THE ISOLATION AND CHARACTERIZATION OF NOVEL POSTSYNAPTIC ANTIBODIES FOR *DANIO RERIO*

By

Yuqing Kou

A THESIS

Presented to the Robert D. Clark Honors College and the

Department of Chemistry and Biochemistry

University of Oregon

May 2012

Approved: _____ Date: _____

Professor Philip Washbourne

University of Oregon, Department of Biology

Thesis Committee:

Primary Thesis Advisor: Professor Philip Washbourne

Secondary Thesis Advisor: Keith Beadle

Clark Honors College Advisor: Professor Daniel Rosenberg

Abstract

The proteins and molecular agents that govern synapse formation in developing organisms are essential for cognitive and neural maturation. Mutations or disruptions in proteins that aid in the formation of a mature synapse have been implicated in developmental and neural disorders such as autism, mental retardation, and schizophrenia. The current body of knowledge on synapses lacks great detail about the processes that occur following the release of neurotransmitters into the synapse, including the events occurring in the synaptic cleft and postsynaptically. The general aim of this project was to generate antibodies that would specifically recognize synaptic targets in order to better characterize the various processes of synaptogenesis, including the establishment of the postsynaptic apparatus and the recruitment of assorted structural proteins and neurotransmitter receptors. Clonal hybridomas were generated by injecting mice with purified fractions of synaptoneurosomes and postsynaptic density. A total of 96 clonal hybridomas were screened for the desired characteristics by enzyme-linked immunosorbent assay, immunolabeling, Western blotting, and immunoprecipitation. A number of hybridomas recognized targets with high affinity and exhibited specificity for neuronal and postsynaptic targets. Notably, a number of subclonal hybridomas labeled neuronal targets strongly in whole-mount immunolabeling. Additionally, immunoprecipitation showed that selected subclonal hybridomas could precipitate antigens from solution, which will allow the isolated antigens to be identified.

Acknowledgements

I would like to thank Professor Philip Washbourne for his guidance and invaluable advice throughout my time as an undergraduate; Keith Beadle for his mentorship, friendship, and advice on all matters science and non-science (also for teaching me how to do almost everything there was to do in the lab); and Professor Daniel Rosenberg for his constructive assistance and instruction during the thesis process and my time in the Honors College. I would also like to thank my fellow Washbourne lab members (both past and present) for their patience and instruction in the lab from PCR to dissecting zebrafish brains. I would like to especially thank Dr. Courtney Easley-Neal for her mentorship when I first joined the Washbourne lab.

I would also like to thank Dr. Gregory Williams and Dr. Michael Koscho for being fantastic professors, for helping me realize the potential I did not know existed, and for making me think I could do science. I also want to thank Mr. Scott Dossa for making science fun and interesting (especially for making molecular orbitals a super fun donut party). I would also like to take this opportunity to express my gratitude towards the various individuals and organizations that have funded my university education and research: the Summerville Fund Presidential Scholarship, the Clarence and Lucille Dunbar Scholarship, and the Edward C. Sargent III Scholarship. Last, but certainly not least, I would like to thank my friends and family for always believing in me and taking care of me. To my rock—thanks for being my partner in science.

Table of Contents

List of Figures and Tables

FIGURES

TABLES

SUPPLEMENTAL TABLES

Introduction

Neurobiology

Understanding the biological basis of human behavior, disease and mental function is one of the central goals of neurobiology. The study of neuronal processes builds on the view that the brain is the ultimate source of all behavior[1]. Neurons are the basic functional subunits of the brain, and are responsible for the signaling capacity of the nervous system. The human brain contains over a thousand types of neurons and approximately 100 billion individual nerve **cells**[i].

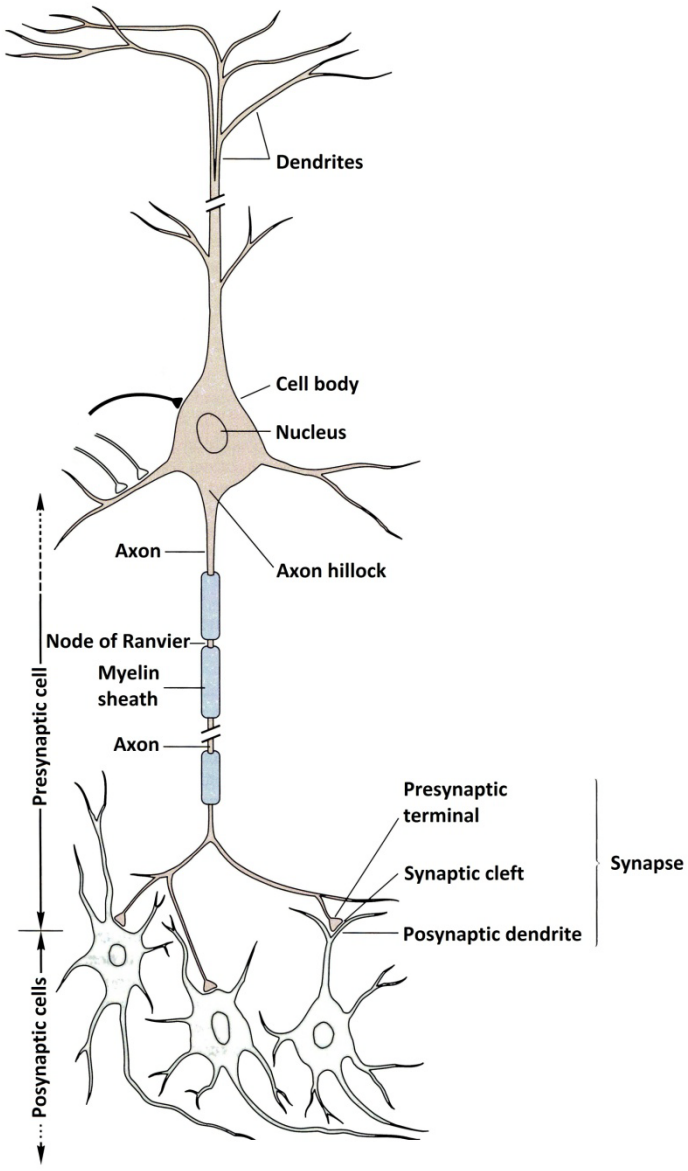

Figure 1. Structure of a neuron[1]. The labeled features are commonalities between most neurons in the vertebrate nervous system.

Despite such diversity, all neurons share the same basic morphology, which consists of four general regions: the dendrites, cell body, axon, and presynaptic terminals (**Figure 1**)[1]. The junction between

[i] Terms in the glossary are indicated in **bold** at their first appearance.

two neurons is called the synapse, and it is the point at which neurons communicate.

The formation of a synapse involves a set of selective contacts between the developing axon and its target, and includes the differentiation of the axon's growth cone into a presynaptic terminal. A mature synapse also requires the establishment of a postsynaptic apparatus, which includes the recruitment of structural **proteins** as well as **neurotransmitter** receptors. All these processes rely heavily on intercellular interactions[1]. **Figure 2** depicts a prototypical chemical synapse, specifically the neuromuscular junction (NMJ), where an electrical signal is converted to a more specific chemical message. The presynaptic cell sends a message through chemical messengers known as neurotransmitters. Neurotransmitters are packaged in the

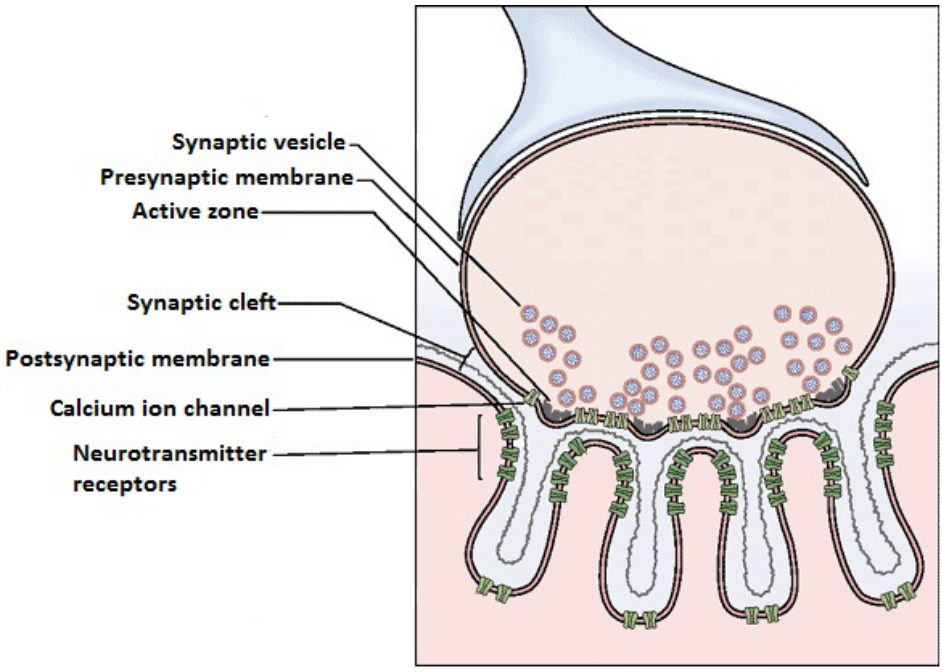

Figure 2. A general representation of the neuromuscular junction (NMJ), which is the synaptic junction between the presynaptic motor axon and postsynaptic muscle cell[1]. Synaptic vesicles fuse with the presynaptic membrane at the active zones to release neurotransmitter molecules, which diffuse across the synaptic cleft and are bound by the appropriate receptors on the postsynaptic membrane. The NMJ is one of the best understood and most completely characterized synapses of the nervous system[1].

presynaptic cell into synaptic vesicles, which cluster at active zones—regions of the presynaptic membrane specialized for transmitter release (**Figure** 2)[1]. During signaling, the synaptic vesicles fuse with the presynaptic membrane and release the packaged neurotransmitters into the synaptic cleft—the space that separates the pre- and postsynaptic cells[1]. As shown in **Figure 3**, when an **action potential** reaches the

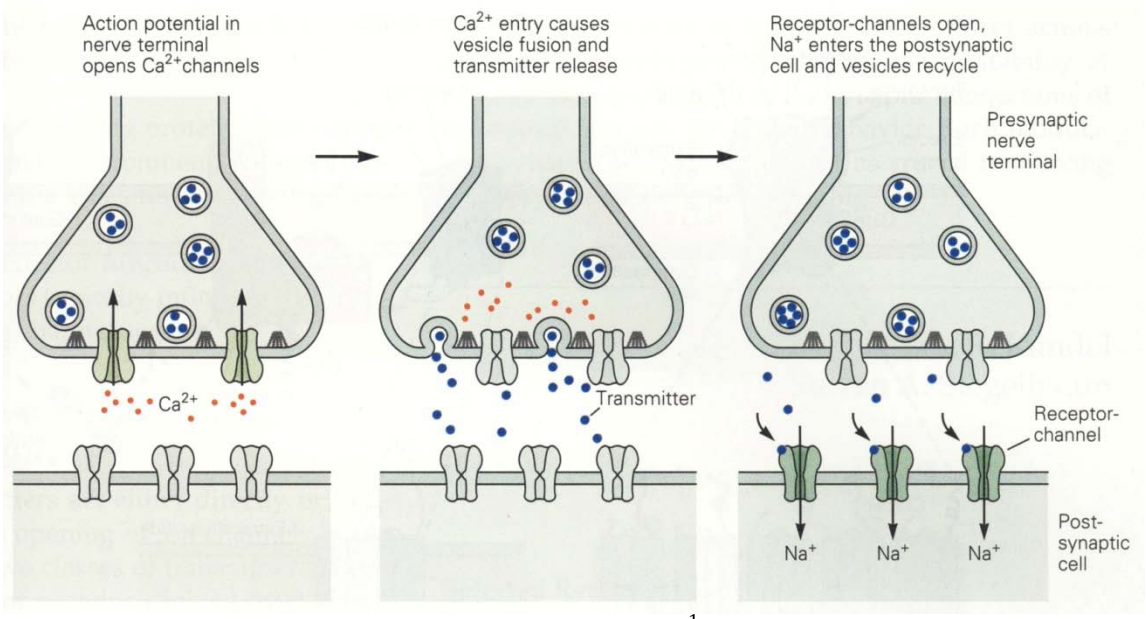

Figure 3. Synaptic transmission at chemical synapses[1].

presynaptic terminal, voltage-gated calcium ion (Ca^{2+}) channels open. The influx of Ca^{2+} induces the fusion of synaptic vesicles to the presynaptic membrane and the release of neurotransmitters into the synaptic cleft.

The nervous system of vertebrates has two major partitions—central and peripheral. The brain and spinal cord compose the central nervous system (CNS), while nerves and **ganglia** outside the brain and spinal cord compose the peripheral nervous system (PNS)[1]. Whereas muscles at the NMJ (**Figure 2**) are innervated by a single

motor neuron and only receive excitatory signals, a central nerve cell can receive both excitatory and inhibitory inputs from hundreds of neurons[1]. Excitatory and inhibitory signals are characterized by whether they increase or decrease the probability of an action potential occurring in the postsynaptic cell. The two different types of signals are mediated by different neurotransmitters and their effects upon binding the appropriate postsynaptic receptors[1].

Synapse Formation

The research of the Washbourne lab encompasses the events that occur at synapses, with an emphasis on synapse formation (termed synaptogenesis). Synaptogenesis is an important process during development, learning, and memory. The formation of a mature synapse requires a number of precise developments in order to establish a tight attachment and precise alignment between the presynaptic and postsynaptic cell[2]. Various synaptic cell adhesion molecules (SynCAMs) that span the synaptic cleft have been implicated in the establishment of a mature synapse. These include neurexins, neuroligins, immunoglobulin (Ig)-**domain** proteins, receptor phosphotyrosine kinases and phosphatases, and several leucine-rich repeat proteins[3,4]. A particular group of Ig-domain proteins called the SynCAM protein family are expressed in developing neurons and promote synapse formation (**Figure 4)**[5]. SynCAM proteins include extracellular regions that are responsible for cell-adhesion interactions and intracellular regions that facilitate binding to the **PDZ domains** of synaptic scaffolding proteins (**Figure 4A**). Four SynCAM proteins are present in mice; although SynCAM1, 2, and 3

are capable of forming **homophilic** interactions, SynCAM1 and 2 preferentially assemble into a **heterophilic** SynCAM1/2 complex (**Figure 4B**)[5]. SynCAM3 and 4 also form heterophilic interactions in an additional SynCAM3/4 complex (**Figure 4B**). These SynCAM complexes promote the formation of functional synapses by increasing the number of active presynaptic terminals and enhancing excitatory neurotransmission[5].

