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Netrin-3: Tracking the Elusive Antimitotic Signal on the Western Frontier

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ABSTRACT

Netrin-3 is a guidance protein expressed throughout the animal kingdom, and involved in the development of branched structures such as the nervous system, lung, and mammary gland. We have previously shown that peptides derived from this protein serve as chemorepellents and mitotic inhibitors in *Tetrahymena thermophila.* Our previous work shows that *Tetrahymena* synthesize and secrete a netrin-3-like protein, as detected by ELISA. In this study, we find that a netrin-3-like protein is present in whole cell extract and secreted protein, as detected by Western blotting. A protein of approximately 48 kD is consistently detected in our Western blots. In addition, we often detect a protein of 52 kD, which may be the netrin-1-like protein of *Tetrahymena* that we have previously described. Further studies will enable us to determine whether the 52-kD protein is indeed the netrin-1 like protein of *Tetrahymena.*

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INTRODUCTION

The netrin family of proteins includes a number of secreted guidance factors (netrin 1, 3, and 4) and several lipid-anchored membrane proteins (G1 and G2). Netrins are involved in a number of homeostatic roles in development, such as neural development, angiogenesis, and the development of lungs and mammary glands (Moore *et al*., 2007). Netrins are also biomarkers for some types of cancer, often signifying a high potential for metastasis and a poor prognosis (Khol *et al.,* 2018).

While all of the secreted netrins have been implicated in developmental signaling, the best-studied netrin is netrin-1. Netrin-1 has been well characterized in many cell types and a netrin-like protein has been characterized in *Tetrahymena thermophila* (Merical *et al.,* 2017).

We have previously described the response of the ciliate, *Tetrahymena thermophila,* to netrin-3 peptides. In this organism, netrin-3 serves as a chemorepellent as well as a growth inhibitor (Khol *et al.,* 2018). Immunofluorescence assays as well as ELISA both suggest that a netrin-3-like protein is secreted from *Tetrahymena thermophila* (Khol *et al.,* 2018).

In our current study, we have attempted to characterize the netrin-3-like protein of *Tetrahymena* through Western blotting. We hope knowing more about the netrin-3-like protein will help us determine whether *Tetrahymena* are a good model system for netrin signaling in vertebrate systems.

METHODS AND MATERIALS

Secreted proteins from *Tetrahymena thermophila* were obtained by 2-day old cells, incubating them in behavioral buffer overnight, and then removing the cells from the buffer by centrifugation. Proteins were concentrated by chloroform-methanol precipitation, reconstituted in Laemmli sample buffer, and run on SDS-PAGE. Proteins were transferred to nitrocellulose using a Turbo-Blot™ apparatus, blocked in 1% BSA overnight, and then exposed to anti-netrin-3 antibody in PBS-Tween containing 1% BSA. After washing, blots were exposed to anti-rabbit-IgG, alkaline phosphatase conjugate, in PBS-Tween containing 1% BSA. Finally, blots were washed four times before being incubated with NBT substrate. After developing, blots were washed in distilled water and allowed to dry.

RESULTS
The good, the bad, and the ugly

Western blotting has given somewhat consistent results; however, some technical challenges have made these results difficult to interpret. When we used a high secondary antibody concentration, we got negative staining of bands (Figures 1 and 2). Our lowest secondary antibody concentration gave us clear bands, but very high background (Figure 4). We consistently see a band right around 50 kD or higher, and a band that is lower than 50 kD. Since the netrin-1-like protein of *Tetrahymena* has previously been characterized by Western blotting at approximately 52 kD (Merical *et al*., 2017), it is possible that the larger band we're seeing represents cross-reactivity of our netrin-3 antibody with the netrin-1-like protein of *Tetrahymena*. Immunolocalization of netrin-3 shows mainly vesicular staining (ER and Golgi) as well as some staining on the cilia. At this point, we are uncertain whether this represents netrin bound to receptors on the cilia, or whether *Tetrahymena*, like mammals, make a membrane-bound form of netrin.

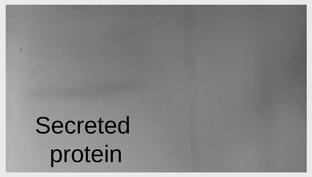

Figure 1. Secreted protein extract from *Tetrahymena thermophila* stained with polyclonal anti-netrin-3 antibody (N-terminal) at 1:1000 and secondary antibody at 1:1000. Inverted image made from negatively stained Western blot. This is the only blot pictured that used the anti-netrin-3 N-terminal antibody.

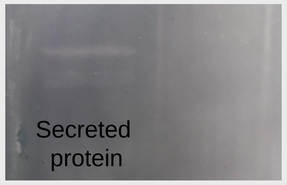

Figure 2. Negatively stained blot with anti-N3 antibody at 1:1000 and secondary antibody at 1:1000.

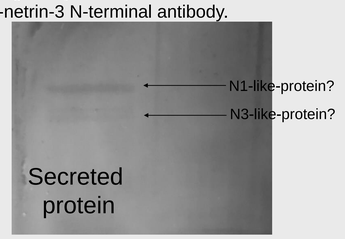

Figure 3. Inverted image of Figure 2.

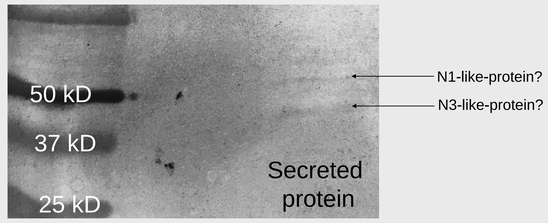

Figure 4. Secreted protein extract from *Tetrahymena thermophila* stained with polyclonal anti-netrin-3 antibody at 1:1000 and secondary antibody at 1:5000.

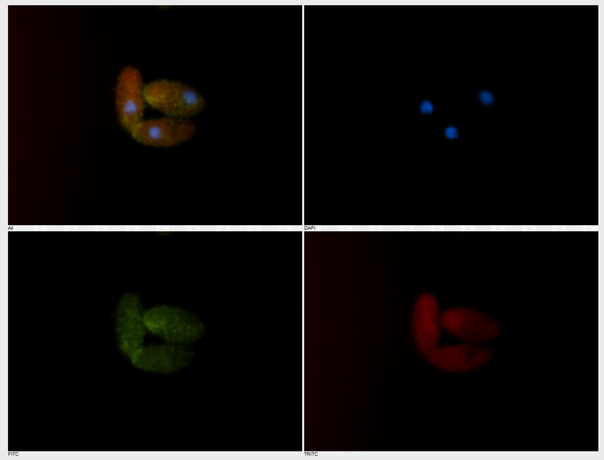

Figure 5. Immunolocalization of netrin-3 in *Tetrahymena thermophila*. Top left: merged image. Top right: DAPI staining (blue). Bottom left: staining with anti-tubulin antibody (green). Bottom right: staining with anti-netrin-3 antibody (red).

CONCLUSIONS

In order to detect netrin-3 levels more reliably, we plan to do the following:

- Use chemiluminescent detection to increase sensitivity.
- Increase our BSA concentration to reduce nonspecific binding, or try using milk as a blocker.
- Purchase a new positive control; ours hasn't shown up in any of the Westerns so far.
- Probe part of our blot with an anti-netrin-1 antibody to see if the netrin-1-like protein of *Tetrahymena* stains with the netrin-3 antibody.

REFERENCES

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