IDENTIFYING PROTEIN PARTNERS OF THE NEURONAL TRANSMEMBRANE PROTEIN NETO2

BY

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A thesis submitted in conformity with the requirements for the degree of Master of Science,
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Abstract

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The neuronal transmembrane proteins Neto1 and Neto2, along with different protein partners, are known to perform multiple roles in diverse processes including axon guidance in the developing nervous system and synaptic plasticity in the adult brain. My project focuses on identifying the membrane bound interacting partners of Neto2 using Membrane Yeast Two Hybrid (MYTH). By performing MYTH screens for the Neto2 molecule using human adult and embryonic whole brain cDNA libraries, I have identified several novel membrane bound putative interacting partners including VAMP associated protein B (VAPB) and Glutamate transporter EAAT3-associated protein (GTRAP3-18), which play diverse functions during the glutamatergic neurotransmission. Initial studies to validate these interactions in vivo are currently underway by co-immunoprecipitation approach using mouse brain tissue. If these two candidate proteins are confirmed to be true interactors, it will open important avenues of research for the Neto2 protein during excitatory neurotransmission in the mammalian central nervous system.
Acknowledgements

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I am deeply indebted to all my friends; particularly, Meera, Vijay, Santhosh, Navin and Arvind. Your mere presence and comradeship will give me the courage and strength to move mountains!!!

My Family!!! How can I ever thank you all? Or even think of it? My dearest Amma, Meera, Radha, Govind and Surabhi, you all hold the key to my life and everything in it. Love you all immeasurably and unconditionally.

Lead us from untruth to the truth.
Lead us from ignorance to knowledge.
Lead us from death to immortality.

And let there be peace, peace and peace!

(An ancient Indian aphorism)
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Foreword

Thesis organisation: This thesis reports the interacting partners of the neuronal transmembrane protein Neto2, by applying an approach called Membrane Yeast Two Hybrid (MYTH), and describes briefly these novel findings. For ease of understanding, the Introduction (Chapter 1) is split into three parts, beginning with a brief sketch and discussion of the structural and molecular organization of synapse. The next part introduces the neuronal protein Neto2 and the current knowledge about its biology. The third part delineates the MYTH approach and the rationale for applying this approach towards the study of Neto2. The Materials and methods section (Chapter 2) details the experimental techniques used in the thesis. The Results and discussion section (Chapter 3) briefly reports the result of the MYTH screens performed, and discusses these findings. Also, this chapter gives a particular emphasis on the important controls that are critical for the different experiments used during the MYTH technique. Finally, the Future directions section (Chapter 4) reviews the two important Neto2-putative interactors identified by the MYTH screen, namely VAMP associated protein B (VAPB) and Glutamate transporter3-18 (GTRAP3-18), and the functional relevance of such interactions with Neto2.
Chapter 1: Introduction
Part 1 - Introduction to the synapse

The human brain is the most complex biological entity known. The complexity and diversity of the nervous system is made possible by numerous interconnections between the nerve cells (neurons). Synapses are the functional connections between neurons, or between neurons and other cell types. The human brain has about $10^{11}$ neurons and each has on average 1,000 – 10,000 synaptic connections to other neurons. It has been estimated that the total number of synapses in an adult brain is about $10^{14}$ to $10^{15}$ (Kandel and Schwartz 1981). Most synapses connect the axon of one neuron to the dendrite of another. There are also other types of synapses including the connections between axon : axon, axon : cell body, and dendrite : dendrite of adjacent neurons (Noback 2005).

There are two types of synapses distinguished on the basis of their mechanism of transmission - electrical and chemical (Table 1.1). At electrical synapses, ions flow through gap junctions which are specialized membrane channels that connect two cells and they are composed of connexin proteins. Connexins form clusters of hemi-receptors and associate with the hemi-receptors of adjacent neurons, forming a continuous link allowing the rapid exchange of ions and thus electrical signals (Connors and Long 2004).

In contrast, chemical synapses enable cell-to-cell communication via the release of chemical agents called neurotransmitters; neurotransmitters released by the presynaptic neuron produce a corresponding effect on the postsynaptic neuron (Purves 2008). The chemical synapse is referred to as excitatory when synaptic activity drives the postsynaptic neuron above its firing threshold. This mode of chemical transmission is mediated through neurotransmitters such as glutamate and dopamine. In contrast, chemical synapses are called inhibitory when synaptic
activity drives the postsynaptic neuron below its firing potential. This mode of chemical transmission is mediated through neurotransmitters such as γ-aminobutyric acid (GABA) and glycine (Kandel and Schwartz 1981).