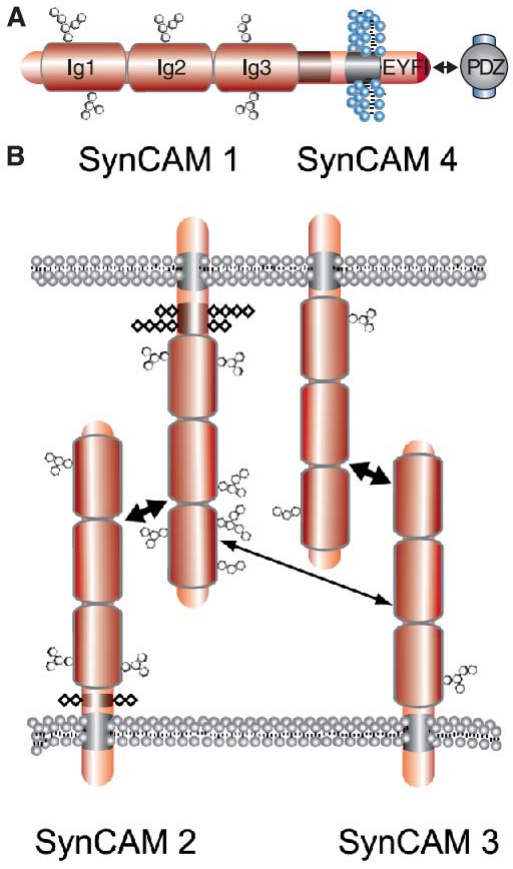

Figure 4. SynCAM structure and assembly. **(A)** Structure of SynCAM1[2]. Ig1, Ig2, and Ig3 are the three immunoglobulin domains. EYFI is a sequence of four carboxyl terminal **amino acids** (Glutamate-Tyrosine-Phenylalanine-Isoleucine) that facilitate binding to the PDZ domains of synaptic scaffolding proteins. **(B)** A model of heterophilic binding between the four SynCAM family members in mice. Strong preferential interactions are shown with bold arrows between SynCAM1/2 and SynCAM3/4; SynCAM1 and 3 can also form a weak interaction, as indicated by the thinner arrow.

Six **orthologs** of SynCAM **genes** have been identified in zebrafish, and their developmental expression patterns have been characterized previously in the Washbourne lab[6]. The coding sequences of the zebrafish SynCAM genes display the same protein domain organization as the SynCAM family members of tetrapod species

(such as mice)[6]. The conservation between these genes in zebrafish and mammals suggested that their functions were also likely conserved. The six SynCAMs were found to be highly regulated during the development of the CNS in zebrafish, and their expression patterns were dynamic[6]. These results suggest that such cell adhesion molecules may play a number of different roles during development, including processes in determining neuronal cell fate and synaptogenesis[6].

Synaptogenesis and mutations associated with SynCAMs are implicated in various neurological disorders such as autism spectrum disorder (ASD), mental retardation, and schizophrenia[3,7,8]. Alterations in neuronal circuitry and signaling have been proposed as the mechanistic agents responsible for the abnormalities in ASD, such as impaired social interactions, communication deficiencies, and restricted or repetitive behaviors[7,8]. ASD is one of the most heritable developmental neurological disorders, and **point mutations** in the family of cell adhesion molecules called neuroligins have been linked to ASD and mental retardation[7,8]. The human neuroligin protein family is encoded by five genes (NLGN1-4 and 4Y)[7]. In particular, point mutations in NLGN3 and 4 can lead to the intracellular retention of mutant proteins, and result in the loss of normal stimulation of presynaptic terminal formation[7]. Two **missense mutations** in the Ig3 domain of the human SynCAM1 gene have also been associated with ASD pathogenesis[8]. The mutant SynCAM1 protein exhibits a lower molecular weight, defective trafficking to the cell membrane, and is more susceptible to protein degradation or cleavage[8]. The mutations also result in morphological abnormalities, including shorter dendrites[9]. These findings support the hypothesis that alterations in

neuronal circuitry, via defective synaptogenesis, are involved in the development of ASD. Thus, the study of proteins involved in synaptogenesis and their functions at mature synapses will further our understanding of neurological development and disease.

Project Overview

The study of synapse formation involves many biochemical, molecular, and cellular techniques. Many of these techniques involve the use of **antibodies**, which can be made to bind a protein of interest specifically[10]. The current body of knowledge on synapses lacks great detail about the processes that occur following the release of neurotransmitters into the synapse, the events occurring in the synaptic cleft, and postsynaptically[11]. The elucidation of the elements that compose the **postsynaptic density (PSD)** is especially necessary[12]. The general aim of the research in the Washbourne lab is to further characterize the processes involved in synapse formation. This includes identifying proteins involved in the recruitment of the necessary components for the formation of a mature synapse. The goal of this project was to generate antibodies that would specifically recognize synaptic targets in order to better characterize the various processes of synaptogenesis, including the establishment of the postsynaptic apparatus and the recruitment of assorted structural proteins and neurotransmitter receptors.

A large number of commercial antibodies that recognize pre- and postsynaptic proteins in mice and other organisms have been screened for their ability to bind the

respective proteins in zebrafish. A significant number of antibodies successfully recognize **epitopes** in zebrafish by **Western blotting**. However, only three antibodies—anti-SV2, anti-Synapsin1/2, and anti-panMAGUK—have been identified as reliable markers in zebrafish **embryos** for **whole-mount staining**. Synapsins are neuron-specific proteins associated with small synaptic vesicles at the presynaptic terminal. Synapsin1 and 2 thus act as specific markers of presynaptic membranes throughout the nervous system[13]. The MAGUKs (membrane-associated guanylate kinase) are a large family of scaffolding proteins that are often associated with cellular junctions. This protein family is characterized by PDZ, **SH3**, and **GUK domains**. The anti-panMAGUK antibody recognizes multiple MAGUK proteins present at the postsynaptic membrane[14]. The combination of Synapsin1/2 and pan-MAGUK labeling allows the identification of the number and location of proximal pre- and postsynaptic structures. However, the locations of other synaptic components cannot be determined without other markers. Antibodies to presynaptic components, proteins of the PSD, and neurotransmitter receptors would help to create a more complete picture of synaptogenesis in developing zebrafish.

Clonal **hybridomas** were generated by injecting mice with purified **fractions** of **synaptoneurosomes** and PSD. A total of 96 clonal hybridomas were screened for the desired characteristics by **enzyme-linked immunosorbent assay** (ELISA), **immunofluorescence** (IF) staining, Western blotting, and **immunoprecipitation** (IP). Generally, many hybridomas recognized targets with high affinity, which translates to efficient recognition and binding of the antigen. A number of clonal and subclonal

hybridomas tested in immunolabeling and Western blotting showed specificity for neuronal and postsynaptic targets. Notably, a number of subclonal hybridomas labeled neuronal targets strongly in whole-mount IF. Additionally, both IP-ELISA and traditional IP showed that select subclonal hybridomas could **precipitate antigens** from **solution**, which allows the isolated antigens to be identified.

Antibodies as Molecular Biology Tools

Special tools and techniques are often required to probe the functions of complex biological systems and their various components. Scientific researchers have harnessed many pre-existing mechanisms from nature to serve as tools for various purposes. The use of antibodies in biological research is one such example. Antibodies are employed in a wide range of applications, including various imaging techniques such as **immunohistochemistry** (IHC) and immunofluorescence (IF) staining. They may also be used to purify a protein of interest in immunoprecipitation (IP). Early studies on the production and use of antibodies in molecular biology demonstrated that antibodies could be conjugated with a small-molecule dye without losing specificity for its antigen[15]. Subsequently, researchers discovered that larger fluorescent compounds could also be attached without loss of activity or specificity[16]. This allowed for the detection of specific antigenic material in animal tissue by optical rather than radiographic or analytic methods[16]. Techniques that utilize antibodies build on the principle that antibodies, also called immunoglobulins (Igs), bind protein targets very specifically[17].

For research use, antibodies can be produced to recognize a single **epitope** (**monoclonal antibodies**) or multiple epitopes of a specific antigen (**polyclonal antibodies**). The general scheme for antibody production is shown in **Figure 5**. First, the host animal (a mouse in this case) is injected with a purified antigen to elicit an

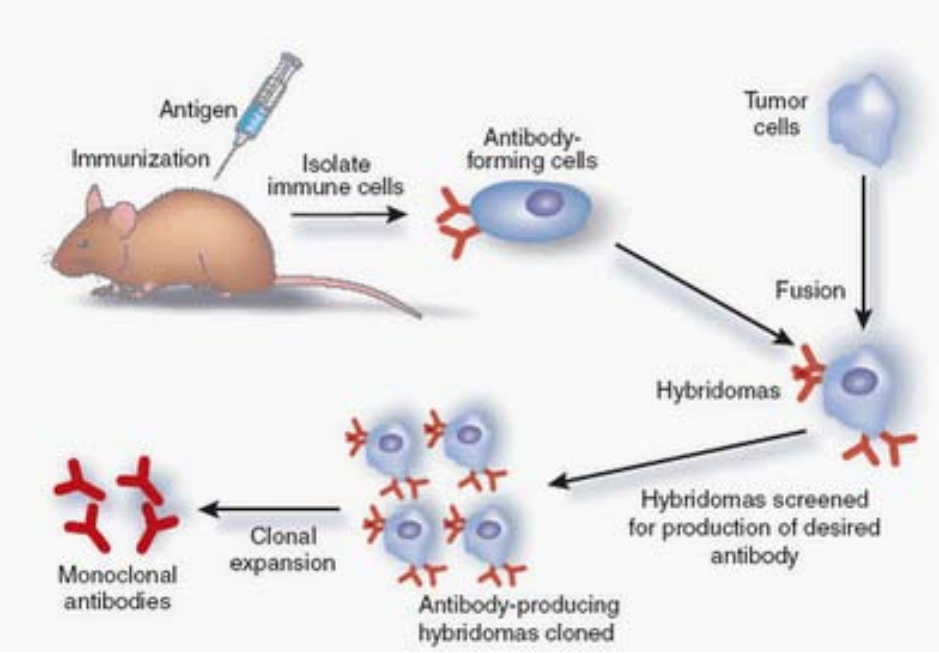

Figure 5. The basic procedure for monoclonal antibody production[ii].

immune response. Subsequently, the antibody-producing immune cells from the mouse are harvested and fused with cells from an immortal cell line that is able to divide indefinitely (usually from a tumor of the same **cell type**) to form a **hybridoma**. The production of hybridomas circumvents the short-lived nature of the antibody-producing cells. The successfully fused hybridomas are screened for production of the antigen of interest and grown in bulk to produce clones and **subclones** of the original antibody-producing cell. The clonal and subclonal hybridoma cells secrete antibodies into the cell

[ii] Figure source: http://biological-discoveries.blogspot.com/2009/04/monoclonal-antibodies.html

culture medium. The hybridoma **supernatants** can be further screened and characterized by various methods to identify positive hybridomas that produce monoclonal antibodies that bind an antigen of interest.

In staining methods such as IHC and IF, an "indirect" system is often used where two antibodies are used for the purposes of signal amplification and visualization[17]. The first antibody added is called the primary antibody, which recognizes and binds a specific protein of interest (e.g. Mouse-anti-SynCAM1: "mouse" indicates that the antibody was generated in mouse and "anti-SynCAM1" means that the antibody binds or is "against" the SynCAM1 protein). Subsequently, the secondary antibody is added, which recognizes and binds the primary antibody. Secondary antibodies typically have a fluorescent dye attached to them so that the location of antibody binding can be visualized (**Figure 6**). Several different types of Igs exist in the immune system and are designated by a letter indicating the class (e.g. IgG or IgA)[17].

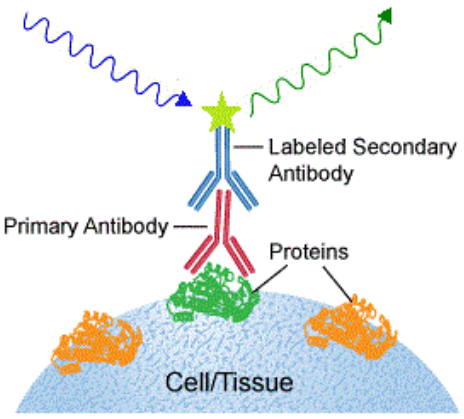

Figure 6. "Indirect" immunostaining. The secondary antibody is depicted with a fluorophore that is excited by blue light and emits green light.

Each Ig class or isotype has a slightly different function in the immune system. Secondary antibodies must only be specific to the isotype. If the primary antibody used was mouse-anti-SynCAM1 IgG, an example of a secondary antibody to use would be goat-anti-mouse IgG Alexa Fluor 488: "goat" indicates that the antibody was produced in goat, "anti-mouse IgG" means that the antibody recognizes

mouse IgGs and finally “Alexa Fluor 488” represents the excitation wavelength of the fluorescent Alexa Fluor dye (incident blue light of 488 nm will produce an emitted signal in the form of green light of ~510 nm). Such fluorescent labeling of antibodies allows for the identification and spatial localization of specific antigens by fluorescence microscopy.

Zebrafish as a Model Organism

Zebrafish (*Danio rerio*) serves as one of the model systems for investigating the processes and proteins involved in synaptogenesis in the Washbourne lab. Antibodies specific for zebrafish neuronal and synaptic proteins needed to be generated in order to visualize the spatiotemporal locations of the proteins that are involved in synapse formation. The relative novelty of the zebrafish as a **model organism** translates to fewer available research tools when compared to older models such as fruit flies and mice. As one of the few labs studying CNS development in zebrafish, we needed to develop tools for examining this system. The development of zebrafish antibodies that could specifically recognize neuronal and postsynaptic targets in the zebrafish nervous system can improve the application of this model organism in neuroscience research and aid in further characterizing synaptogenesis.

The zebrafish is a relatively novel model organism developed at the University of Oregon by George Streisinger. The zebrafish was being used as a developmental and embryological model as early as the 1930s[18]. However, zebrafish did not become a commonplace model organism until Streisinger pioneered the development of various

genetic techniques for zebrafish in the 1980s. Large-scale **genetic screens** of mutants in the 1990s further advanced the zebrafish as a mainstream developmental and genetic model organism[18]. Zebrafish are extremely useful for studying developmental phenomenon such as synaptogenesis. Because the fish embryos develop outside the body of the mother (i.e. *ex utero*), researchers have access to early embryonic stages beginning with the fertilized zygote (**Figure 7**)[18]. For developmental studies, fish embryos confer a large advantage over mice, which are placental mammals that must develop inside the uterus[18]. This *in utero* development of mouse embryos limits the number and types of genetic manipulations possible during early stages of development because of the necessity of the placenta[18]. Observation of early developmental

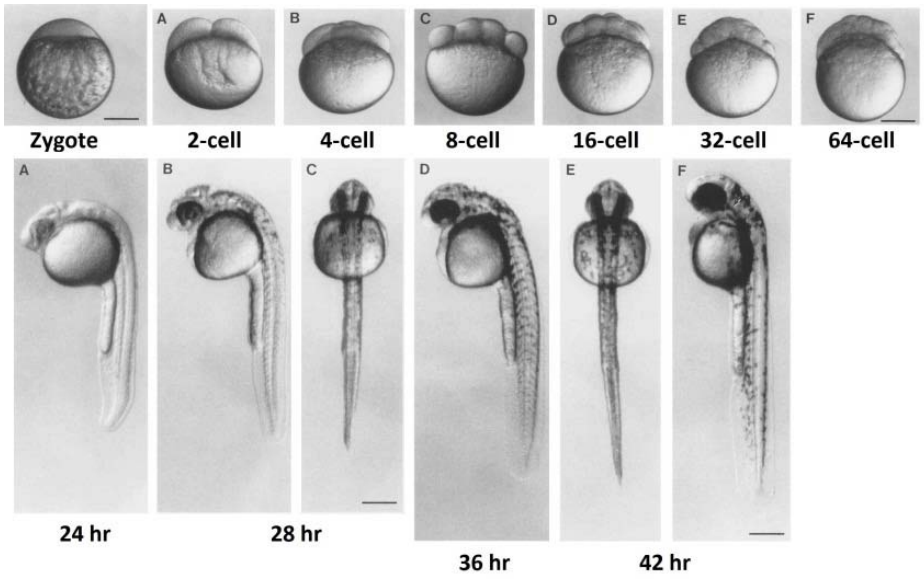

Figure 7. Select stages of zebrafish embryonic development[19]. The easily-accessible fertilized zygote allows for convenient genetic manipulations and the translucent embryo permits the monitoring of early embryonic development.

phenomena are also difficult in placental animals. In addition to *ex utero* embryonic development, the optical transparency of zebrafish embryos allows researchers to study

real-time maturation of specific structures or systems of interest, such as the growth and formation of synapses in the nervous system[18]. Zebrafish also develop on a relatively fast timescale and reproduce abundantly, which allows for large-scale genetic manipulations to investigate the biological process or system of interest[18]. The optical transparency and *ex utero* embryonic development are unique traits of zebrafish that confer a distinctive benefit to employing the zebrafish as a neurodevelopmental model organism.

