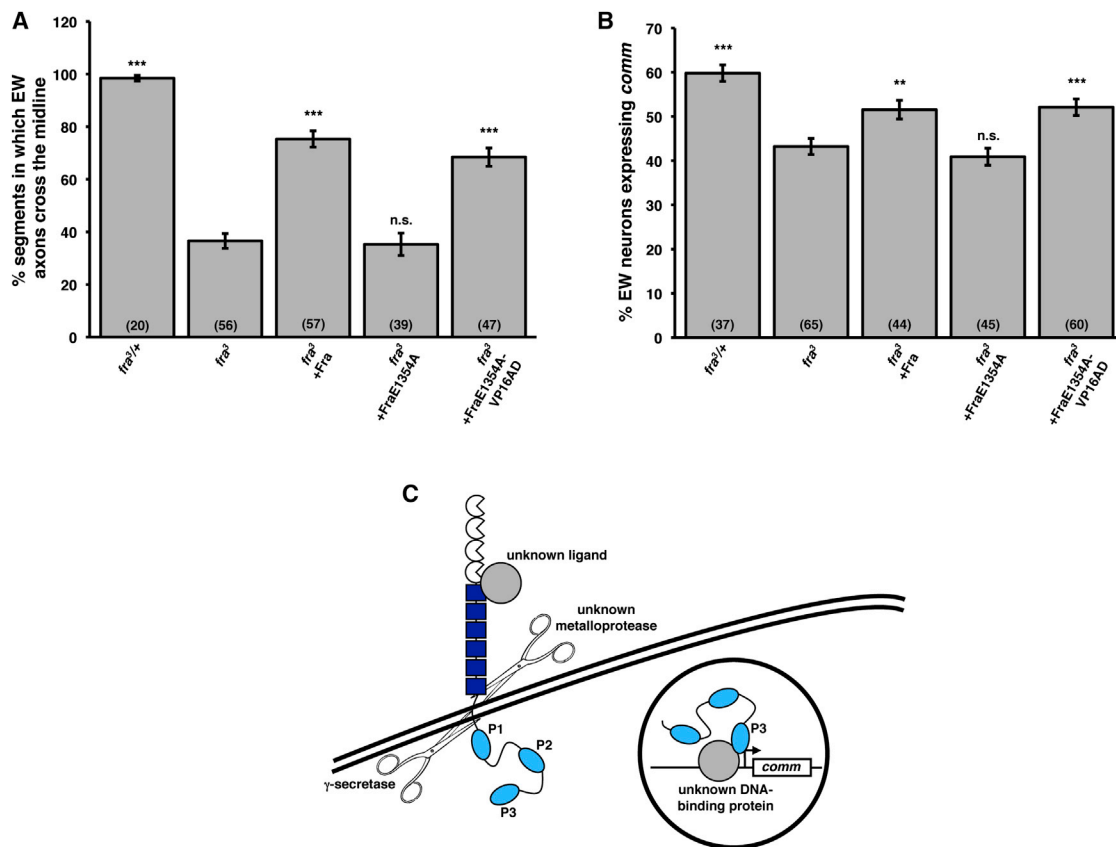


Figure 6. Fra's Transcriptional Activation Function Is Required for Its Ability to Regulate Midline Crossing and *comm* Expression

(A) Quantification of EW axon crossing in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's *t* test. ****p* < 0.0001, compared to *fra³* mutants. n.s. indicates *p* > 0.05, compared to *fra³* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored. Note that the data for *fra³* heterozygotes and *fra³* mutants are also shown in Figures 2B and S3.

(B) Quantification of *comm* expression in EW neurons in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's *t* test. ****p* < 0.0001, compared to *fra³* mutants. ***p* < 0.005, compared to *fra³* mutants. n.s. indicates *p* > 0.05, compared to *fra³* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored. Note that the data for *fra³* heterozygotes and *fra³* mutants are also shown in Figures 2C and S2 and that the data for *fra³* mutants rescued with wild-type Fra are also shown in Figure S2.

(C) A model for Fra-dependent *comm* expression. Full-length Fra is cleaved by γ -secretase, likely in response to an unknown ligand, which stimulates metalloprotease cleavage. The soluble ICD then translocates to the nucleus, where it functions as a transcriptional activator to induce *comm* expression, either directly or indirectly. The Fra ICD probably associates with DNA by interacting with one or more unknown DNA-binding proteins. P3 functions as a transcriptional activation domain.

See also Figure S3.

arms in an UNC-40-dependent manner (Seetharaman et al., 2011; Chan et al., 2014). The function of the *Drosophila* ortholog of MADD-4, Nolo, has not been investigated, nor has its ability to bind to Fra.