Table 1.1 Distinguishing properties of electrical and chemical synapses

<table>
<thead>
<tr>
<th>Type of synapse</th>
<th>Distance between pre and post synaptic membranes</th>
<th>Ultrastructural components</th>
<th>Synaptic delay</th>
<th>Agent / Direction of Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrical</td>
<td>3.5 nm</td>
<td>Gap-junction channels</td>
<td>Virtually absent</td>
<td>Ion current/ Bidirectional</td>
</tr>
<tr>
<td>Chemical</td>
<td>20 – 40 nm</td>
<td>Presynaptic vesicles and active zones; postsynaptic receptors</td>
<td>Significant: 0.3 - 5 ms long</td>
<td>Chemical transmitter/ Unidirectional</td>
</tr>
</tbody>
</table>

Modified from (Kandel and Schwartz 1981)
1.1.1 The structure of the chemical synapse

The majority of the synapses in the nervous system follow the chemical mode of nerve impulse transmission (Greengard 2001) and thus, chemical transmission between neurons is the first level of complexity towards the understanding of higher-order brain functions like learning, memory, and other complex behaviour (Kandel 2001). A chemical synapse is characterized by unidirectional transmission of information directly from the presynaptic cell to the postsynaptic cell. In an axodendritic chemical synapse, the presynaptic terminal or the synaptic boutons are specialized regions of the termination of the presynaptic axon that contain chemical messengers called neurotransmitters enclosed within membrane-bound synaptic vesicles (SV). These vesicles are docked at the presynaptic plasma membrane at regions called active zones (AZ) (Rettig and Neher 2002). Directly opposite to the synaptic bouton is the dendritic membrane of the postsynaptic cell that contains neurotransmitter receptors. Immediately behind the postsynaptic membrane is an electron dense region called the postsynaptic density (PSD), which contains several multi-protein complexes (Sheng and Kim 2002) found in specialized protrusions from the main dendritic shaft called dendritic spines (Kennedy 2000). The presynaptic and the postsynaptic membranes are separated by a synaptic cleft that is 20nm wide (Figure 1.1).

Nerve impulse transmission at the chemical synapse is based on a series of sequential events as described in Figure 1.2. The process begins when an action potential reaches the presynaptic axon terminal and alters the local resting membrane potential. This change results in the opening of a type of voltage-sensing ion channels present on the presynaptic membrane that allow a huge influx of Ca\(^{2+}\) ions into the presynaptic axon terminal, and leads to a transient increase in the Ca\(^{2+}\) concentration in the cytoplasm. The elevated Ca\(^{2+}\) concentration triggers the
fusion of synaptic vesicles with the plasma membrane of the presynaptic neuron. The Ca\textsuperscript{2+}-dependent fusion of the synaptic vesicles with the terminal membrane causes the release of neurotransmitters into the synaptic cleft and marks the conversion of the electrical signal into a chemical signal at the presynaptic terminal - a hallmark of the chemical mode of synaptic transmission (Purves 2008).

Following exocytosis, the neurotransmitters diffuse across the synaptic cleft to bind to specific receptors on the membrane of the postsynaptic terminal of the neuron. Following the neurotransmitter binding, a complex interplay between multiple ion channels, transporters, pumps and several other proteins lead to ion fluxes within the postsynaptic terminal. This ion flux alters the membrane potential in such a way that it increases or decreases the probability for that neuron to fire an action potential. For example at an excitatory synapse, the binding of excitatory neurotransmitters to their cognate receptors at the postsynaptic membrane leads to a net increase of Na\textsuperscript{+} ions within the postsynaptic terminal to produce an excitatory postsynaptic potential (EPSP). Conversely, at an inhibitory synapse, the binding of inhibitory neurotransmitters to their cognate receptors at the postsynaptic membrane leads to a net increase of Cl\textsuperscript{−} ions within the postsynaptic terminal to produce an inhibitory postsynaptic potential (IPSP). Thus, the chemical signal is converted back into an electrical signal at the postsynaptic terminal and re-establishes the propagation of nerve impulse transmission (Purves 2008).
**Figure 1.1** - An electron micrograph of an excitatory synapse showing the presynaptic bouton, synaptic vesicles, active zone, synaptic cleft, postsynaptic density and dendritic spine.

**A**. A presynaptic terminal forming chemical synapses with two dendritic spines. Synaptic vesicles carrying neurotransmitters can be seen in the presynaptic terminal. The electron dense-post synaptic density (PSD), which can be seen at the dendritic spines, contains several multi-protein complexes involved in neurotransmission. Scale bar - 400 nm.

**B**. Tracing of the electron micrograph, identifying the major structures present at the chemical synapse. The dendritic shaft and the spines contain cytoskeletal elements like microtubules and actin that are involved in the transport of proteins and other cargo from the cell body towards the PSD.

(Modified from Kennedy 2000)
Figure 1.2 – A schematic representation for the mechanism of nerve impulse transmission at a excitatory chemical synapse.