Materials and Methods

The approach of this project was composed of three general phases: antigen isolation, antibody production, and antibody screening/characterization. The first phase involved purifying synaptoneurosomes and PSDs from adult zebrafish. The NeuroMab Facility at the University of California, Davis completed the second phase of the process by injecting the purified synaptoneurosomes and PSDs into mice to produce an immune response. Finally, the screening and characterization were accomplished through various methods, as described below.

Isolation of Antigen

The production of monoclonal antibodies was inspired by previously published methods[20,21]. The immune stimulus in this case was purified zebrafish PSD, which were isolated according to methods adapted from Villasana et al.[22].

Isolation of Synaptoneurosomes

Brain tissue was dissected from adult zebrafish and homogenized in synaptoneurosome **buffer** at 4 °C using a Teflon-glass electrical tissue grinder. The **homogenate** was diluted in a 1:1 ratio with synaptoneurosome buffer and briefly sonicated with three pulses on output power 1 on a 60Sonic dismembrator (Fisher Scientific). The sample was placed in a 60-milliliter (mL) Luer-lock syringe and strained twice through three layers of pre-wetted 100-micrometer (μm) pore 13-millimeter (mm) diameter nylon filters. The primary **filtrate** was again strained through a pre-wetted 5-μm pore 13-mm hydrophilic filter in small amounts. The resulting filtrates were pooled in a 50-mL

polycarbonate tube and **centrifuged** at 1000 times the force of gravity (1000×g) for 10 minutes. The resulting pellet contained the synaptoneurosome fraction and was resuspended in 5 mL of 0.32 molar (M) sucrose and 1 millimolar (mM) sodium bicarbonate ($NaHCO_3$) at **pH** 7.0 and stored at -20 °C.

Isolation of Postsynaptic Densities

Isolated synaptoneurosomes were diluted 1:1 with PSD dilution buffer. Samples were stirred in open-top tubes for 15 minutes at 4 °C and centrifuged at 33000×g for 20 minutes. The pellet was resuspended in 50 microliters (μL) synaptoneurosome buffer and layered onto a sucrose gradient containing 1.0 mL 1.0 M sucrose-1 mM $NaHCO_3$ and 1.0 mL 1.5 M sucrose-1 mM $NaHCO_3$ and centrifuged for 2.5 hours at 167000×g in a swing-bucket rotor. The cloudy band between the two layers was removed and suspended in 3.0 mL of a solution containing a 1:1 ratio of synaptoneurosome buffer and a detergent additive (1% Triton X-100 and 0.15 M KCl). The sample was again centrifuged at 237000×g for 15 minutes. The final pellet containing the PSD fraction was resuspended in 600 μL synaptoneurosome buffer.

Antibody Production

The NeuroMab facility at the University of California, Davis produced clonal hybridomas according to the general method described in the Introduction. Mice were injected with purified PSD, and the spleen was harvested for antibody-producing cells. The spleen cells were fused with an immortal cell line to produce hybridomas. A total of 96 clonal hybridomas were sent to the University of Oregon to be screened.

Screening and Characterization of Antibodies

The hybrdomas generated at NeuroMab were screened using a variety of methods, as described below.

Immunoprecipitation/Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was used in conjunction with the principle of immunoprecipitation (IP) to measure the strength of the interaction between the antibody and antigen and to test whether the antibodies were able to pull the antigen out of solution (this screening approach will be referred to as IP-ELISA) (**Figure 8**). All incubations were completed in humid boxes and all washes were for 45 minutes in three separate changes of solution. 96-well microplates were coated with goat anti-mouse **serum** (100µL/well) and incubated overnight at 4 °C. The wells were washed with 300 µL **PBST** and incubated with Roche blocking reagent (RBR) [23] in **PBS** for 2 hours at room temperature (RT). The wells were washed again and a different antibody serum (100µL per well) was added to each set of three wells (i.e. each antibody was tested in triplicate). The plates were incubated for 2 hours at RT and washed again with PBST. Biotinylated zebrafish PSD diluted in

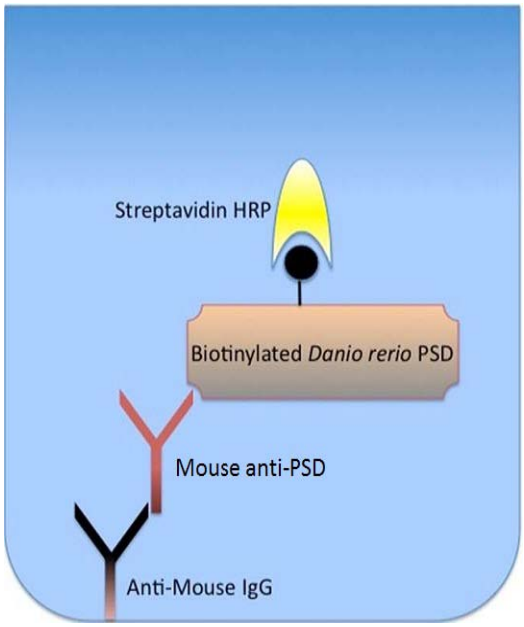

Figure 8. A schematic diagram of a single well in the IP-ELISA assay. The interaction of interest (in this case) is the interaction between the mouse-anti-PSD antibody (from the hybridoma) and the PSD protein sample. The strength of the interaction is detected as the absorbance at 450 nm.

diluents buffer (0.015% lauryl maltoside (LM)/1X RBR/PBS) to 12 μg/mL was added (100μL/well) and incubated at RT for 1 hour then washed with 0.015% LM/PBS. High sensitivity streptavidin-horseradish peroxidase (**HRP**) was diluted 1:6000 in diluents buffer and incubated at RT for 1 hour and washed with 0.015% LM/PBS. 3,3',5,5'-tetramethylbenzidine (TMB)-stabilized chromogen was added (100μL/well) to begin the oxidation reaction, which produced a blue color change. 1 M HCl (100μL/well) was added to stop the oxidation reaction, which produced a color change from blue to yellow. The extent of binding corresponded to the intensity of the yellow color, which was determined by measuring the absorbance at 450 nm with a **spectrophotometer**. The monoclonal antibodies were tested against several controls: anti-panMAGUK, heart bleed, normal mouse serum (NMS), anti-HA.11, anti-SP2, anti-GST, and a blank control in which no clonal antibody was added[iii]. The IP-ELISA assay was performed for all clonal and subclonal hybridomas.

Immunofluorescence Staining and Imaging

Both tissue sections and whole zebrafish embryos were used to identify some of the structural targets of the antibodies in the brain and spinal cord. Immunofluorescence (IF)

[iii] Anti-panMAGUK recognizes multiple MAGUK proteins at the postsynaptic membrane (obtained from Millipore). Heart bleed is blood from the immunized mouse (N286) after the immunization process. The sample was provided by NeuroMab. Blood drained from the heart of the immunized mouse should contain antibodies that recognize proteins from zebrafish. NMS is the pre-immunization (normal) serum of the N286 mouse, which should not contain any antibodies that would recognize zebrafish proteins (provided by NeuroMab). Anti-HA.11 binds a sequence of twelve amino acids (CYPYDVPDYASL) from the influenza hemagglutinin (HA) (obtained from Covance). Anti-SP2 recognizes the Sp2 transcription factor present in a number of organisms including humans, mice, and zebrafish (obtained from Abcam). Anti-GST recognizes a small protein called Glutathione-S-Transferase (GST) present in a large number of organisms (obtained from Millipore).

staining employs the specific binding of an antibody to its target together with fluorescent dyes to allow the researcher to visualize the anatomical location of the target. Mueller and Wullimann's developmental atlas of the zebrafish brain was used to help identify important regions[24]. IF experiments with **coronal** sections and whole-mount embryos were completed for N286/9, 21, 52, 74, 76, 79, 9.2, 9.4, 21.9, 21.11, 52.1, 52.3, 74.1, 74.4, 76.1, 76.3, 79.2, and 79.5. Zebrafish embryos at five days-post-fertilization (dpf) were fixed in 4% paraformaldehyde (PFA) in 1X fix buffer (5.33% sucrose, 0.2 μM $CaCl_2$, 0.133 M PO_4 buffer, pH = 7.3)[25] overnight and washed in three changes of PBS for 15 minutes before cryosectioning. The embryos were cryosectioned either through the **sagittal** or coronal plane into 16 μm slices and embedded in agarose on glass slides. The slides were washed for 5 minutes in PBS and incubated with 10% normal goat serum (NGS), 0.5% RBR/1X PBS for at least 1 hour at RT. Individual clonal antibodies (Mouse IgG) were added at a 1:5 concentration with synapsin-1,2 (Rabbit IgG) at a 1 :750 concentration. The primary antibodies were incubated overnight at 4 °C and washed for 45 minutes in three changes of PBS. The secondary antibodies goat-anti-mouse IgG Alexa Fluor 488 and goat-anti-rabbit IgG Alexa Fluor 546 were each added at 1:500 and incubated for 2 hours at RT. The tissue was washed for 45 minutes in three changes of PBS again and mounted with Vectashield Hard Set Mounting Fluid with a coverslip.

For whole-mount IF, **wild type** zebrafish embryos at ~26 hours-post-fertilization (hpf) were fixed in 4% PFA/1X zebrafish fix buffer[25] for 2 hrs at 4 °C. 2% NGS in **PBDTx** was added and incubated for 2 hrs at RT. Individual clonal antibodies (mouse

IgG) were added at a 1:10 concentration with anti-synapsin-1,2 (rabbit IgG) at a 1:1000 concentration or anti-snap25 (rabbit IgG) at a 1:500 concentration. Antibodies were incubated with the embryos overnight at 4 °C. The tissue was washed for 1 hour in three changes of PBDTx at RT. The secondary antibodies goat-anti-mouse IgG Alexa Fluor 488 and goat-anti-rabbit IgG Alexa Fluor 546 were each added at 1:500 and incubated with the tissue at RT for 5 hours in the dark. The samples were washed for 30 minutes in two changes of PBDTx at RT. The stained embryos were added to 80% glycerol in PBS and allowed to sink overnight. Embryos were subsequently mounted on glass slides with single-bridged coverslips and imaged by confocal fluorescence microscopy.

SDS-PAGE/Western Blotting

Sodium dodecyl sulfate polyacrylamide **gel electrophoresis** (**SDS-PAGE**) and Western blotting were used to analyze the approximate size of the antigen(s) for each antibody and to screen for the antibodies that specifically bound neuronal and/or PSD targets. In a given protein preparation, SDS-PAGE separates the proteins by size and Western blots show whether an antibody of interest binds to any of the proteins in the mixture. Whole-brain and muscle tissue were dissected from adult zebrafish and homogenized using a tissue grinder. The protein concentration of these and previously prepared PSD samples were determined by the Bio-Rad protein assay[26]. Mixtures containing 5 μg of protein were loaded into the SDS-Polyacrylamide gel and electrophoresed at 80 V. Because the polyacrylamide gel is extremely fragile, the proteins from the gel were transferred onto a nitrocellulose membrane with an electric current of 110 V. The nitrocellulose membrane was washed for 15 minutes in three changes of PBS and

incubated with 3% milk in PBST for 1 hour at RT on an orbital shaker. The clonal antibodies were added at a concentration of 1:150 and incubated overnight at 4 °C. The membrane was washed for 45 minutes in three changes of PBST and secondary antibodies (HRP-conjugated donkey anti-mouse IgG in 3% milk) were added at a concentration of 1:1000 and incubated for 2 hours at RT. The membrane was again washed for 45 minutes in three changes of PBST and Pierce enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Scientific) was added for 1 minute[27]. The membrane was dried and exposed to film, which was developed to visualize the binding pattern.

Immunoprecipitation

Immunoprecipitation (IP) was used to further determine the specificity of the interaction between antibodies and their antigens. IP can allow for antigens to be isolated and identified by taking advantage of the specific interactions between the antibody and its antigen. **Figure 9** below depicts the basic steps of a traditional IP. Protocols from Abcam and GE Healthcare were followed with some adaptations [28,29]. Brain tissue was dissected from adult zebrafish and homogenized in **RIPA buffer** at 4 °C using an electrical tissue grinder. The homogenized sample (~5 mg/mL tissue) was placed on an orbital shaker at 4 °C for 2 hours and centrifuged for 20 min at 14,000×g. The supernatant was aspirated into a fresh tube. To reduce non-specific interactions between the sepharose beads and the neuronal tissue sample, Protein G Sepharose 4 Fast Flow beads (GE Healthcare) were washed for 15 minutes in three changes of PBS and added to the homogenized tissue at a concentration of 1:20. The mixture was placed on an

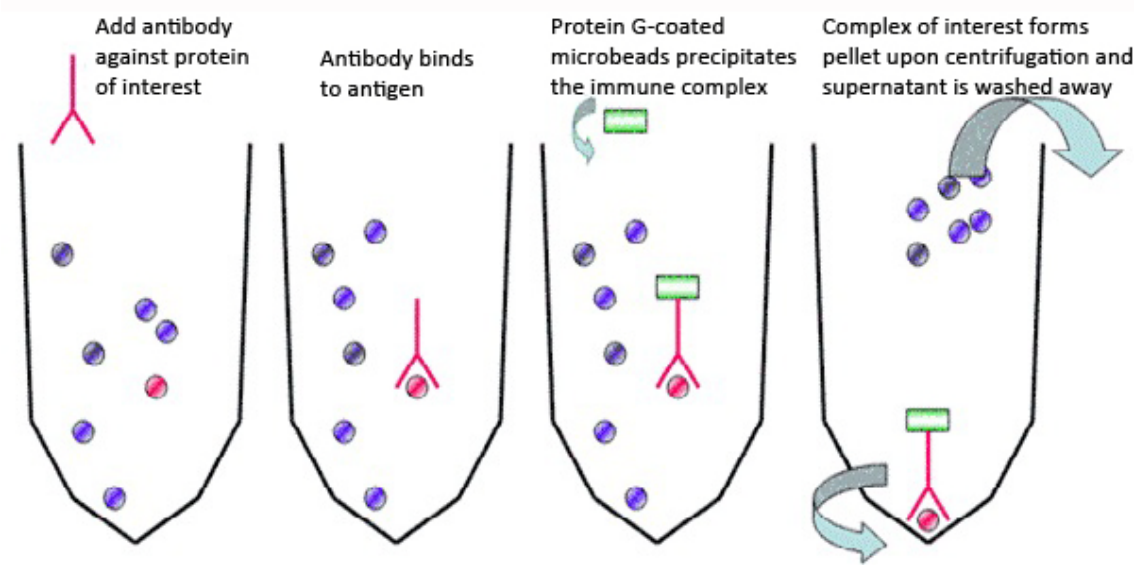

Figure 9. Diagram of a traditional IP assay.

orbital shaker at 4 °C for 1 hour and centrifuged at 12,000×g for 20 seconds in a tabletop microcentrifuge. The supernatant was aliquoted into new tubes. The antibody serum of interest was added at a 1:6 concentration and incubated overnight at 4 °C. Protein G Sepharose beads were added at a 1:10 concentration and mixed gently for 1 hour at 4 °C. The mixture was centrifuged at 12,000×g for 20 seconds, and the pellet was washed five times with five times the sample volume of RIPA buffer. Alternatively, the pellet was washed three times with RIPA buffer and twice with 1 M NaCl. The samples were centrifuged at 12,000×g for 20 seconds between each wash step. The final pellet was suspended in 30 µL SDS-PAGE sample buffer and heated to 95 °C for 3 minutes. The sample was centrifuged at 12,000×g for 20 seconds and the supernatant was analyzed by SDS-PAGE followed by Western blotting and/or Silver staining.