It seems unlikely that the transcriptional activity of the Fra ICD is controlled at the level of nuclear localization. When we express Fra ICD Δ P3 (lacking a NES) in the commissural EW neurons in vivo, it accumulates in the nucleus at the earliest developmental stages that we can observe (data not shown), suggesting that the Fra ICD is constitutively imported into the nucleus. We observe nuclear accumulation of full-length Fra ICD (with a NES) only occasionally (Figures 4B and 4C), implying that after the Fra ICD translocates to the nucleus, it is rapidly exported. The fact that Fra's NES and activation domain are both encoded by P3 raises the possibility that when Fra is engaged in transcrip-

tional activation, the association of co-activators with P3 might prevent it from associating with nuclear export machinery, coupling Fra's nuclear activity to its nuclear retention.

Mechanism of Fra's Function as a Transcription Factor

Our finding that Fra's ability to regulate *comm* expression depends on its function as a transcriptional activator seems to imply that the Fra ICD can associate with chromatin, but the Fra ICD does not contain an obvious DNA-binding domain. A Neo DNA-binding domain has not been identified either, but chromatin immunoprecipitation experiments have demonstrated that the Neo ICD associates with chromatin in vitro (Goldschneider et al., 2008). The Fra ICD's DNA-binding activity and specificity probably arise from associations between the Fra ICD and DNA-binding partners, as is the case with Notch. The

Notch ICD has no DNA-binding activity of its own and associates with DNA as part of a complex including an obligate CSL (CBF1/RBPjk, Su(H), Lag-1) DNA-binding partner (Nam et al., 2006; Wilson and Kovall, 2006). If the Fra ICD can associate with multiple DNA-binding proteins, it might allow the Fra ICD to regulate the expression of many different target genes, depending on which of its DNA-binding partners are expressed in particular cell types or developmental contexts.

The observation that a structurally intact P3 is required for Fra-dependent transcription (Figure S2) suggests that P3 plays another role in Fra's transcriptional output besides its function as an activation domain. One possibility is that P3 is required for Fra's association with chromatin, perhaps by functioning as a binding interface for Fra's DNA-binding co-factors. This idea is supported by our observation that FraE1354A antagonizes midline crossing in both *fra* mutants and heterozygotes, while FraΔP3 has only a mild effect (Figures 6A, 6B, and S3). Perhaps the ICD of FraE1354A inhibits midline crossing by occupying chromatin sites that are normally targets of both Fra and other transcriptional activators that act in a parallel pathway; the ICD of FraΔP3 would not have this effect if P3 is required for Fra's association with chromatin. FraE1354A is not likely to be inhibiting endogenous Fra in our rescue experiments, as *fra*³ is either a strong hypomorphic or null allele (Kolodziej et al., 1996; Yang et al., 2009). This model predicts that Fra has other transcriptional targets in EW neurons that are relevant for commissural axon guidance. It will be informative to identify additional transcriptional targets of Fra both in embryonic commissural neurons and in other cell types. In the retina, R8 photoreceptor axons have targeting defects that are much milder in *Netrin* mutants than in *fra* mutants (Timofeev et al., 2012), raising the possibility that the Netrin-independent output of Fra signaling in this system might be through the transcriptional pathway we have identified.

Proteolytic Regulation of Axon Guidance Receptor Signaling

Cleavage of axon guidance receptors has been shown to regulate the activities of these receptors in a number of different ways. Degradation of axon guidance receptors can provide temporal control of axonal sensitivity to guidance cues. In vertebrates, this mode of regulation controls axonal responsiveness to members of the class 3 family of secreted Semaphorins (Sema3s), which signal repulsion through Neuropilin (Nrp)/Plexin (Plex) co-receptors. Calpain proteolysis of PlexA1 in pre-crossing spinal commissural neurons reduces their sensitivity to Sema3B, which is expressed in the ventral spinal cord as these axons are growing toward the ventral midline (Nawabi et al., 2010). ADAM metalloprotease cleavage of Nrp1 reduces the sensitivity of proprioceptive sensory axons to Sema3A allowing them to terminate in the ventral spinal cord, where Sema3A expression is high (Romi et al., 2014). In addition, γ -secretase proteolysis of DCC in vertebrate motor neurons inhibits their responsiveness to midline-derived Netrin, preventing them from ectopically projecting toward the midline (Bai et al., 2011).

Proteolytic processing has also been implicated as a requisite step in local repulsive Robo signaling in *Drosophila* (Coleman et al., 2010). The Robo ectodomain is cleaved by the ADAM metalloprotease Kuzbanian and this proteolytic event is required for

Robo's ability to transduce repulsive signals in vivo and for Slit-dependent recruitment of effectors of local Robo signaling in vitro. As γ -secretase-dependent intramembrane proteolysis is typically constitutive following ectodomain shedding, and occurs subsequent to metalloprotease processing of the human Robo1 receptor (Seki et al., 2010), it is likely that *Drosophila* Robo is cleaved to produce a soluble ICD. The observation that Robo proteolysis is required for local Slit-Robo signaling does not exclude the possibility that the Robo ICD may also have a nuclear function that contributes to axon guidance, but this possibility has not yet been explored.