(A) The arrival of an action potential at the synaptic bouton opens voltage-gated Ca²⁺ channels. Ca²⁺ ions flow into the synaptic bouton and vesicles are tethered to the presynaptic membrane.

(B) Upon calcium influx, the synaptic vesicles docked to the presynaptic membrane release the neurotransmitters into the synaptic cleft by exocytosis.

(C) The neurotransmitters bind to their corresponding receptors located on the postsynaptic membrane. This causes an influx of Na⁺ ions (in excitatory synapses) to produce an excitatory post synaptic potential (EPSP) or the influx of Cl⁻ ions (in inhibitory synapses) to produce an inhibitory post synaptic potential (IPSP), thus transmitting the nerve impulse.

(Modified from Kandel and Schwartz 1981)
1.1.2 Ion channels and receptors at the synapse

Apart from mediating neural communication, chemical synapses are also involved in altering the strength of connection between adjacent neurons, a phenomenon termed synaptic plasticity. More than 50 years ago, Donald Hebb postulated that the modulation of synaptic strength underlies complex cognitive functions like learning and memory. He proposed that the synapses between neurons are strengthened during concerted pre-and postsynaptic neuronal activity, and they are weakened by non-coincidental firing (Turrigiano and Nelson 2000; Purves 2008). It is now clear that the repeated neural activity which is the basis for plasticity as postulated by Hebb is mediated through the different types of neurotransmitter receptors, ion channels, pumps and other proteins present on the neuronal membrane (Figure 1.3) (Malenka and Nicoll 1999; Greengard 2001; Sheng and Kim 2002; Voglis and Tavernarakis 2006).

There exist different types of synaptic membrane proteins that mediate the two categories of communication between the neurons, referred to as slow and fast synaptic transmission. Slow neurotransmission employs G-protein coupled (metabotropic) receptors, and occurs over periods of hundreds of milliseconds to minutes. Activation of metabotropic receptors leads to the activation of downstream signalling cascades, which in turn results in multiple cellular changes including the transcription of genes required for plasticity and alteration of the properties of other molecules involved in neurotransmission. (Greengard 2001). On the other hand, rapid neurotransmission employs ionotropic receptors to mediate neural communication within 1/1000th of a second. Ionotropic receptors are specialised synaptic ion channels that are gated by the binding of specific neurotransmitters. Upon activation these ionotropic receptors together with other ion channels cause ion fluxes within the postsynaptic terminal leading to a fast
excitation or inhibition (depending upon the neurotransmitter employed) (Voglis and Tavernarakis 2006). Some important synaptic ion channels and receptors which are known to directly facilitate synaptic plasticity are reviewed in Table 1.2 (Voglis and Tavernarakis 2006). Additionally, there are other classes of membrane proteins like neurotransmitter transporters and cell adhesion molecules that indirectly regulate synaptic plasticity by modulating the dynamics of the released neurotransmitters within the synapse (Amara and Fontana 2002; Tzingounis and Wadiche 2007) and by regulating the activities of neurotransmitter receptors (Dityatev, Bukalo et al. 2008).

Of particular relevance to this thesis is glutamatergic neurotransmission and the synaptic membrane proteins involved in this particular neurotransmission at excitatory synapses. The ionotropic glutamate receptors are the principal class of molecules that directly mediate glutamatergic neurotransmission. These receptors are multimeric assemblies of individual subunits. The ionotropic receptors are subdivided into three groups, the N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid (AMPA) receptors and the kainate receptors, each named after their ability to be activated by their respective synthetic glutamate analogs. These receptors are ligand-gated cation channels that allow the flow of K⁺, Na⁺ and Ca²⁺ in response to glutamate binding (Ozawa, Kamiya et al. 1998). These receptors are associated with multiple scaffolding proteins like PSD 95, SAP 97, GRIP and PICK1 that anchor the receptors within the PSD. They are also associated with several signalling molecules that perform various cellular processes leading to synaptic plasticity at excitatory synapses (Sheng and Kim 2002).

In addition to the ionotropic glutamate receptors, the other important class of synaptic membrane proteins that regulate glutamatergic neurotransmission are the glutamate transporters
belonging to the family of excitatory amino acid transporters (EAAT). The 5 subtypes of EAAT (1-5) are \( \text{Na}^+ \)-dependent, high-affinity glutamate transporters that clear glutamate from synapses released during neurotransmission. Glutamate mediates normal neurotransmission only when its concentration is regulated at the synapse. Excess glutamate can excessively activate glutamate receptors leading to neuronal death, a phenomenon termed excitotoxicity (Sheldon and Robinson 2007). Hence, the EAATs act as molecular buffers for synaptic glutamate, thereby preventing neuronal cell death by glutamate excitotoxicity (Amara and Fontana 2002; Tzingounis and Wadiche 2007). Recent studies also indicate that a subtype of EAAT indirectly modulates synaptic plasticity by regulating the recruitment of extrasynaptic and perisynaptic NMDARs in hippocampal CA1 neurons (Scimemi, Tian et al. 2009).