Silver Staining

The Fast Staining Protocol from the Invitrogen SilverQuest™ silver staining kit was used to stain polyacrylamide gels from SDS-PAGE[30]. Following electrophoresis, the gel was placed in a clean microwaveable staining tray and rinsed briefly with ultrapure water. The gel was placed in fixative (40% ethanol, 10% acetic acid in ultrapure water), microwaved at high power for 30 seconds, and gently agitated at RT for 5 minutes. The fixative was decanted and 30% ethanol was added. The gel was microwaved at high power for 30 seconds, gently agitated for 5 minutes at RT, and the ethanol was decanted. Sensitizing solution (30% ethanol, 10% Sensitizer in ultrapure water) was added; the gel was microwaved at high power for 30 seconds and agitated for 2 minutes at RT. The sensitizing solution was decanted and the gel was washed twice with ultrapure water (the gel was microwaved at high power for 30 seconds and agitated for 2 minutes at RT for each wash). Staining solution (1% Stainer in ultrapure water) was added; the gel was microwaved at high power for 30 seconds and agitated at RT for 5 minutes. After decanting the staining solution, the gel was gently agitated in ultrapure water for ~30 seconds. The water was decanted and the gel was incubated with Developing solution (10% Developer, 1 drop/100mL Developer Enhancer in ultrapure water) for ~5 minutes at RT with gentle agitation until the desired exposure was achieved. Without decanting the Developing solution, 10% Stopper solution was added directly and the gel was gently agitated for 10 minutes at RT. Finally the gel was washed with ultrapure water for 10 minutes at RT.

Results

A variety of characterization methods were employed in this project to screen the large number of clonal hybridomas generated. The richness and variety of PSD proteins increased the probability of finding multiple monoclonal antibodies that would recognize specific targets when mice were immunized against adult zebrafish PSDs. The production of antibodies that would recognize synaptic proteins in zebrafish allows for further characterization of the components involved in recruiting the necessary apparatus for the formation of a mature synapse. As a model organism, zebrafish provide researchers with unprecedented versatility in genetic manipulations and allow the study of various developmental phenomena in a vertebrate system. The relative novelty of the zebrafish served as the motivation for improving and finding more molecular tools in order to gain a fuller understanding of development in zebrafish and in vertebrate organisms in general.

A total of 96 hybridoma supernatants were received from NeuroMab. Each hybridoma was derived from clones of a single cell, and will be referred to as N286/x, where N286 designates the mouse from which the antibody-producing immune cells were harvested and x is a number corresponding to the specific antibody-producing hybridoma. Clonal hybridomas were analyzed by IP-ELISA, IF, and Western blotting. Clones were ranked based on their performance in each screen, and the highest ranking and/or most unique clones from each screen were chosen to be expanded, or subcloned. Selected clonal and subclonal hybridomas were further analyzed via IP-ELISA, sectional and whole-mount IF, and IP.

Preliminary Analysis of Clonal Antibodies via IP-ELISA

Upon receiving the hybridomas from NeuroMab, the supernatants were screened by IP-ELISA, a method through which the degree of antigen binding and the ability of the antibody to "fish" the antigen out of solution could be measured. All 96 clonal supernatants were tested in triplicate twice (see **Figure 10** for a sample 96-well plate). The absorbance at 450 nm (A_{450}) was averaged over the six wells for each sample, and the average A_{450} measurements ($\langle A_{450} \rangle$) for the 96 parent hybridoma supernatants are given in **Supplemental Table I-IV**. The clonal hybridomas were tested against

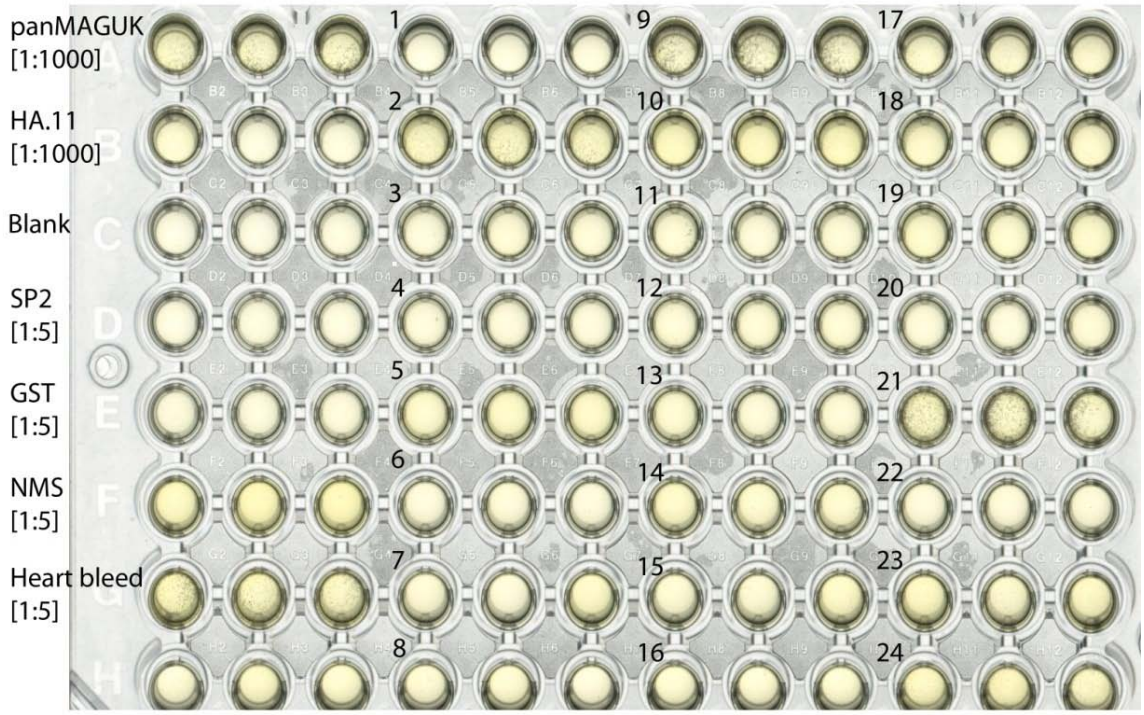

Figure 10. IP-ELISA screening for clones N286/1-24. Each clonal hybridoma and control was tested in triplicate to acquire the most precise measurement. The blue to yellow color change produced at the end of the assay was measured by a spectrophotometer as the intensity of the absorbance at 450 nm.

two positive controls[iv]—anti-panMAGUK and heart bleed—and four negative controls[v]: normal mouse **serum** (NMS), anti-HA.11, anti-SP2, and anti-GST (**Figure 10**). An additional negative control ("blank") with no clonal antibody was also included. For each plate, a standard deviation (σ_-) was calculated from all the negative control samples according to the following equation:

$$\sigma_- = \sqrt{\frac{\sum_n (x - \langle x \rangle)^2}{(n-1)}} \qquad [1]$$

In equation 1, x, $\langle x \rangle$, and n represent the absorbance reading of a sample, the average absorbance reading of all samples, and the total number of samples, respectively. The capital sigma (Σ) denotes a summation of the terms following the symbol, which in this case is over all n, meaning that each x value for the n samples was added. Each clone was ranked based on a value **α** given by equation 2:

$$\alpha = \langle A_{450} \rangle / 3\sigma_- \qquad [2]$$

α expresses the ratio of the average absorbance reading $\langle A_{450} \rangle$ to three times the standard deviation of the negative controls ($3\sigma_-$). Multiplying σ_- by three ensured that high values of α would be significant and would indicate a strong interaction between

[iv] Anti-panMAGUK recognizes multiple MAGUK proteins at the postsynaptic membrane (obtained from Millipore). Heart bleed is blood from the immunized mouse (N286) after the immunization process. The sample was provided by NeuroMab. Blood drained from the heart of the immunized mouse should contain antibodies that recognize proteins from zebrafish.

[v] NMS is the pre-immunization (normal) serum of the N286 mouse, which should not contain any antibodies that would recognize zebrafish proteins (provided by NeuroMab). Anti-HA.11 binds a sequence of twelve amino acids (CYPYDVPDYASL) from the influenza hemagglutinin (HA) (obtained from Covance). Anti-SP2 recognizes the Sp2 transcription factor present in a number of organisms including humans, mice, and zebrafish (obtained from Abcam). Anti-GST recognizes a small protein called Glutathione-S-Transferase (GST) present in a large number of organisms (obtained from Millipore).

the antibody and antigen. IP-ELISA results were synthesized with IF and Western blotting data to determine which hybridomas would be expanded to produce subclones. The expanded clones all met the criteria of $\alpha > 3$ (**Table 1**).

Table 1. IP-ELISA Results for Selected Clonal Hybridomas

Clone	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$	Clone	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
2	1.7171	3.8057	62	0.3221	3.1395
9	2.9972	6.6430	73	0.5025	3.6952
21	3.1106	6.8943	74	2.7494	20.2164
29	1.8293	14.6452	76	1.8927	13.9172
52	2.4022	23.4133	79	2.4821	18.2504
57	1.8720	18.2460	81	1.2294	9.0395

Absorbance values were measured with a spectrophotometer at 450 nm and averaged over multiple sample wells for each antibody. The standard deviation (σ_{-}) of the negative controls (blank, anti- NMS, anti-HA.11, anti-SP2, and anti-GST) was calculated for each IP-ELISA experiment. The value α gives a quantitative comparison of the $\langle A_{450} \rangle$ for each antibody with respect to the negative controls.

Preliminary Immunofluorescence Staining

The 96 clonal antibodies were analyzed by IF staining to determine which clones possessed a sufficiently robust and specific interaction to garner a strong signal in this assay. The N286 clones were applied as the primary antibody to sagittal sections of 5 dpf zebrafish embryos. Goat-anti-mouse IgG Alexa Fluor 488 was added as the secondary antibody to visualize the location of the primary antibodies. The stained tissue sections were imaged via confocal fluorescence microscopy. All clones were scored qualitatively based on overall fluorescence intensity and specificity of staining in neuronal structures. The scores and rankings of selected clones are shown in **Table 2,** all others are shown in **Supplemental Table IX**. **Figure 11** shows two example images of IF stains. This evaluation showed that a number of clonal hybridomas appeared to

label specific layers of the eye and the optic tectum (TeO). These areas exhibited a higher relative intensity of staining when compared to background levels, and the qualitative nature of this scree did not require further quantitative image analysis.

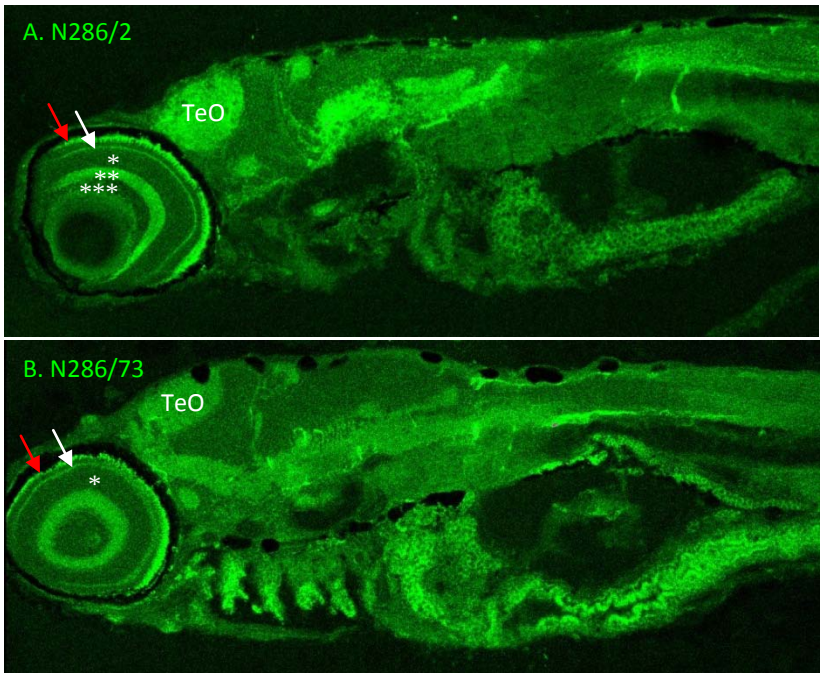

Figure 11. Immunofluorescence staining of sagittal sections of 5 dpf zebrafish with hybridomas N286/2 **(A)** and N286/73 **(B)**. Staining in the different layers of the eye and the optic tectum were observed, suggesting that these hybridomas specifically neuronal targets.

Table 2. Preliminary Immunofluorescence Rankings for Selected Clones

Clone	IF Score (1-5)	IF Rank	Clone	IF Score (1-5)	IF Rank
2	3	11	62	3	16
9	3	12	73	5	1
21	4	5	74	5	2
29	0	67	76	4	8
52	4	6	79	5	3
57	3	15	81	0	89

Tissue sections were imaged by confocal microscopy, and each clone was evaluated qualitatively based on fluorescence intensity and specificity of staining in regions of high synaptic density. Each clone was given a score of 1-5 (with 5 as the highest score), and ranked accordingly.