Proteolysis has also been identified as a regulator of contact-mediated axonal repulsion. Eph receptors signal repulsion in response to their transmembrane ephrin ligands; ephrins can also function as receptors, signaling repulsion in response to Eph binding. Metalloprotease and subsequent γ -secretase cleavage of both Ephs and ephrins have been demonstrated, providing a mechanism through which adhesive interactions can be broken to allow for repulsive signaling (Hattori et al., 2000; Janes et al., 2005; Tomita et al., 2006; Litterst et al., 2007; Lin et al., 2008; Gatto et al., 2014). The importance of this mode of regulation for axon targeting has not yet been established in vivo and a recent study using an EphA4 variant that is insensitive to metalloprotease cleavage suggests that EphA4 proteolysis is not required for EphA4-dependent motor axon targeting (Gatto et al., 2014).

Here, we have identified a new way in which axon guidance receptor proteolysis can influence axon responsiveness to guidance cues. γ -secretase-dependent processing of Fra releases its ICD, which translocates to the nucleus, where it functions as a transcription factor to regulate the guidance of commissural axons (Figure 6C). We propose that the ability to signal from the nucleus may be a common property of axon guidance receptors and may serve as a general mechanism through which axon guidance receptors regulate their own activities or the activities of other proteins. Human Robo1 is processed by sequential metalloprotease and γ -secretase cleavage and its ICD localizes to the nucleus in vitro (Seki et al., 2010). It remains to be seen whether the ICDs of Ephs and ephrins, which are cleaved by γ -secretase, and of Plexins, which are proteolytically processed, but have not yet been identified as γ -secretase substrates, translocate to the nucleus as well. It will also be interesting to determine whether the ICDs of Fra and other axon guidance receptors signal from the nucleus to regulate aspects of neuronal morphogenesis and function besides axon pathfinding. Finally, recent work indicating that the cleaved C terminus of the *Drosophila* Wnt receptor Frizzled translocates to the nucleus and contributes to the establishment of postsynaptic structures by regulating RNA export (Mathew et al., 2005; Mosca and Schwarz, 2010; Speese et al., 2012) serves as a reminder that the trafficking of cell surface receptor fragments to the nucleus may allow these fragments to signal not only by regulating transcription, but in other ways as well.

EXPERIMENTAL PROCEDURES

Molecular Biology

See Supplemental Experimental Procedures for details about plasmid construction.

Genetics

See [Supplemental Experimental Procedures](#) for details about mutant alleles and transgenic lines used in this study.

Immunostaining of Embryos

Embryo fixation and staining were performed as described (Kidd et al., 1998a). See [Supplemental Experimental Procedures](#) for details.

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization was performed as previously described (Labrador et al., 2005) and antisense, digoxigenin-labeled *comm* probes were generated as previously described (Yang et al., 2009).

Cell Culture and Immunostaining

Drosophila S2R+ cells were cultured and stained as previously described (Evans et al., 2015). See [Supplemental Experimental Procedures](#) for details.

Imaging and Phenotypic Analysis

Images of embryos and S2R+ cells were acquired using a spinning disk confocal system (Perkin Elmer) built on a Nikon Ti-U inverted microscope using a Nikon OFN25 60× objective with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head with Velocity imaging software. Images were processed using ImageJ. When scoring EW crossing, a segment was considered to have a crossing defect if one or both bundles of EW axons failed to reach the midline. When scoring ap crossing, a segment was considered to have an ectopic cross if it contained at least one continuous projection that extended across the midline and reached the lateral bundle of ap axons on the contralateral side. *comm* expression was scored using Velocity imaging software. Embryos expressed *UAS-Tau-Myc-GFP* and EW or ap neurons were identified by anti-Myc immunostaining. If the cell body of a neuron could be detected by the in situ signal, that neuron was scored as positive. Crossing and *comm* expression were scored in EW neurons at stage 14 and in ap neurons at stages 16–17. For all analyses, segments A1–A7 were scored. Midline crossing phenotypes and *comm* mRNA expression were scored blind to genotype whenever possible.

Biochemistry

Embryo lysates were generated and immunoprecipitations and western blots were performed as previously described (Evans et al., 2015). See [Supplemental Experimental Procedures](#) for details.

Yeast Transformations

The yeast strain used for both nuclear localization and activation reporter assays was Y860 [α *his3-11*, 15 *leu2-3*, 112 *trp1-1 ade2-1 can1-100 ura3-1::URA3:lexAop-ADE2*] (a gift from Erfei Bi). Yeast were grown overnight at 30°C in liquid YPD media until log phase ($OD_{600} = 0.4$ – 0.6) and transformed using the PEG/lithium acetate method (Ito et al., 1983). Yeast were then plated onto solid SD media lacking histidine and grown at 30°C for 2–3 days.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2015.08.006>.

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