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Characterization of a Netrin-4 Like Protein in *Tetrahymena thermophila*

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Abstract and Introduction

The netrin family of proteins has homeostatic roles in vertebrate development and angiogenesis, and pathophysiological roles in the progression and metastasis of cancer. We have previously characterized a netrin-1-like protein in *Tetrahymena thermophila*, and have shown that vertebrate netrin-1, netrin-3, and netrin-4 all serve as chemorepellents in this organism. We are currently using Western blotting and immunofluorescence to further characterize the netrin-like proteins in *Tetrahymena.* Western blotting with our anti-netrin-4 antibody shows a band that is clearly visible in whole cell extract, but shows little reactivity with secreted protein, indicating that most of our netrin-4-like protein remains within the cell. Western blotting of whole cell extract with anti-netrin-1, netrin-3, and netrin-4 antibodies shows a clear band at 50 kD that stains with all three antibodies. Some lower molecular weight bands are also evident in all three blots, possibly due to proteolytic activity. Immunolocalization with an anti-netrin-4 antibody shows some colocalization with netrin-1, netrin-3, and ER Tracker™. Our anti-netrin-4 antibody localizes to the oral groove, basal bodies, and nuclei of cells, indicating a possible structural role for the netrin-4-like protein in this organism. Further research will involve determining the primary amino acid sequence of the 50 kD protein and comparing it with the *Tetrahymena thermophila* proteome database to help ascertain the physiological role of this protein.

Materials and Methods

Immunofluorescence

Immunofluorescence was carried out using a modified protocol obtained from cellsignal.com. Cells were washed twice and fixed for 15 min at 20°C. Cells were then rinsed 3 times before being blocked for 60 min. After washing off blocking buffer, cells were incubated overnight at 20°C in primary antibody at a dilution of 1:100. After rinsing 3 times, cells were incubated in fluorochrome-containing secondary antibody at 1:100 dilution for 2 hours at 20°C in the dark. Cells were then rinsed 3 times in PBS. 10 µl of cell suspension was then applied to a slide and mixed with 10 µl DAPI, then covered with a coverslip and observed under a fluorescence microscope at 400X.

Western Blotting

Protein extracts were prepared from 2-day old *Tetrahymena* cultures and run on a 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and Western blots were performed using a 1:1000 dilution of anti-netrin-1, 4, or 4 IgG as the primary antibody and a 1:1000 dilution of alkaline phosphatase conjugated secondary secondary antibody. NBT substrate was used to show alkaline phosphatase activity.

Results—Western Blots

Figure 1. Western blotting with an anti-netrin-4 antibody reacts with a 50 kD protein in whole cell extract. Some staining is seen in the secreted protein lane.

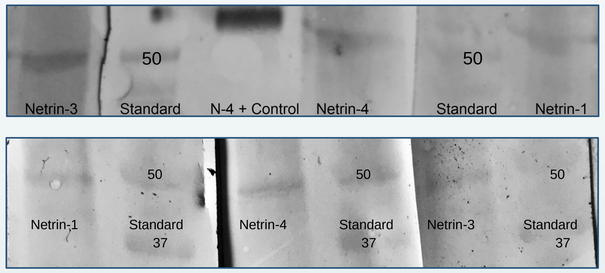

Figure 2. Western blotting of whole cell extract with anti-netrin-1, anti-netrin-3, and anti-netrin-4 antibodies shows that all three anti-netrin antibodies react with a 50 kD protein. The mouse netrin-4 positive control (Lane 3; top panel) runs at approximately 60 kD.