Western Blot Analysis of Clonal Antibodies

Western blotting was used to estimate the size of the antigen and to screen for the clonal hybridomas that specifically recognized neuronal and PSD proteins (**Figure 12**). SDS-PAGE was used to separate the proteins of three tissue types by size. The three different tissue samples were non-neuronal homogenate derived from adult zebrafish muscle, neuronal homogenate derived from whole adult zebrafish brains, and purified PSD derived from the differential centrifugation of whole-brain neuronal homogenate. After transferring the proteins from an acrylamide gel to a nitrocellulose membrane, the clonal hybridoma supernatants were applied. The application of a secondary antibody allowed the locations of the primary antibodies to be visualized as a blot or band on light-sensitive film. These bands correspond to locations of antigens in the polyacrylamide gel that were recognized by the primary antibody. Each of the 96 parent

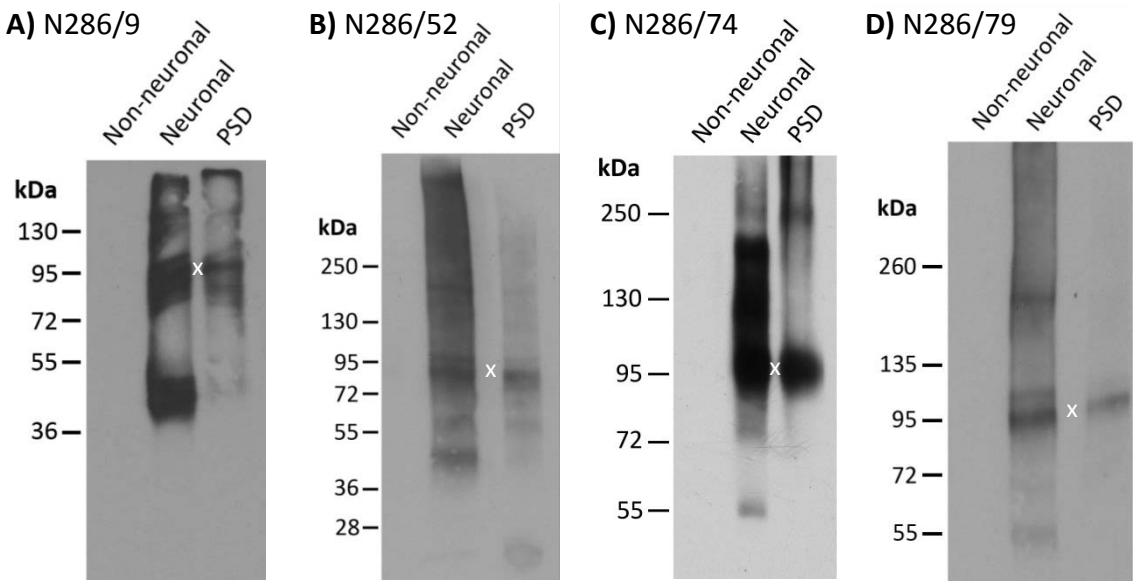

Figure 12. Western blots of N286/9, 52, 74, and 79. The dark bands present on the developed film correspond to the sizes of antigens recognized by the hybridoma for each blot. The banding patterns show that the antibodies exhibit specificity for neuronal tissue. The "x" marks the bands of interest at ~95-100 kDa.

clones were tested in Western blot analysis, and the results of four clonal antibodies that were further characterized are shown below in **Figure 12**. In all four blots, a prominent band was observed in both the neuronal homogenate and PSD at ~95-100 **kilodaltons** (kDa). The banding pattern of the PSD sample as compared to the neuronal homogenate indicates that the antibody recognizes the antigen in the purified PSD fraction with higher specificity.

Further Characterization of Selected Clones and Subclones

Based on the results of the preliminary analyses, twelve clones were expanded to obtain subclonal hybridomas. A total of 78 subclones were obtained from NeuroMab as hybridoma supernatants produced from the parent clones N286/2, 9, 21, 29, 52, 57, 62, 73, 74, 76, 79, 81. Similar to the initial characterization of the parent clones, all subclones were subject to analysis by IP-ELISA to determine the ability of the derived antibodies to recognize the antigen in solution and to quantify the affinity of the antibodies for their particular antigen. The IP-ELISA results for all expanded clones and corresponding subclones are shown in **Supplemental Table V-VIII**. Data for selected clones and subclones are included below in **Table 3**. The subclones were also analyzed by Western blotting. Similar to the clonal antibody experiments, the subclonal antibodies were used to detect proteins in non-neuronal, neuronal, and PSD tissue samples. **Figure 13** below shows the Western blots of selected subclones (blots for other subclones are not shown). Similar to the parent clones, these subclones also recognized a protein of ~95-100 kDa.

Table 3. IP-ELISA Results for Selected Clones and Subclones

N286/-	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$	N286/-	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
9	1.6550	143.0902	74	1.5877	79.8347
9.2	1.6495	142.6176	74.1	1.5200	76.4338
9.4	1.6607	143.5859	74.4	1.5384	77.3590
21	1.0300	69.0197	76	0.0118	1.0203
21.9	1.2515	83.8829	76.1	0.0165	1.4238
21.11	1.2599	84.4437	76.3	0.0304	2.6284
52	0.8157	54.6738	79	2.0986	205.0447
52.1	0.2433	16.3047	79.2	2.0356	198.8861
52.3	0.4137	27.7279	79.5	2.0905	204.2533

Absorbance values were measured with a spectrophotometer at 450 nm and averaged over multiple sample wells for each antibody. The standard deviation (σ_{-}) of the negative controls (blank, anti-HA.11, anti-SP2, and anti-GST) was calculated for each IP-ELISA experiment. The value α gives a quantitative comparison of the $\langle A_{450} \rangle$ for each antibody with respect to the negative controls. Multiple experiments are represented in this table; please refer to **Supplemental Table V-VIII** for the specific σ_{-} values for each IP-ELISA experiment.

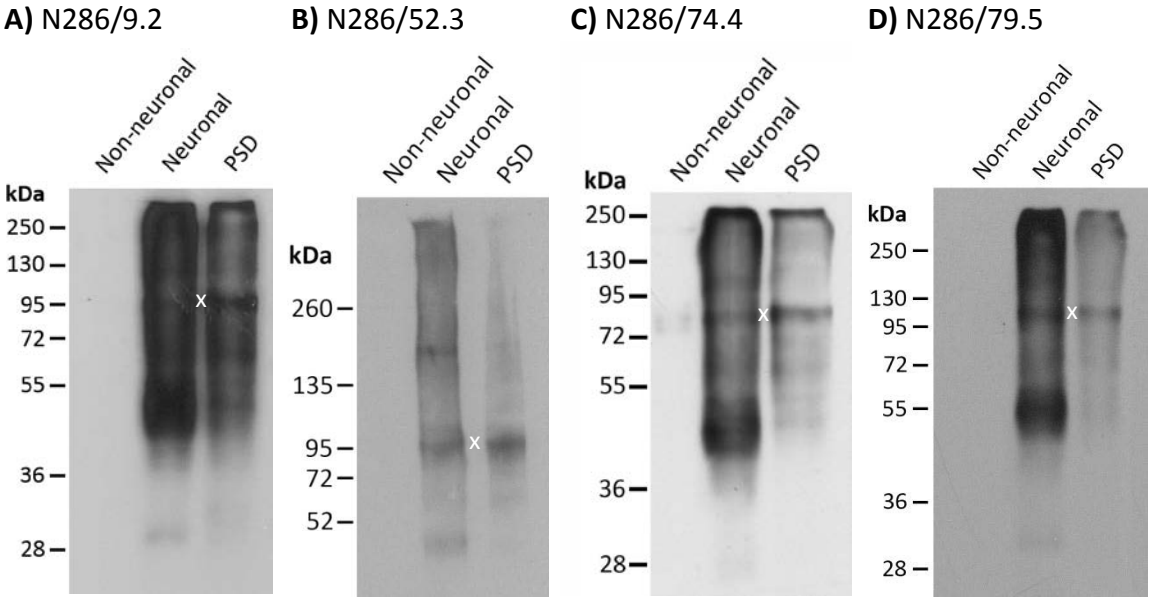

Figure 13. Western blots of N286/9.2, 52.3, 74.4, and 79.5. The banding patterns show that the subclonal antibodies also exhibit specificity for neuronal tissue. The "x" marks denote the bands of interest at ~95-100 kDa.

Select clones and the corresponding subclones were further analyzed via immunolabeling (**Figure 14-16**). For sectional stains, embryos at 5 dpf were fixed, the head was cryosectioned, and the tissue was mounted on glass slides. For whole-mount immunofluorescence (IF), entire embryos at ~26-28 hpf were fixed and used for whole-mount staining. In both types of stainings, the typical two-tiered antibody detection system was used with the clone or subclone acting as the primary antibody. In the IF experiments shown in **Figure 14-16**, a second primary antibody that binds known targets was used to identify overlapping targets (a technique called colocalization). The presynaptic marker synapsin1,2 was combined with the hybridomas to help locate synapses.

In the coronal sections, prominent staining was observed in the optic tectum (TeO) and the layers of the eye, including the photoreceptor layer and inner plexiform layer[24]. The whole-mount embryos at 10X magnification exhibited prominent staining in various structures, including the **dorsal** root ganglion (DRG), sympathetic ganglia, dorsal longitudinal fasciculus (DLF), and Rohon-Beard (RB) sensory neurons. At 60X magnification, RB cell bodies, dendrites, and axons showed clear labeling by the subclonal antibodies. RB neurons are a class of neurons that are only present and function during embryonic development in the spinal cord of fish and amphibians. In embryonic zebrafish, RB cells are responsible for sensing touch. The dendrites of RB neurons reach into the dorsal surface of the skin, and their axons form a bundle that extends into the DLF[31]. RB cells synapse onto commissural primary ascending (CoPA)

interneurons, which are the next cells in the neuronal network that relays sensory information from RB cells to the CNS[32].

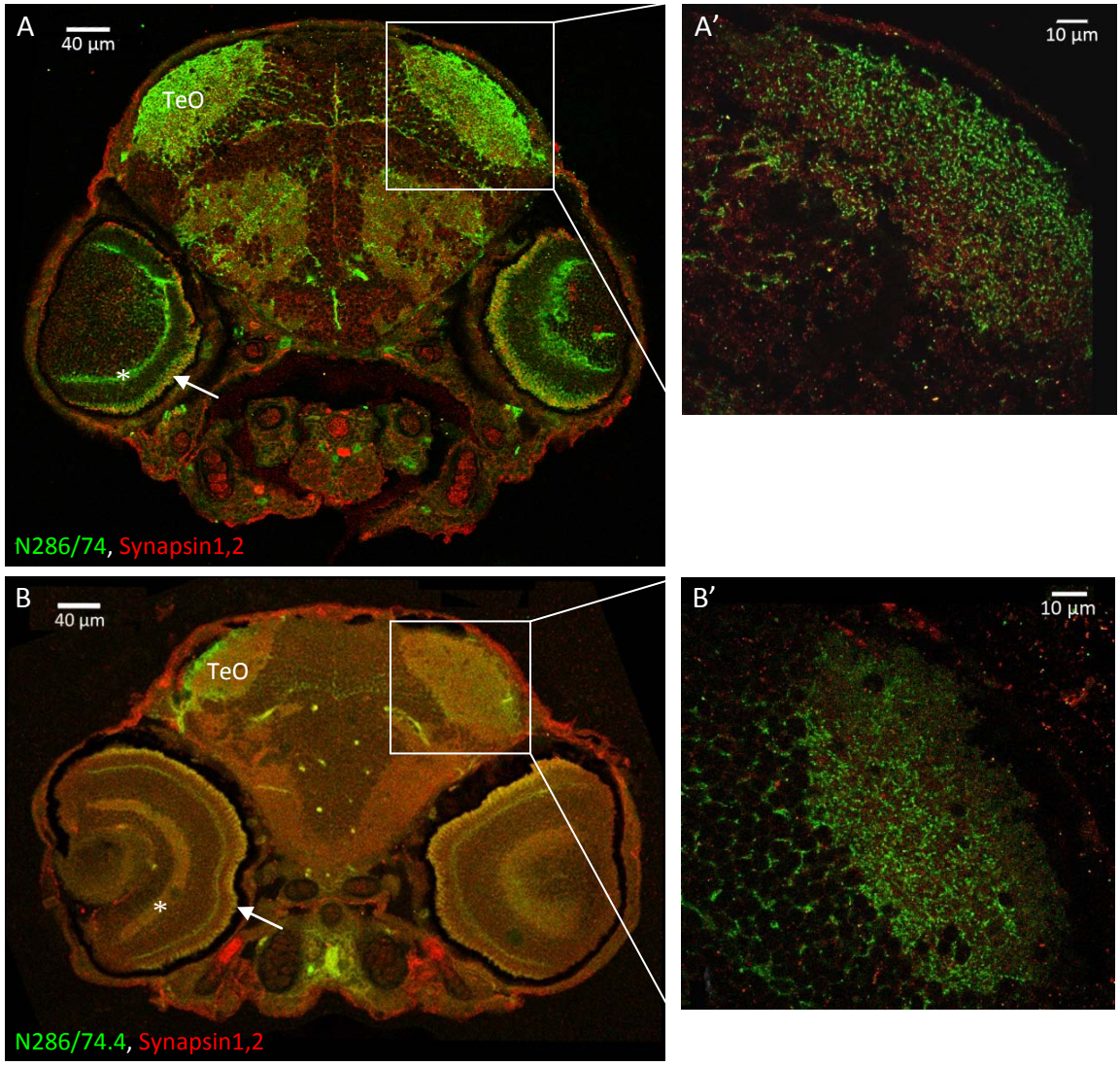

Figure 14. Coronal sections of zebrafish embryos at 5 dpf. **(A)** N286/74 and synapsin1,2 costaining at 20X and 100X **(A')**. **(B)** N286/74.4 and synapsin1,2 costaining at 20X and 100X **(B').** At 20X, the photoreceptor layer (arrow) and inner plexiform layer (*), exhibit prominent staining by the antibodies of interest[24]. The optic tectum (TeO), the main processing unit of visual input in fish, is expanded in **A'** and **B'** at 100X magnification[24]. The synapse-rich axonal projections of retinal ganglion cells and dendrites of tectal cells can be seen at both 20X and 100X. Colocalization between synapsin1,2 and the hybridoma of interest represents an overlap of synaptic targets.

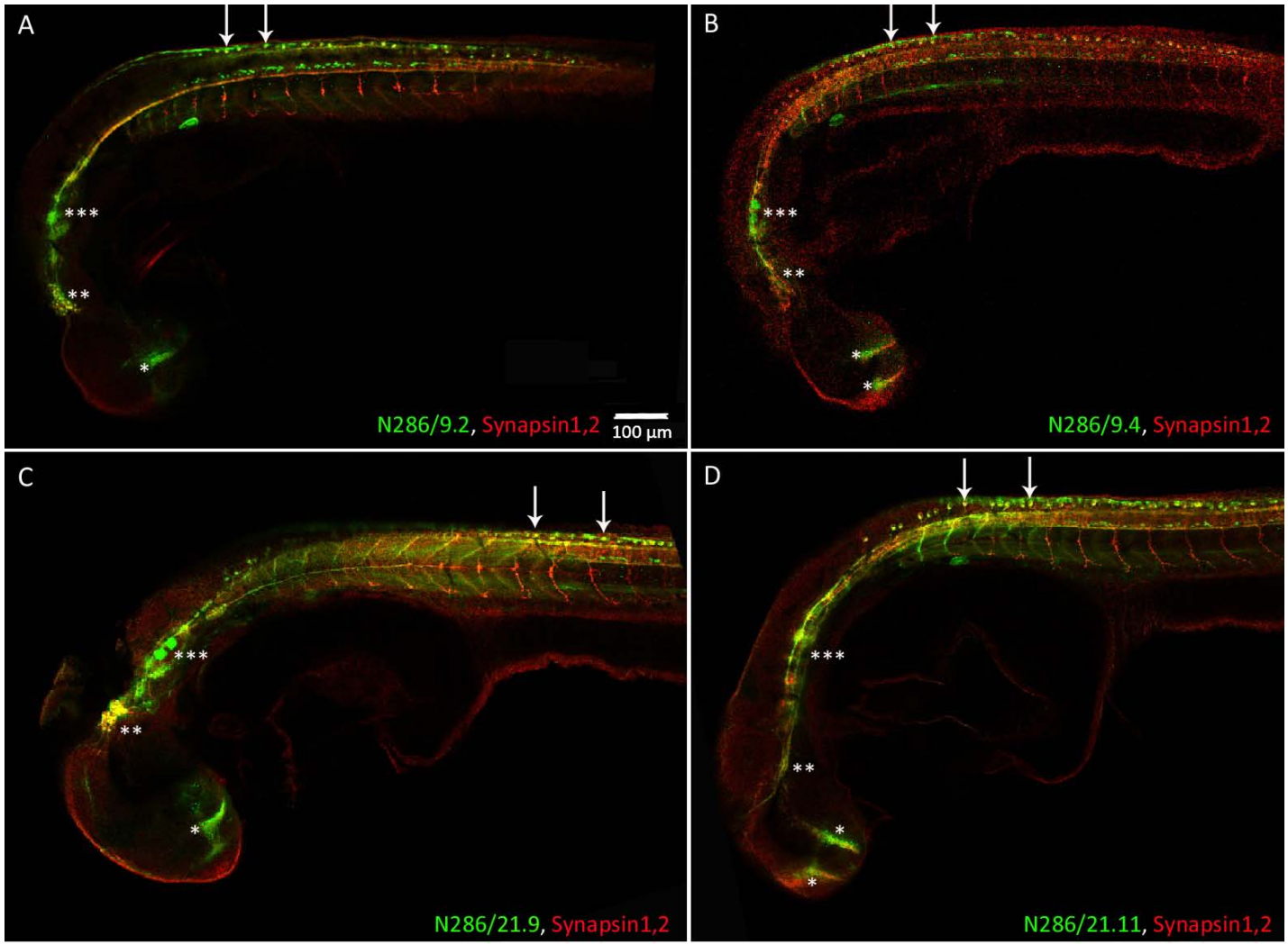

Figure 15. Whole-mount IF of 28 hpf embryos with **(A)** N286/ 9.2, **(B)** N286/9.4, **(C)** N286/21.9, **(D) N**286/21.11 costained with synapsin1,2 at 10X magnification. Prominent staining by the subclones can be seen in the olfactory bulbs (*), the DRG (**), sympathetic ganglia (***), and RB neurons in the spinal cord (arrows). Colocalization can be visualized in the RB cell bodies, dendrites, and axon bundles in the dorsal spinal cord.