The defective individual functions of ionotropic glutamate receptor complexes or their individual components, along with the defective function of glutamate transporters are important causes of several central nervous system disorders including dementia, psychiatric illnesss, and epilepsy (Lau and Zukin 2007; Bowie 2008; Vincent and Mulle 2009), as well as neurodegenerative diseases (Sheldon and Robinson 2007). It is likely that a broader understanding of the biology of glutamate-mediated neurotransmission and its individual components, will contribute to understanding pathobiology.
**Figure 1.3** - Different types of membrane proteins at the neuronal membrane

Concerted activities of numerous synaptic membrane proteins belonging to the class of ionotropic receptors and metabotropic receptors together with numerous other classes of membrane proteins like ion channels, ion pumps, neurotransmitter transporters and cell adhesion molecules facilitate synaptic plasticity at the chemical synapse.

(Modified from Malenka and Nicoll 1999; Greengard 2001; Sheng and Kim 2002; Voglis and Tavernarakis 2006).
Table 1.2 Important synaptic ionotropic receptors and ion channels that are known to facilitate synaptic plasticity

<table>
<thead>
<tr>
<th>Ion channel</th>
<th>Ligand, activator</th>
<th>Conducted ion</th>
<th>Relevant expression</th>
<th>Synaptic localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA receptor</td>
<td>L-Glutamate, NMDA</td>
<td>Na⁺, Ca²⁺</td>
<td>Hippocampus</td>
<td>Presynaptic, postsynaptic</td>
</tr>
<tr>
<td>AMPA receptor</td>
<td>L-Glutamate, AMPA</td>
<td>Na⁺, K⁺</td>
<td>Hippocampus, cerebellum</td>
<td>Presynaptic, postsynaptic</td>
</tr>
<tr>
<td>Kainate receptor</td>
<td>L-Glutamate, kainate</td>
<td>Na⁺, Ca²⁺, K⁺</td>
<td>Hippocampus, thalamocortical system</td>
<td>Presynaptic, postsynaptic</td>
</tr>
<tr>
<td>nACh receptor</td>
<td>Acetylcholine, nicotine</td>
<td>Na⁺, K⁺, Ca²⁺</td>
<td>Hippocampus</td>
<td>Presynaptic, postsynaptic</td>
</tr>
<tr>
<td>GABA receptor</td>
<td>γ-aminobutyric acid</td>
<td>Cl⁻</td>
<td>Hippocampus</td>
<td>Postsynaptic</td>
</tr>
<tr>
<td>L-type VGCC</td>
<td>Membrane potential</td>
<td>Ca²⁺</td>
<td>Amygdala, hippocampus</td>
<td>Postsynaptic, postsynaptic</td>
</tr>
<tr>
<td>P/Q-type VGCC</td>
<td>Membrane potential</td>
<td>Ca²⁺</td>
<td>Hippocampus, Purkinje cells</td>
<td>Presynaptic</td>
</tr>
<tr>
<td>R-type VGCC</td>
<td>Membrane potential</td>
<td>Ca²⁺</td>
<td>Hippocampus</td>
<td>Presynaptic</td>
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<tr>
<td>BK channel</td>
<td>Ca²⁺</td>
<td></td>
<td>Hippocampus, Purkinje cells</td>
<td>Presynaptic</td>
</tr>
<tr>
<td>SK channel</td>
<td>Ca²⁺</td>
<td>K⁺</td>
<td>Amygdala, hippocampus</td>
<td>Postsynaptic</td>
</tr>
</tbody>
</table>

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; BK, large conductance calcium-gated potassium channel; GABA, γ-aminobutyric acid; LTP, long-term potentiation; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; SK, small conductance calcium-gated potassium channel; VGCC, voltage-gated calcium channel.

(Modified from Voglisi and Tavarnarakis 2006)
1.1.3 Additional regulatory protein partners for the ion channels and receptors

Ionotropic receptor function at synapses is modulated by multiple factors, including alternate splicing of the individual subunits, subunit composition of the heteromer, and post-translational modification of the receptor (Wilding and Huettner 2001; Cull-Candy and Leszkiewicz 2004; Greger, Ziff et al. 2007). Also, receptor trafficking to the surface, timing of expression at the neuronal membrane, endocytosis, and abundance within the PSD are additional factors that further fine tune receptor functions (Shepherd and Huganir 2007; Coussen 2009; Groc, Bard et al. 2009). The emerging theme in this field is that the core subunit of these ligand-gated synaptic ion channels and receptors associate with additional transmembrane protein partners to regulate receptor function (Gally, Eimer et al. 2004; Zheng, Mellem et al. 2004; Milstein and Nicoll 2008; Coombs and Cull-Candy 2009; Ng, Pitcher et al. 2009; Sager, Tapken et al. 2009; Zhang, St-Gelais et al. 2009).