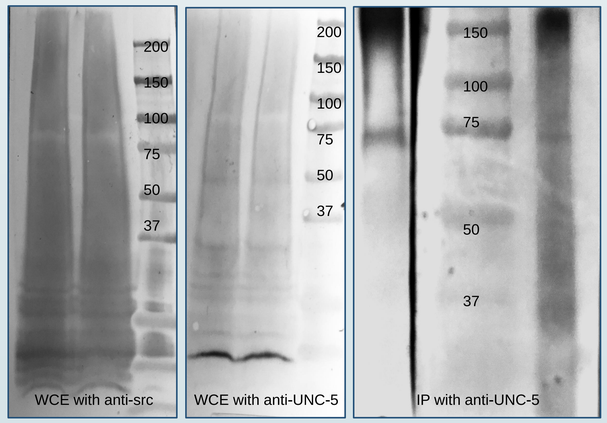

Figure 3. Western blotting of whole cell extract with anti-src and anti-UNC5 antibodies, as well as an immunoprecipitate done with antibodies to UNC-5 suggest that immunologically similar proteins to both src and UNC-5 are present in *Tetrahymena thermophila*. This is consistent with our previous immunofluorescence studies. The netrin receptor, UNC-5, and the tyrosine kinase, src, are instrumental in netrin signaling in the nematode, *Caenorhabditis elegans*, as well as in vertebrates.

Results--Immunofluorescence

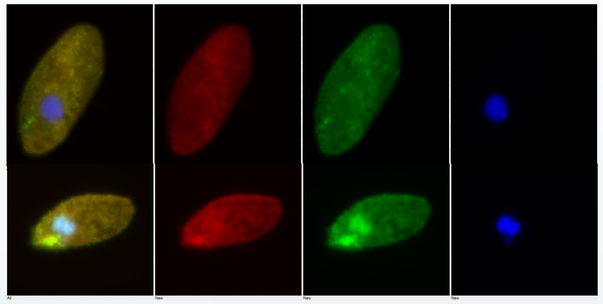

Figure 4. Merged Images (far left) show evidence that anti-netrin-1 (red) and anti-netrin-4 (green) antibodies colocalize.

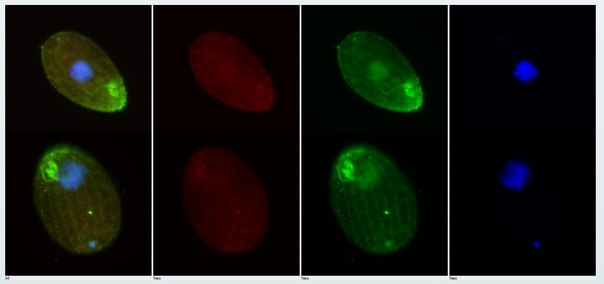

Figure 5. Merged Images (far left) show that anti-netrin-3 (red) and anti-netrin-4 (green) antibodies colocalize.

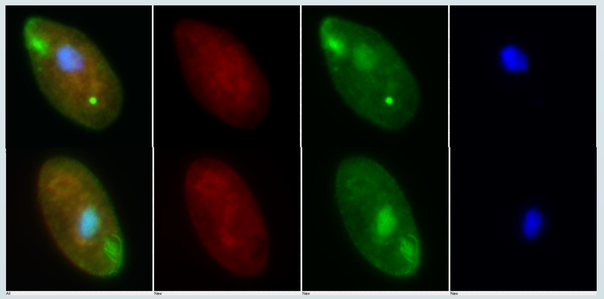

Figure 6. Merged Images (far left) show evidence that ER Tracker™ colocalizes with the anti-netrin-4 antibody (green).

Conclusions

- Western blotting of whole cell extract with anti-netrin-1, anti-netrin-3, and anti-netrin-4 antibodies suggests that we have found a single netrin-like protein of 50 kD, which cross-reacts with all three antibodies.
- Western blotting of whole cell extract with anti-src and anti UNC-5 antibodies suggests that *Tetrahymena* may share signaling machinery with organisms in the animal kingdom, including *C. elegans* and vertebrates.
- Immunolocalization of netrin-like proteins shows significant overlap in staining between all of the three antibodies, again suggesting a single protein could be responsible for the signal.
- All of the anti-netrin antibodies stain the endoplasmic reticulum, as expected for a secreted, membrane, or ciliary protein.
- Anti-netrin antibodies also stain structures containing cilia, such as the oral groove and basal bodies, as well as the nuclear lamina. This suggests that our netrin-like-protein may serve a structural role in this organism.
- We plan to sequence the 50 kD protein which stains with all three anti-netrin antibodies and compare the sequence with the proteomic data in the *Tetrahymena* genome database.

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