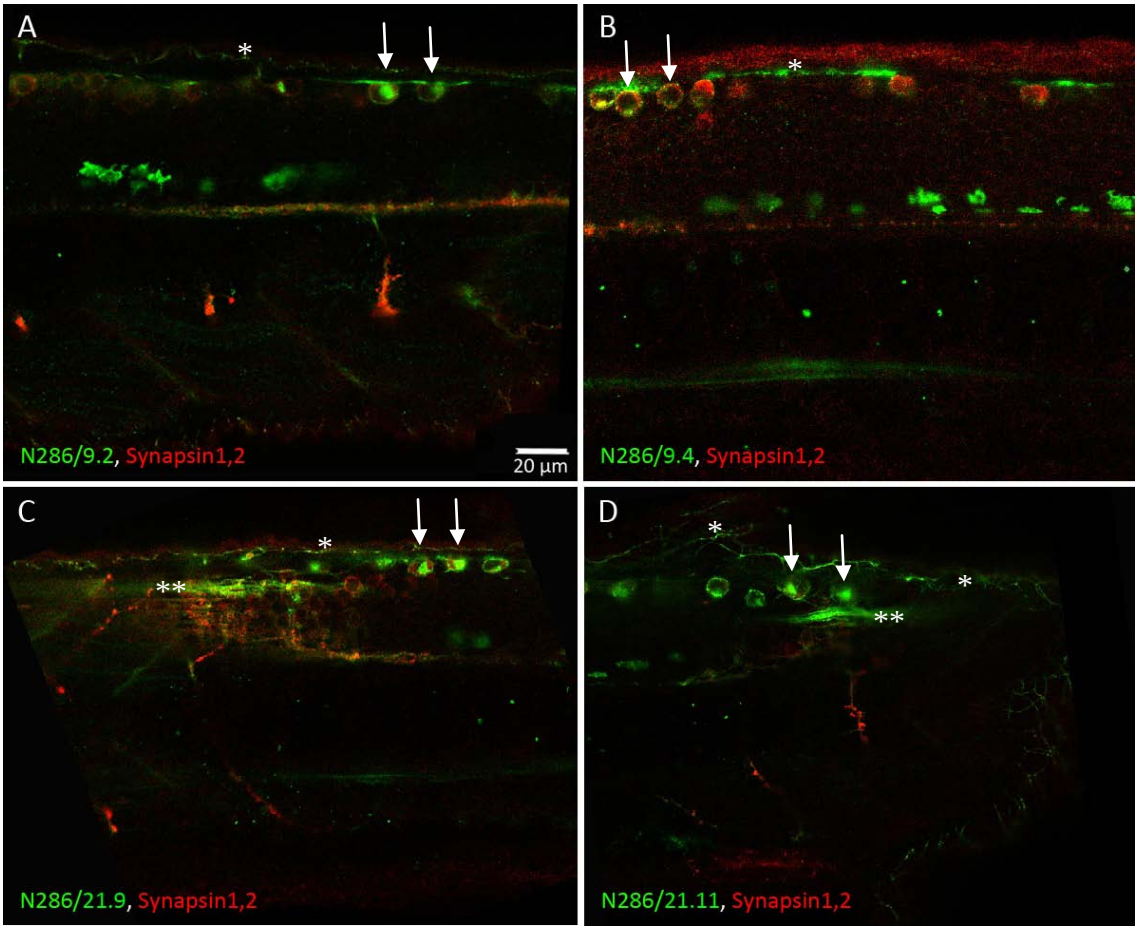

Figure 16. Whole-mount IF of 28 hpf embryonic spinal cord with **(A)** N286/ 9.2, **(B)** 9.4, **(C)** 21.9, and **(D)** 21.11 costained with synapsin1,2 at 60X magnification. Prominent staining by the hybridomas can be seen in the RB cells bodies (arrows), dendrites (*), and DLF (**) where the axons of RB neurons synapse onto CoPA interneurons in the dorsal spinal cord[31].

Finally, N286/9.2, 74.1, and 74.4 were also analyzed by immunoprecipitation followed by Western blotting. Immunoprecipitation detects specific interactions between an antibody and its antigen. IP uses that specific interaction to “pull” the antigen out of solution. Analysis of immunoprecipitated proteins by Western blotting verifies whether the proteins bound by the antibody in the IP assay are recognized as an antigen in the Western blot assay. In each IP experiment, the antibody of interest was

allowed to first form a complex with its antigen in neuronal homogenate or PSD solution. Secondly, microbeads coated with an IgG-binding protein (Protein G) were added to the solution, which precipitated the immune complex with the antigen-bound antibody. The bead/antibody/antigen complex was isolated from the solution upon centrifugation (**Figure 9**). Subsequently, the antigen(s) that were precipitated can be analyzed by SDS-PAGE and Western blotting. N286/74.4 was tested against two known postsynaptic antibodies—anti-PSD95 (**Figure 17A**) and anti-panMAGUK (**Figure 17B**). As noted previously, anti-panMAGUK recognizes a number of different

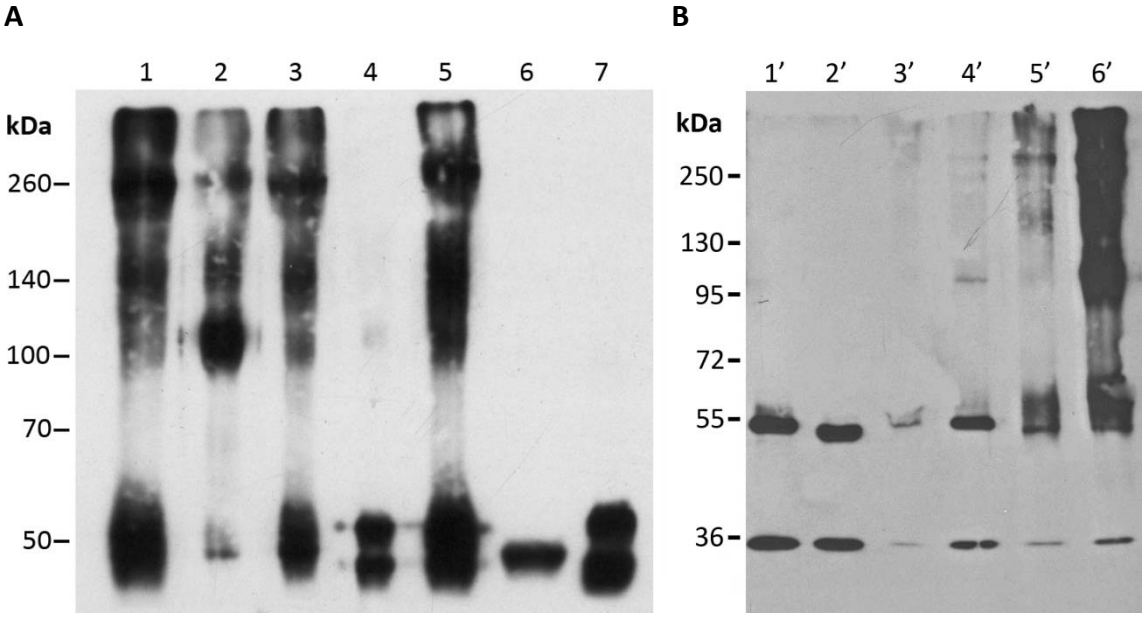

Figure 17. Immunoprecipitated samples were analyzed by SDS-PAGE/Western blotting. N286/74.4 was used as the primary antibody for the Western blot in both **A** and **B**. The IP experiment shown in blot **A** used anti-PSD95 as a control antibody; anti-panMAGUK was used as the control antibody in blot **B**.
A) 1: Neuronal homogenate, **2:** PSD, **3:** Neuronal homogenate IP with N286/74.4, **4:** PSD IP with N286/74.4, **5:** Neuronal homogenate IP with α-PSD95, **6:** PSD IP with anti-PSD95, **7**: N286/74.4 (antibody only)
B) 1': α-panMAGUK (antibody only), **2':** N286/74.4 (antibody only), **3':** PSD IP with anti-panMAGUK, **4':** Neuronal homogenate IP with anti-panMAGUK, **5':** PSD IP with N286/74.4, **6':** Neuronal homogenate IP with N286/74.4

MAGUK proteins present at the postsynaptic membrane[14]. Anti-PSD95 specifically recognizes PSD95, a member of the MAGUK family of proteins that acts as a structural support at the PSD. PSD95 also aids in the clustering of neurotransmitter receptors through its PDZ domain[33].

In **Figure 17**, the neuronal IP with N286/74.4 in both blots exhibit a band at ~100 kDa (see lanes 3 and 6'), similar to previous Western blots with the same antibody. Curiously in lanes 5 and 4', the neuronal IPs with anti-PSD95 and anti-panMAGUK, respectively, also exhibit a band of approximately the same size (~95-100 kDa). Additional bands were observed concomitantly with the main band of interest at ~100 kDa. Lane 7 in **Figure 17A** suggests that the lower weight bands around 50 kDa likely correspond to antibody self-recognition (i.e. the antibody used as the Western blot primary is recognizing unbound versions of itself from the IP sample). The higher weight bands may be due to protein-protein interactions between the antigen of interest and other proteins in the neuronal homogenate. Such interactions would result in protein complexes that appear as higher molecular weight bands.

Immunoprecipitation also allows the antigen of an unknown or novel antibody to be isolated and identified. In **Figure 18**, samples from an IP of neuronal homogenate with N286/74.4 were analyzed by Western blotting with N286/74.4 and Silver staining in parallel. The Silver stain allows the visualization of all the proteins present in the gel. Performing a Western blot in tandem to the Silver stain permits the identification of proteins specifically recognized by the antibody of interest. Different amounts of the IP samples were loaded in the SDS-PAGE gel to determine the optimal concentration of

protein for a clear banding pattern. In addition, samples in the odd-numbered lanes were washed with 1 M NaCl prior to analysis by SDS-PAGE to disrupt non-specific or relatively weak protein-protein interactions. As in previous Western blots, a prominent band was observed at ~95-100 kDa. This band also appeared noticeably in lane 6 of the Silver stained gel and as a faint band in lanes 2 and 4.

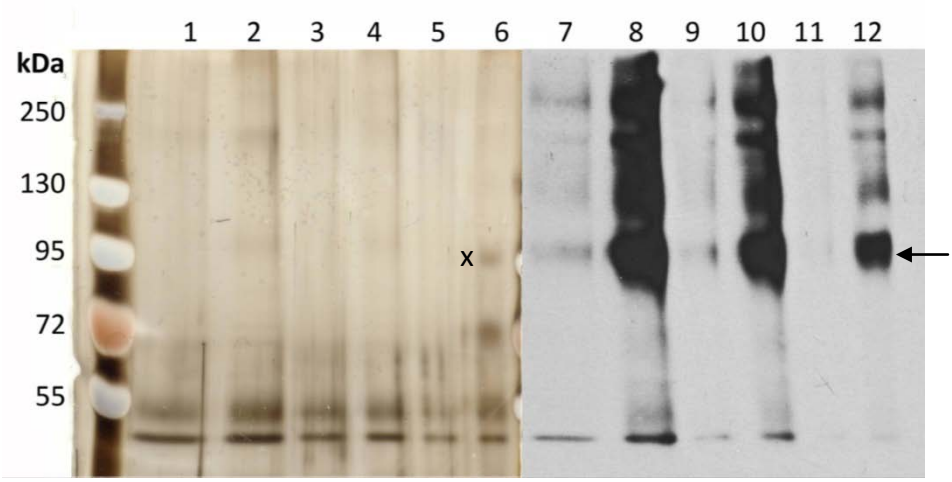

Figure 18. Neuronal homogenate immunoprecipitated with N286/74.4 were analyzed by SDS-PAGE followed by Silver staining (lanes 1-6) and Western blotting with N286/74.4 (lanes 7-12). The odd-numbered lanes show samples that were washed with additional volumes of 1 M NaCl. The "x" mark and the arrow denote the corresponding band of interest at ~95-100 kDa in the Silver stain and Western blot, respectively. Additionally, different amounts of the IP sample were loaded to determine the optimal protein concentration for this assay. 15 µL were loaded in lanes 1, 2, 7, and 8; 7 µL in lanes 3, 4, 9, and 10; 3 µL in lanes 5, 6, 11, and 12.

Discussion

We sought to generate monoclonal antibodies that recognize precise synaptic targets in zebrafish in order to aid the general study of synapse formation and the application of zebrafish as a neurodevelopmental model organism. More specifically, we hoped to identify reliable markers of postsynaptic proteins that would display clear labeling in whole-mount immunofluorescence experiments. The antibody production approach of injecting mice with purified synaptoneurosomes and PSD fractions led to a large number of successfully fused clonal hybridomas. The hybridomas were screened and characterized by IP-ELISA, IF, and Western blotting to identify positive hybridomas that produced antibodies with specific affinities for a single epitope (i.e. monoclonal antibodies). Generally, many hybridomas recognized targets with high affinity, as demonstrated by IP-ELISA. A number of clonal and subclonal hybridomas tested in immunofluorescence and Western blotting assays showed specificity for synaptic targets. Additionally, both IP-ELISA and traditional IP showed that certain subclonal hybridomas were capable of precipitating specific antigens from solution, which allows the antigens to be isolated and identified.

Hybridomas Exhibited High Affinities for Antigens

The IP-ELISA showed that a number of antibodies possessed a high affinity for their antigen. The results from the IP-ELISA were combined with preliminary IF stainings and Western blots of clonal hybridomas to choose twelve clonal hybridomas to expand into stable subclonal cell lines. As **Supplemental Tables I-IV** show, the α-values for

the expanded clonal hybridomas were all relatively high compared to other clones, which corresponds to a higher affinity for and tighter binding of the antigen in the selected clones. The selection criterion for this step of the screening and characterization process was an α-value greater than three, which was present in all subcloned hybridomas (**Table 1**).