One widely studied example of this association occurs with AMPARs and the transmembrane AMPA receptor regulatory proteins (TARPs). Directly interacting with the AMPAR subunits, TARPs are thought to act like chaperones for AMPARs, assisting their proper folding and assembly within the endoplasmic reticulum. More importantly, the TARPs participate in delivery and synaptic localisation of AMPARs, facilitate stabilisation within the PSD, as well as their lateral diffusion between the extrasynaptic and synaptic regions. Also, TARPs alter the biophysical properties of AMPARs by increasing the glutamate-evoked current and by reducing their desensitization following glutamate binding (Collingridge, Isaac et al. 2004; Sager, Tapken et al. 2009). Therefore the AMPAR and TARP complex, along with several
other AMPAR associated proteins, perform various cellular processes leading to synaptic plasticity (Figure 1.4).

Although critical interacting partners for the core subunit of other ionotropic glutamate receptors have been identified (Zheng, Brockie et al. 2006; Ng, Pitcher et al. 2009; Zhang, St-Gelais et al. 2009), a much more elaborate study to understand the precise functional role of such interactions is required in order to have a clearer picture of their precise roles during glutamatergic neurotransmission. These studies will not only broaden our understanding of normal brain function, but also increase knowledge about different pathological states.
**Figure 1.4** - Known AMPAR associated proteins at the PSD

The core AMPAR subunit is associated with TARP, which regulates the biophysical properties of AMPAR and its subcellular and synaptic localizations. Together with several associated proteins the AMPAR-TARP complex plays a crucial role during synaptic plasticity.

(Modified from Collingridge, Isaac et al. 2004)
Part 2 - Introduction to the Neto proteins

1.2.1 Neto1 and Neto2

Previously, in an approach to utilize the existing expressed sequence tag (EST) data to attribute potential functions for novel proteins, an *in silico* screen of human retinal ESTs was performed. Two molecules encoding the CUB domain protein:protein interaction motif were identified in the screen. These proteins were designated neuropilin-1/tolloid-like (Neto), based on their sequence similarity with the known axon guidance protein neuropilin, (He and Tessier-Lavigne 1997) and the dorso-ventral patterning protein tololoid (Shimell, Ferguson et al. 1991). The *Neto1* and *Neto2* genes encode two closely related, evolutionarily conserved type I transmembrane neuronal proteins with their C-terminus facing the cytoplasm and N-terminus facing the extracellular space at the neuronal membrane (Stohr, Berger et al. 2002; Michishita, Ikeda et al. 2003; Michishita, Ikeda et al. 2004). Neto1 (533 amino acids) and Neto2 (525 amino acids) contain two extracellular CUB domains (for Complement C1r/C1s, Uegf, and Bmp 1), one LDLa motif (for low-density lipoprotein receptor domain class A), a single pass transmembrane domain, and a cytoplasmic tail (Figure 1.5). The cytoplasmic tails of Neto1 and Neto2 are 168 and 157 amino acids long respectively.

The CUB1, CUB2 and LDLa domains (ectodomain) of vertebrate Neto1 and Neto2 share 63%, 71%, and 83% amino acid identity respectively, while their cytoplasmic domain (endodomain) shares only 39% identity. Both Neto1 and Neto2 have C-terminal PDZ ligands. While Neto1 has a class I PDZ tripeptide, Neto2 terminates in a putative class II PDZ tripeptide. This similarity suggests that Neto1 and Neto2 may bind to similar proteins on their extracellular side but may associate with different intracellular partners. There are also Neto-like proteins in
invertebrates that have similar domain organisation to the Neto proteins such as Q9XUU2 in *C. elegans* and Q9VYC7 in *Drosophila melanogaster* (Zhang, St-Gelais et al. 2009) and other CUB domain proteins with less similar domain organisation to the Neto proteins like SOL-1 and LEV-10 in *C. elegans* (Gally, Eimer et al. 2004; Zheng, Mellem et al. 2004).