Clones and Subclones Displayed Specificity for Synaptic Targets

Preliminary IF stains with clonal hybridomas showed that select clones were able to specifically recognize neuronal targets, such as the synaptic layers of the eye and the spinal cord (**Figure 11**). The synaptic layers of the eye include the photoreceptor layer and the inner plexiform layer. The photoreceptor layer, as the name suggests, contains rod and cone **photoreceptor cells** while the inner plexiform layer contains the axons of amacrine and bipolar cells from the inner nuclear layer and the dendrites of ganglion cells from the ganglion cell layer[34]. Labeling of these structures suggested that the clonal hybridomas were recognizing synaptic proteins. Western blotting with three tissue types ensured that the clonal hybridomas were specific for neuronal tissue and also showed that the size of a possible antigen was ~95 kDa (**Figure 12**). A comprehensive evaluation of the clonal hybridomas based on IP-ELISA, IF, and Western blotting led to the subcloning of N286/2, 9, 21, 29, 52, 57, 62, 73, 74, 76, 79, and 81.

Further IF analysis of selected clones and subclones showed that the hybridomas were recognizing synaptic targets in the eye, midbrain, and spinal cord (**Figure 14-16**).

Colocalization with synapsin1,2 demonstrated that several subclonal hybridomas recognized synaptic targets specifically, and could clearly tag them in whole-mount IF. Labeling of the synaptic layers of the eye and the optic tectum of the midbrain were observed in the stained coronal sections of 5 dpf embryos (**Figure 14**)[24,34]. The optic tectum (TeO) is the central processing unit of visual input in zebrafish and the structure forms the dorsal portion of the midbrain[24]. The strong staining of neuronal structures and colocalization with synapsin1,2 strongly suggested again that the selected clones and subclones recognized specific synaptic proteins.

Whole-mount IF also showed that N286/9.2, 9.4, 21.9, 21.11, 74.1, 74.4, 79.2, 79.5 recognized a number of neuronal structures, including the olfactory bulbs, dorsal root ganglia, sympathetic ganglia, and the spinal cord (**Figure 15**). Labeling of the RB sensory neurons in the spinal cord could be visualized at 60X in **Figure 16**. A number of the assayed subclonal hybridomas appeared to have a high affinity for the RB cells, which previous research in the Washbourne has shown to be involved in an important early developmental phenomenon in zebrafish called the touch response[32]. RB neurons are responsible for sensing mechanical touch or pressure and delivering this sensory information to other neurons, leading to a neuronal circuit that activates a contralateral coiling of the tail in response to touch (i.e. a C-shaped bending of the tail towards the opposite side of the body as the stimulus)[32]. Pietri et al. found that sensory input from the RB cells is routed through CoPA interneurons in the **anterior** spinal cord before reaching the motor neurons that activate the contralateral coil observed in the touch response. Glutamate receptors were necessary for the touch-evoked coiling response,

and electrophysiology recordings showed that the synapses between RB and CoPA neurons were characterized by **glutamatergic transmission**[32].

The immunolabeling experiments demonstrated that selected subclones were able to bind synaptic targets with specificity and high affinity in both sectional and whole-mount assays. The clear labeling of RB cells by the assayed hybridomas in IF show that monoclonal antibodies produced from these hybridomas could be used to further characterize the transduction of sensory information from RB cells to CoPA interneurons and to further elucidate the nature of glutamatergic transmission in the touch-response circuit. The nature of many synaptic connections in the zebrafish spinal cord remains unclear[32]. Antibody staining of RB cells could illuminate contacts between RB cells and other interneurons to further characterize synapses and neuronal networks in developing zebrafish.

The glutamatergic RB-CoPA synapse is only one of many synaptic connections in the developing zebrafish spinal cord[31]. Synapses between sensory neurons (such as RB cells) and other interneurons could be involved in circuits that respond to different sensory inputs, perhaps temperature or pressure changes in the surrounding aquatic environment. Additionally, other synaptic connections could be inhibitory instead of excitatory. In general, the detailed mechanisms of synapse formation and synaptic transmission at different types of synapses remain undiscovered. The monoclonal antibodies from positive hybridomas could be applied towards probing unknown functions of sensory neurons and their synaptic connections with various neuronal cell

types in order to gain a better understanding of neural circuit formation and synapse development.

The Antigen of Interest Appears in Electrophoresis Gels at ~100 kDa

Western blots showed that the size of the antigen recognized by multiple clones and subclones was ~95-100 kDa. **Figure 12-13** show that the band at 95-100 kDa was the most prominent and consistently present band for clones N286/9, 52, 74, 79 (**Figure 12**) and subclones N286/9.2, 52.3, 74.4, and 79.5 in the PSD lane (**Figure 13**). Similar results were observed for clones N286/9, 21, 76 and subclones N286/9.4, 21.9, 21.11, 52.1, 74.1, and 79.2 (data not shown). In a number of blots, additional bands and smearing was observed in the neuronal lanes. This phenomenon may have been due to an overloaded gel lane. An overabundance of protein could have led to non-specific interactions between the proteins in the neuronal homogenate and the antibody, resulting in a smear instead of clear banding. IP-Western blotting experiments with anti-PSD95 and anti-panMAGUK controls showed some similarities between the size of the proteins precipitated by these control antibodies and N286/74.4 (**Figure 17**). The proteins precipitated by the control antibodies were also recognized by N286/74.4 in the Western blot analysis following the IP assay. These results also suggest that perhaps N286/74.4 recognizes some of the same or similar epitopes in neuronal and PSD proteins as anti-panMAGUK and anti-PSD95. Although we have narrowed down the approximate size of the potential antigen, many possibilities for the identity of the antigen still remain (given the large number of proteins present at the PSD). A number

of synaptic proteins have been isolated and identified at this point, as illustrated by **Figure 19**, but the functions of many PSD and trans-synaptic proteins are still uncertain.

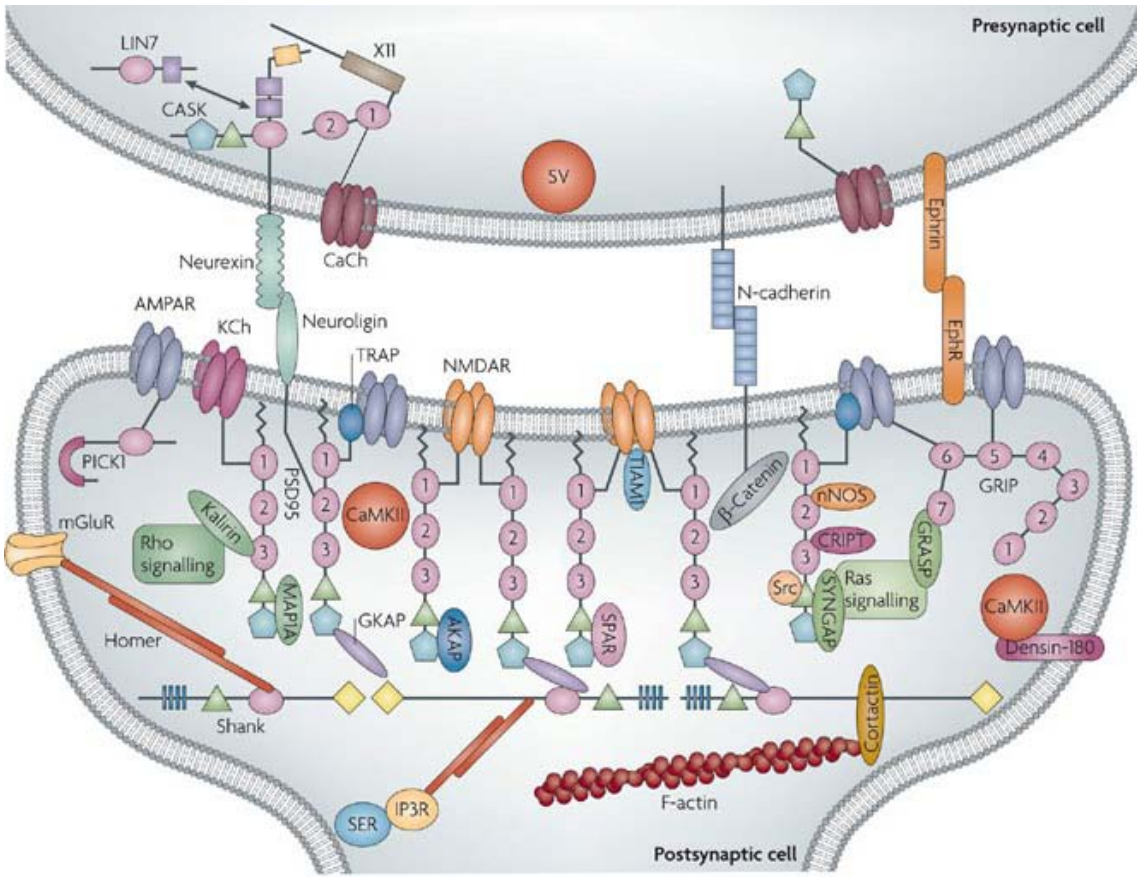

Figure 19. The PSD consists of many proteins, including neurotransmitter receptors, ion channels, scaffold and adaptor proteins (e.g. PSD95), signaling proteins, cell-adhesion molecules (e.g. neurexin/neuroligin), and components of the cytoskeleton[35].

Future Directions: Isolating and Identifying the Antigens

The next step in this project is to establish the identity of the antigen for each subclone that demonstrated desirable characteristics in the screening process. IP experiments need to be completed for each subclonal hybridoma to determine whether the antibodies produced by the hybridoma can precipitate a specific antigen from a sample of neuronal homogenate or PSD. Completed IPs have shown that N286/9.2, 74.1, and 74.4 can

precipitate antigens from solution (**Figure 17-18**), which is the prerequisite for identifying the antigen. IP samples can be run on an SDS-polyacrylamide gel and stained for proteins. The band of interest can be excised, destained, and analyzed for amino acid sequence by mass spectrometry. Potential obstacles in protein sequence analysis are the high sensitivity to contamination and the requirement for relatively high concentrations of the antigen of interest. The resultant monoclonal antibodies obtained from the stable hybridoma cell lines could be applied towards gaining a deeper understanding of zebrafish and vertebrate neural development and function.

Appendix I: Glossary of Terms and Abbreviations (Alphabetical)

Action potential:	A rapid and transient electrical nerve impulse of about one millisecond that is initiated at the axon hillock. Action potentials are propagated down the length of the axon until they reach the presynaptic terminal, at which point the electrical impulse triggers the influx of Ca^{2+} and the release of neurotransmitters at the synapse[1].
Amino acid:	Molecules with a backbone composed of carbon, nitrogen, oxygen, and hydrogen that are the building blocks of proteins[10].
Anterior:	An anatomical term that refers to the regions located towards or near the head of an organism's body.
Antibody:	Large proteins produced by the immune system to recognize and neutralize foreign objects such as bacteria or viruses[10].
Antigen:	A substance that triggers the production of antibodies by the immune system when introduced into the body. Antigens are usually foreign proteins from an organism other than the host[10].
Assay:	A procedure for testing or measuring the activity or function of a macromolecule or chemical.
Buffer solution:	A liquid solution in which a mixture of a weak acid and its corresponding base are dissolved in water to keep the pH of the solution relatively constant. Buffer solutions are very important in biological research because living organisms have a limited tolerance for pH changes.
Cell type:	A form of cell with distinct morphology or function.
Cell:	The basic functional unit of living organisms.
Centrifuge:	(noun) A piece of equipment that rotates an object around a fixed axis and through the acceleration in the curved path, applies a force perpendicular to the axis that separates substances based on density. (verb) To subject an object to the force of a centrifuge.

Coronal plane:	The plane that divides the body into anterior and posterior sections. In zebrafish, the coronal plane runs along the shorter dorsal-ventral axis. See also: dorsal and ventral.
Differential centrifugation:	A procedure that uses a centrifuge to separate parts of a homogenized tissue sample based on density, producing different fractions of the whole tissue sample.
Domain (protein):	A globular cluster of a protein that is structurally and functionally independent of the rest of the protein[10].
Dorsal:	An anatomical term that refers to the region located towards or near the back of an organism's body. In zebrafish and other vertebrates, dorsal refers to the location of or near the vertebral column.
ELISA:	Stands for enzyme-linked immunosorbent assay. This assay is used to detect the presence of an antibody or an antigen in a sample preparation. The assay can be carried out in various ways to detect the presence of a specific species in solution or to detect a desired interaction. See also: **enzyme**.
Embryo:	An animal in the very early stages of growth that are characterized by cell division, the development of fundamental tissues and primitive organs.
Enzyme:	Proteins that increase the rates of chemical reactions, but do not participate as a reactant[10].
Epitope:	The part of an antigen that is recognized by antibodies[10]. See also: monoclonal antibodies and polyclonal antibodies.
Filtrate:	The liquid produced after filtering a mixture of solid and liquid.
Fraction:	Please see "***Differential centrifugation***"
Ganglion:	(plural: ***ganglia***) A mass of nerve cell bodies that act as relay points between the central and peripheral nervous systems.
Gel electro-phoresis:	An analytical technique commonly used in biochemistry that separates charged molecules based on their abilities to move through

	a gel matrix when an electric field (or voltage) is applied. This technique can be used with nucleic acids or protein. Because smaller particles can generally move through the matrix faster during this process, the separation corresponds roughly with the size of the molecule.
Gene:	A unit of heredity that encodes for a functional product, as in a protein or functional ribonucleic acid.
Genetic screen:	A procedure to identify and select for individuals that possess a phenotype of interest. In biology, genetic screens usually involve techniques such as polymerase chain reaction and gel electrophoresis.
Glutamatergic transmission:	Synaptic transmission via the neurotransmitter glutamate. Synapses that employ glutamatergic transmission (called glutamatergic synapses) can involve different types of receptors that bind glutamate and other amino acid derivatives that mimic the action of glutamate[1].
GUK domain:	The guanylate kinase domain is an enzymatic protein domain that converts guanosine monophosphate to guanosine diphosphate.
Heterophilic binding:	A type of cell-cell adhesion interaction that involves a CAM on one cell directly binding to the same kind of CAM on an adjacent cell[17]. See also: **homophilic binding**.
Homogenate:	Tissue that has been mechanically disrupted (e.g. by a grinder) to yield a mixture with homogeneous consistency.
Homophilic binding:	A type of cell-cell adhesion interaction that involves a CAM on one cell directly binding to the same kind of CAM on an adjacent cell[17]. See also: heterophilic binding.
HRP:	Horseradish peroxidase, an enzyme found in horseradish that catalyzes the conversion of color-producing substrates (chromogens) through an oxidation reaction with hydrogen peroxide.