In the mature mouse brain, *Neto1* and *Neto2* display both overlapping and distinct patterns of expression. Both are strongly expressed in the cerebral cortex, olfactory bulb, olfactory tubercle, pons and hippocampus. However, in cerebellum *Neto1* is only expressed in Purkinje cells, whereas *Neto2* is expressed in both Purkinje and granule cell layers. In the hippocampus *Neto1* mRNA is seen throughout the pyramidal and granule cell layer with strong expression in CA3 pyramidal cells. In contrast, *Neto2* is uniformly expressed in the pyramidal cells and weakly expressed in the granule cells (Michishita, Ikeda et al. 2003; Michishita, Ikeda et al. 2004; Ng, Pitcher et al. 2009; Zhang, St-Gelais et al. 2009).
Figure 1.5 - Domain organisation of the Neto proteins and related proteins in vertebrates and invertebrates
To elucidate the biological functions of the Neto proteins, a previous graduate student in the lab (David Ng) generated mice lacking each of the *Neto1* and *Neto2* genes (*Neto1*\(^{-/-}\) mice and *Neto2*\(^{+/-}\) mice) and observed several biochemical changes and behavioral phenotypes in the knockout animals. Both *Neto1*\(^{-/-}\) and *Neto2*\(^{+/-}\) mice showed axon-guidance defects during development (Ng 2006), synaptic plasticity defects (Ng, Pitcher et al. 2009) and neuronal excitability defects in adults (Ng 2006).

The developmental defects in Neto-null mice can be ascribed to the axon guidance functions of the Neto proteins, similar to the phenotypes observed in loss of function mouse mutants of the molecules of the Neuropilin family. The more surprising adult phenotypes such as learning and memory impairments and myoclonic seizures seen in Neto-null mice further indicated that the Neto proteins might be involved in a wide variety of CNS functions, both during early development and in adults. Several biochemical fractionation tests were performed to identify the precise subcellular localisation of the Neto proteins *in vivo*, and the results indicated that both Neto1 and Neto2 are present at the postsynaptic membrane and within the PSD of excitatory synapses in the mouse brain, and that each interacts with multiple components of the PSD (Ng, Pitcher et al. 2009; Zhang, St-Gelais et al. 2009). These fractionation tests also revealed that Neto2 is a component of the presynapse (Tang, Unpublished), but the presynaptic protein partners of Neto2 and its precise role at the presynapse are currently unknown.
1.2.2 Neto proteins and the glutamate receptors

In a recent study published from our lab (Ng, Pitcher et al. 2009), it was shown that Neto1 physically interacts with the NR2A and NR2B subunits of the NMDAR, and that this interaction is mediated by the ectodomain of Neto1. In addition, Neto1 interacts with PSD-95 through its PDZ ligand, in both hippocampal crude synaptosome fractions and in transfected HEK293 cells.

The NR2A and NR2B subunits of the NMDAR and PSD-95 are important components of the NMDAR complex. The NMDAR complex is the principal ionotropic receptor complex that is critical for glutamatergic neurotransmission at excitatory synapses. They are heterotetrameric assemblies between the glycine-binding NR1 subunits and the glutamate-binding NR2 subunits. Upon glutamate binding and a concurrent voltage-dependent activation, the ion channel of the NMDAR opens leading to the influx of Na\(^+\) and Ca\(^{2+}\) and an efflux of K\(^+\). This in turn leads to downstream signalling cascades that regulate several cellular functions. The NR2A and NR2B subunits are therefore critical core components of the NMDAR which modulate receptor activity. PSD-95 is an important scaffolding protein of the NMDAR complex which anchors several ion channels and receptors in the PSD (including the NMDAR) with cytoskeletal and signalling proteins. It is well known that these components of the NMDAR complex are critical molecular players for hippocampal-dependent synaptic plasticity as well as spatial learning and memory (Cull-Candy and Leszkiewicz 2004; Nakazawa, McHugh et al. 2004) (Fig 1.6a).

While the Neto1 interaction with the NMDAR complex through NR2 subunits and PSD-95 has important physiological and behavioural consequences in mice, the molecular mechanisms remain unknown. The absence of Neto1 results in a reduction in the abundance of NR2A but not NR2B subunits in the hippocampal PSD of Neto1-null mice. However, no change was observed
in the overall abundance of both NR2A and NR2B subunits in whole brain extracts or in their surface expression in the hippocampus (Ng, Pitcher et al. 2009). These findings imply that Neto1 is critical for the delivery or stability of NR2A-NMDARs at the synapse. As a consequence of the reduction of NR2A subunits in hippocampal PSDs, Neto1-null mice showed a decrease in NR2A-mediated currents and an overall reduction in long term potentiation (LTP) at Schaffer collateral-CA1 synapses of the hippocampus (Ng, Pitcher et al. 2009). LTP is an important phenomena underlying synaptic plasticity that is considered one of the major cellular mechanisms underlying learning and memory (Malenka and Nicoll 1999). Correspondingly, Neto1-null mice also showed impairment in hippocampus-dependent spatial learning in Morris water maze tasks. Comparable to the TARPs that control AMPAR targeting to the synapse, Neto1 thus represents a new protein that functions to maintain synaptic NMDARs and NMDAR-mediated synaptic transmission and learning.

Another group recently published that Neto2 directly interacts with the kainate type of ionotropic glutamate receptor (KAR) subunit GluR6 in rat cerebellar lysates (Zhang, St-Gelais et al. 2009). They showed that Neto2 modulates the functional properties of kainate receptors including glutamate-evoked currents (mediated by the LDLa domain of Neto2) and glutamate sensitivity but not the surface trafficking of GluR6 subunits in *Xenopus laevis* oocytes injected with Neto2 and GluR6 cRNA. They also showed that Neto2 modulates kainate-receptor-mediated synaptic transmission in cerebellar granule cell cultures cotransfected with Neto2 and GluR6 cDNA. Although the investigations did not extend to Neto2 null mice, additional experiments from our lab clearly highlight the association of Neto2 and KAR subunits in multiple brain regions *in vivo* (Tang, unpublished) (Figure 1.6A).
While the ionotropic glutamate receptors that interact with vertebrate Neto1 and Neto2 are known, the binding partners of Neto-like molecules in invertebrates are not well characterized. However, other invertebrate CUB domain proteins like SOL-1 and LEV-10 in *C. elegans*, are known to physically interact with ionotropic receptors in the nervous system. SOL-1 was identified as an auxiliary subunit that modulates the gating of *C. elegans* GLR-1-type glutamate receptors (Zheng, Mellem et al. 2004; Zheng, Brockie et al. 2006), while LEV-10 is involved in clustering of acetylcholine receptors (AchRs) at the *C. elegans* neuromuscular junction (Figure 1.6B).

A clear picture emerging out of these studies is that ionotropic receptors, particularly glutamate-binding ionotropic receptors in the CNS, require additional regulatory binding partners for their proper function, and that CUB domain proteins are an important class of proteins that mediate protein:protein interactions with ionotropic receptors and contribute to multiple aspects of their function. It is well known that the ionotropic glutamate receptors are involved in several important cellular and physiological processes, and aberration in their function leads to pathological states including psychiatric illness, epilepsy, or dementia (Lau and Zukin 2007; Bowie 2008; Vincent and Mulle 2009). Therefore it is important to gain more understanding about the precise molecular mechanisms by which Neto1 and Neto2 modulate neurotransmission.
Figure 1.6 – (A) Association of vertebrate Neto1 and Neto2 with components NMDA-type and kainate-type ionotropic GluRs respectively (B) Association of invertebrate CUB domain proteins SOL-1 and LEV-10 with GluR-1 and AchR respectively.

Identification of CUB-domain-containing protein Neto1 and Neto2 in vertebrates and SOL-1 and LEV-10 in invertebrates as ion channel-binding accessory proteins indicate a conserved role for the CUB-domain-containing proteins as ion channel subunits throughout nature. The precise molecular mechanisms by which these CUB-domain proteins particularly Neto1 and Neto2, modulate neurotransmission through these interactions, and possibly through other interacting protein partners, are not yet understood.
1.2.3 Known interacting partners of the Neto proteins

Several studies were conducted in the McInnes lab to identify the interacting protein partners of the Neto proteins to gain a better understanding of their biological function. A candidate approach was undertaken to identify the protein partners of the Neto proteins during axon-guidance in the developing brain, based on the knowledge of known interacting partners of the neuropilins. This approach identified PlexinD1 as a strong interactor of Neto1 in the presence of the semaphorin3F-axon-guidance ligand (Gingrich, unpublished). In parallel, a candidate approach to identify the interacting partners of the Neto-C-terminal PDZ ligand with known PDZ domain proteins was also attempted. These experiments identified Glutamate Receptor Interacting Protein (GRIP) as an interacting partner of Neto2 through its class II PDZ ligand (Tang, unpublished) (Figure 1.7).

Unbiased approaches like yeast two-hybrid (YTH) and glutathione-S-transferase (GST) pull down were also applied to identify the interactome of the Neto proteins (Tang, Ivakine, unpublished) (Figure 1.7). However, these approaches relied on the soluble cytoplasmic domain of the Neto proteins. Since it is well established from the literature that CUB domains also mediate protein:protein interactions, and that several CUB domain proteins including Neto1 and Neto2 regulate the function of some important ion channel receptors, it remained possible that other membrane-bound interactors of the Neto proteins may not have been identified by these approaches (unless the cytoplasmic region of the Neto proteins was exclusively required for an interaction, as in the K⁺Cl⁻-cotransporter KCC2-Neto2 interaction identified through the GST pull down approach (Ivakine, unpublished)).
Supporting this idea, there are other phenotypes in the Neto2-null mice that are not clearly explained by the known interacting partners to date. For example, an interesting phenotype observed in the Neto2-null mice is the presence of recurrent myoclonic seizures. These mice also show abnormal brain discharges as observed in electroencephalogram (EEG) recordings, which is a characteristic of aberrant neuronal excitability. This phenotype suggests that Neto2 might associate with additional molecules that are involved directly or indirectly in the maintenance of neuronal membrane excitability. If these additional protein interactions were mediated through the Neto2-ectodomain, they may not have been identified by the previous approaches.