Hybridoma:	A hybrid cell line formed by fusing a specific antibody-producing cell to a cancer cell that is able to divide indefinitely in vitro. This process allows the normally short-lived antibody-producing cell to survive and continue making antibody proteins.
Immunofluore-scence (IF):	A staining technique that involves the binding of antibodies to specific targets in a biological tissue. However, immunofluorescence staining also employs the use of fluorescent dyes and allows visualization by fluorescence microscopy without the need for sectioning the tissue.
Immunohisto-chemistry (IHC):	A staining technique that involves the binding of antibodies to specific targets in the cells of a tissue section. This allows for visualization by light microscopy.
Immuno-precipitation (IP):	A technique that uses antibodies to precipitate or pull a protein of interest out of solution. IP procedures typically involve the use of polysaccharide polymer beads to precipitate the antibody-antigen immune complexes.
Kilodaltons (kDa):	A Dalton (Da) is a unit of mass for the atomic or molecular scale. It is equal to one unified atomic mass unit, defined as one twelfth of the rest mass of an unbound atom of carbon. Its value is approximately 1.660539×10^{-27} kg^{10}. A kilodalton (kDa) is a common unit of mass for macromolecules such as proteins and DNA, and 1 kDa = 1000 Da.
Missense mutation:	A point mutation that results in an amino acid change in the gene's coding sequence. See also: point mutation.
Model organism:	A non-human species, usually with significant orthology with humans that is used to study particular biological phenomena, especially human diseases. Model organisms are employed when human experimentation would be unfeasible or unethical.
Molarity:	A measure of the concentration of a substance, defined as the amount

	of the substance divided by the volume of the solvent solution; 1 Molar (M) = 1 mole (6.022×10^{23} molecules) of a substance per 1 liter volume of solution (i.e. 1 mol/L)
Monoclonal antibodies:	Antibodies derived from clones of an individual parent cell that are specific for a single epitope.
Neurotrans-mitter:	A molecule that binds to specific receptors in the postsynaptic cell membrane to transmit chemical signals.
Ortholog:	Genes in different species that originated from a single gene in a common ancestor[17].
PBDTx:	An aqueous solution containing 10% 1X PBS, 1% BSA, 1% DMSO, pH 7.3, 0.1% Triton X-100 (a non-ionic detergent that helps dissolve proteins).
PBS:	Phosphate-buffered saline solution
PBST:	Phosphate-buffered saline solution with 0.05% Tween 20 (a non-ionic detergent).
PDZ domain:	A common protein domain of 80-90 amino acids found in structural proteins at the synapse that help anchor transmembrane proteins such as cell adhesion molecules.
pH:	A measure of the acidity or basicity of a solution.
Photoreceptor cell:	A type of neuron found in the retina that is specialized for transducing light[1].
Point mutation:	A type of genetic mutation that causes the replacement of a single nucleotide base with another in the genetic code (DNA or RNA). Such mutations can result in various consequences including a non-functional or deleterious protein product.
Polyclonal antibodies:	A mixture of antibodies derived from different antibody-producing parent cells that recognize different epitopes of a specific antigen[10].
Posterior:	An anatomical term that refers to the regions directed towards or near the tail of an organism's body.

Postsynaptic density (PSD):	The protein-dense region on the internal surface of the postsynaptic membrane[1].
Precipitate:	(noun) The solid substances in a liquid solution. (verb) To separate the solid substances from the liquid in a mixture.
Protein:	A biochemical macromolecule that is made of a single linear polymer of amino acids folded into a globular or fibrous form. Proteins perform enzymatic catalysis and assist biological reactions in the cell.
RIPA buffer:	150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM Complete, Mini, EDTA-free protease inhibitor cocktail (Roche).
Sagittal plane:	The plane that divides the body of a bilaterally symmetric organism into mirror-image left and right portions or any plane parallel to it. In zebrafish, the sagittal plane runs along the long anterior-posterior axis. See also: anterior and posterior.
SDS-PAGE:	Stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis, which is a form of gel electrophoresis that is specialized for proteins.
Sequencing:	To determine the identities and sequence of subunits in a biopolymer such as deoxyribonucleic acid (DNA) or protein.
Serum (blood):	The serum of an organism's blood is the component that contains neither blood cells nor clotting factors.
SH3 domain:	The SRC-homology-3 domain is typically found in proteins that interact with other proteins; the domain is thought to mediate the assembly of protein complexes.
Solution:	A homogeneous mixture consisting of only one distinguishable state of matter (e.g. solid, liquid, or gas) with a solute (the dissolved substance) and a solvent (the substance that is dissolving the solute). An aqueous solution is a liquid solution where water is the solvent.

Solvent:	A substance that dissolves another solid, liquid, or gas.
Spectrophoto-meter:	Instruments that measure the transmittance of a specific wavelength of light (e.g. yellow is 570–590 nm) through a sample solution (usually aqueous).
Subclone (antibodies):	A subclone refers to an antibody-producing cell line that is cloned from a parent clonal hybridoma by expanding the growth of the parent clone. Subclonal hybridomas tend to be more stable than parent clones. After a subclonal hybridoma tests positive for the relevant antibodies, the antibodies generated from that hybridoma are called a monoclonal antibodies.
Supernatant:	The liquid above the solid pellet after centrifugation. See also: **centrifuge**.
Synapto-neurosomes:	Synaptosomes with attached resealed postsynaptic sacs. This entity was identified in electron microscopy studies by Hollingsworth et al.[36].
Synaptosomes:	Isolated and detached presynaptic sacs containing synaptic vesicles[36].
Ventral:	An anatomical term that refers to the region located towards or near the lower surface of an organism's body. In zebrafish and other vertebrates, ventral refers to the location of or near the abdomen.
Western blotting:	Western blotting (also known as immunoblotting) is a common analytical technique used to identify the presence of specific proteins in a tissue sample. This analysis uses antibodies to identify certain protein targets in a mixture of proteins from a tissue or cell homogenate.
Whole-mount staining:	The staining of tissue or embryos without first sectioning the tissue onto glass slides. This staining approach allows the visualization of whole zebrafish embryos at various stages in development to determine the location and expression of specific proteins of interest.

Appendix II: Supplemental Data[vi]

Supplemental Table I. IP-ELISA Results for N286/1-24

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_-$
MAGUK	2.6961	-
HA	0.0690	-
BLANK	0.0000	-
SP2	0.0247	-
GST	0.0473	-
1	0.0810	0.1795
2	**1.7171**	**3.8057**
3	0.0916	0.2031
4	0.0723	0.1603
5	0.2411	0.5343
6	0.0808	0.1791
7	0.1930	0.4278
8	0.1550	0.3435
9	**2.9972**	**6.6430**
10	0.5103	1.1311
11	0.0965	0.2140
12	0.1137	0.2519
13	0.1163	0.2579
14	0.2218	0.4917
15	0.1936	0.4291
16	0.1770	0.3923
17	1.1137	2.4685
18	0.2017	0.4472
19	0.2090	0.4633
20	0.0721	0.1599
21	**3.1106**	**6.8943**
22	0.0938	0.2079
23	0.6764	1.4992
24	1.0646	2.3597

For this 96-well plate: $\sigma_- = 0.1504$, $3\sigma_- = 0.4512$.

Supplemental Table II. IP-ELISA Results for N286/25-48

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_-$
MAGUK	3.3490	-
HA	0.0158	-
BLANK	0.0000	-
SP2	0.0051	-
GST	0.0892	-
25	0.2361	1.8901
26	0.0663	0.5309
27	0.0245	0.1964
28	-0.0102	-0.0815
29	**1.8293**	**14.6452**
30	0.1465	1.1727
31	-0.0006	-0.0052
32	0.0334	0.2677
33	0.1352	1.0827
34	0.3243	2.5959
35	0.0022	0.0177
36	1.3946	11.1649
37	1.4164	11.3394
38	1.7875	14.3106
39	-0.0196	-0.1570
40	1.2880	10.3113
41	1.0447	8.3641
42	0.5946	4.7601
43	-0.0852	-0.6825
44	0.1363	1.0908
45	0.0602	0.4820
46	1.2611	10.0961
47	0.0578	0.4626
48	0.0786	0.6295

For this 96-well plate: $\sigma_- = 0.0416$, $3\sigma_- = 0.1249$

[vi] For Supplemental Tables I-IV, the standard deviation of the absorbance reads for negative controls (σ_-) was calculated according to Equation 1. The bolded values represent clonal hybridomas that were subcloned.

Supplemental Table III. IP-ELISA Results for N286/49-72

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
MAGUK	2.4073	-
HA	-0.0010	-
BLANK	0.0000	-
SP2	0.0355	-
GST	0.0160	-
49	0.0364	0.3545
50	0.0313	0.3046
51	0.0555	0.5404
52	**2.4022**	**23.4133**
53	1.7274	16.8358
54	0.3697	3.6036
55	0.0408	0.3980
56	1.8225	17.7627
57	**1.8720**	**18.2460**
58	1.2816	12.4908
59	0.0205	0.2001
60	1.6694	16.2708
61	2.4123	23.5121
62	**0.3221**	**3.1395**
63	0.0617	0.6017
64	0.8546	8.3298
65	2.3020	22.4364
66	0.0113	0.1098
67	0.3959	3.8584
68	0.0427	0.4162
69	2.5107	24.4711
70	0.0540	0.5260
71	1.0151	9.8941
72	0.1624	1.5824

For this 96-well plate: $\sigma = 0.0342$
$3\sigma = 0.1026$

Supplemental Table IV. IP-ELISA Results for N286/73-96

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
MAGUK	2.8518	-
HA	0.0184	-
BLANK	0.0000	-
SP2	-0.0080	-
GST	0.0581	-
73	**0.5025**	**3.6952**
74	**2.7494**	**20.2164**
75	0.0453	0.3332
76	**1.8927**	**13.9172**
77	1.6396	12.0562
78	0.1642	1.2076
79	**2.4821**	**18.2504**
80	0.1972	1.4504
81	**1.2294**	**9.0395**
82	-0.0111	-0.0813
83	0.8452	6.2150
84	2.4807	18.2407
85	-0.0613	-0.4506
86	1.6039	11.7932
87	-0.0896	-0.6586
88	0.5581	4.1040
89	-0.1184	-0.8702
90	-0.0454	-0.3336
91	-0.0891	-0.6548
92	2.1288	15.6532
93	1.8155	13.3491
94	-0.1542	-1.1335
95	-0.1451	-1.0668
96	-0.1494	-1.0984

For this 96-well plate: $\sigma = 0.0453$
$3\sigma = 0.1360$

Supplemental Table V. IP-ELISA Results for N286/2, 9, 73, 76, and respective subclones

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
2	0.0306	2.6486
2.1	0.0179	1.5506
2.2	0.0177	1.5333
2.3	0.0451	3.8965
2.4	0.0140	1.2076
2.5	0.0134	1.1557
2.6	0.0157	1.3603
2.7	0.0273	2.3575
2.8	0.0113	0.9742
9	1.6550	143.0902
9.1	0.1022	8.8335
9.2	1.6495	142.6176
9.3	1.5768	136.3318
9.4	1.6607	143.5859
9.5	0.0209	1.8042
9.6	1.5785	136.4759
9.7	0.9761	84.3976
9.8	1.0983	94.9573
9.10	1.6015	138.4645
73	0.0345	2.9801
73.1	0.0063	0.5447
73.2	0.0107	0.9252
73.3	0.0137	1.1874
76	0.0118	1.0203
76.1	0.0165	1.4238
76.2	0.0214	1.8474
76.3	0.0304	2.6284

For this 96-well plate: $\sigma = 0.00386$
$3\sigma = 0.0116$

Supplemental Table VI. IP-ELISA Results for N286/21, 52, 57, and respective subclones

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
21	1.0300	69.0197
21.1	1.1860	79.4735
21.2	1.2083	80.9830
21.3	1.2831	86.0009
21.4	1.0407	69.7498
21.5	1.1824	79.2493
21.6	0.0634	4.2516
21.7	1.2489	83.7042
21.8	1.0782	72.2632
21.9	1.2515	83.8829
21.10	1.2240	82.0375
21.11	1.2599	84.4437
57	0.0172	1.1528
57.1	0.0165	1.1059
57.2	0.0226	1.5125
57.3	0.0203	1.3584
57.4	0.0134	0.8981
57.5	0.0262	1.7538
57.6	0.0236	1.5840
57.7	0.0230	1.5416
57.8	0.0206	1.3807
57.9	0.0293	1.9638
57.10	0.0213	1.4254
57.11	0.0283	1.8945
52	0.8157	54.6738
52.1	0.2433	16.3047
52.2	0.0703	4.7140
52.3	0.4137	27.7279

For this 96-well plate: $\sigma = 0.00497$
$3\sigma = 0.0149$

Supplemental Table VII. IP-ELISA Results for N286/29, 62, 79 and respective subclones

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
29	0.8112	79.2556
29.1	0.7757	75.7857
29.2	0.7304	71.3597
29.3	0.7261	70.9396
29.4	0.6871	67.1356
29.5	0.6585	64.3348
29.6	0.7057	68.9496
29.7	0.7793	76.1374
29.8	0.6949	67.8944
29.9	0.7611	74.3624
29.10	0.7754	75.7564
29.11	0.7107	69.4414
29.13	0.7889	77.0754
29.14	0.7515	73.4277
29.15	0.7620	74.4471
29.16	0.7421	72.5061
29.17	0.7057	68.9496
62	0.0337	3.2926
62.1	0.0356	3.4750
79	2.0986	205.0447
79.1	2.1220	207.3310
79.2	2.0356	198.8861
79.3	2.0555	200.8272
79.4	2.0150	196.8702
79.5	2.0905	204.2533
79.6	0.1636	15.9876
79.7	2.0343	198.7559

For this 96-well plate: $\sigma = 0.00341$
$3\sigma = 0.01024$

Supplemental Table VIII. IP-ELISA Results for N286/74, 81, and respective subclones

Sample	$\langle A_{450} \rangle$	$\alpha = \langle A_{450} \rangle / 3\sigma_{-}$
74	1.5877	79.8347
74.1	1.5200	76.4338
74.2	1.5384	77.3590
74.3	1.5211	76.4891
74.4	1.4807	74.4543
74.5	1.4502	72.9223
81	0.0275	1.3828
81.1	0.0326	1.6376

For this 96-well plate: $\sigma = 0.00663$
$3\sigma = 0.01989$

Supplemental Table IX. Preliminary IF Scores and Rankings for N286 Clones

Clone	Score (1-5)	Rank	Clone	Score (1-5)	Rank	Clone	Score (1-5)	Rank
1	2	18	33	0	70	65	3	17
2	**3**	**11**	34	1	36	66	2	27
3	0	53	35	0.5	50	67	0	84
4	0	54	36	0.5	51	68	4	7
5	0	55	37	0	71	69	2	28
6	0	56	38	1	37	70	0	85
7	1	33	39	2	22	71	0	86
8	1	34	40	0.5	52	72	2	29
9	**3**	**12**	41	2	23	**73**	**5**	**1**
10	1	35	42	1	38	**74**	**5**	**2**
11	0.5	45	43	1	39	75	2	30
12	0	57	44	2	24	**76**	**4**	**8**
13	0	58	45	0	72	77	1	41
14	0	59	46	3	13	78	0	87
15	0	60	47	0	73	**79**	**5**	**3**
16	0	61	48	0	74	80	0	88
17	2	19	49	1	40	**81**	**0**	**89**
18	2	20	50	0	75	82	0	90
19	0	62	51	0	76	83	0	91
20	0	63	**52**	**4**	**6**	84	4	9
21	**4**	**5**	53	3	14	85	1	42
22	0	64	54	0	77	86	0	92
23	2	21	55	0	78	87	0	93
24	0.5	46	56	0	79	88	0	94
25	0	65	**57**	**3**	**15**	89	2	31
26	0.5	47	58	0	80	90	0	95
27	0.5	48	59	2	25	91	1	43
28	0	66	60	2	26	92	4	10
29	**0**	**67**	61	0	81	93	5	4
30	0	68	**62**	**3**	**16**	94	0	96
31	0.5	49	63	0	82	95	1	44
32	0	69	64	0	83	96	2	32

The clonal hybridomas were analyzed by immunolabeling of sagittal sections of 5 dpf embryos. Clones were given a score of 1-5 (with 5 as the highest score) based on a qualitative assessment of relative specificity and intensity of staining. Clones in bold were expanded to obtain subclonal hybridomas.

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