Thus I undertook to identify the membrane-bound and possibly, the ectodomain mediated interacting partners of the Neto proteins to understand their full range of biological functions. My project focused on identifying the membrane-bound, interacting partners of the Neto2 protein using a novel approach called Membrane Yeast Two Hybrid (MYTH).
Figure 1.7 - Some of the known interacting partners of Neto1 and Neto2 identified in our lab using different candidate and unbiased approaches.

The Neto1-NR2A/B interaction was reported by a previous graduate student David Ng from our lab (Ng, Pitcher et al. 2009); The Neto2-GluR6 interaction was reported by (Zhang, St-Gelais et al. 2009)
Part 3 - Introduction to Membrane Yeast Two Hybrid (MYTH)

In the traditional yeast two-hybrid (YTH) system, the bait and the prey molecules are fused to split-transcription factor components. Therefore, the interacting partner fusions must be relocated into the nucleus to activate the reporter genes. This system poses a considerable challenge for proteins that contain high hydrophobicity and/or transmembrane domains. Therefore, the only way to study membrane-anchored proteins using traditional YTH is to limit the baits to individual cytosolic and extracellular domains (Auerbach, Thaminy et al. 2002; Stagljar and Fields 2002). This separation of peptides from their natural context may complicate or detract from functions. To circumvent this problem, a split-ubiquitin based Membrane Yeast Two Hybrid system was developed for identifying interacting partners of integral membrane proteins and membrane associated proteins (Fetchko and Stagljar 2004; Miller and Stagljar 2004; Thaminy, Miller et al. 2004).

1.3.1 Split-ubiquitin based MYTH – A novel approach to identify the interactome of the neuronal transmembrane protein Neto2

The principle behind MYTH is based on the observation that ubiquitin can be separated into two fragments and that ubiquitin can functionally reconstitute when both ubiquitin parts are presented in close proximity. Ubiquitin is an evolutionarily conserved protein which marks proteins for degradation by the proteasome system. In the MYTH approach, the membrane protein of interest (Neto2), called the “bait protein” was fused to the C-terminal half of split-ubiquitin ($C_{ub}$), along with an artificial transcription factor that consists of the bacterial LexA-DNA binding domain and the *Herpes simplex* *VP16* transactivator protein. The putative interacting proteins, called “prey-proteins” are fused with the N-terminal half of split-ubiquitin
(N\textsubscript{ub}) and can be either membrane-bound or cytosolic proteins (Fetchko and Stagljar 2004; Miller and Stagljar 2004; Thaminy, Miller et al. 2004).

Due to a high affinity between the two halves of split-ubiquitin, they naturally reconstitute and are recognized by ubiquitin binding proteases (UBPs). In order to prevent a rapid reconstitution, glycine (G) is substituted for an isoleucine (I) at position 13 in the wildtype N\textsubscript{ub} fragment to produce N\textsubscript{ub}G. Only upon the interaction between the split-ubiquitin-associated bait and the prey protein, C\textsubscript{ub} and N\textsubscript{ub}G are brought into close proximity resulting in the reconstitution of split-ubiquitin. The reconstituted ubiquitin is then recognized by UBPs resulting in the cleavage of the linker-polypeptide chain between C\textsubscript{ub} and the LexA-VP16 part of the fusion-bait protein. As a result, the artificial transcription factor is released from the membrane bound-bait and translocates into the nucleus where it binds to the LexA operator region situated upstream of the reporter genes \textit{HIS3}, \textit{ADE2} and to an additional LexA operator region situated upstream of the reporter gene \textit{lacZ}. The VP16 transactivator domain then recruits the RNA polymerase II complex to the transcriptional start of the reporter gene and results in its transcriptional activation and gene expression. A common promoter activates the reporter genes \textit{HIS3} and \textit{ADE2} while a separate promoter activates the \textit{lacZ} reporter gene. The \textit{HIS3} and \textit{ADE2} genes are two auxotrophic growth markers, which encode enzymes for the histidine and adenine biosynthetic pathways, while the \textit{lacZ} gene encodes the enzyme β-galactosidase. Thus, upon the interaction between two proteins at the membrane of yeast, the activation of the reporter genes are translated into a transcriptional readout resulting in growth of yeast on selective media and color development in a β-galactosidase assay (Thaminy, Miller et al. 2004; Iyer, Burkle et al. 2005) Figure 1.8.
**Figure 1.8 - Overview of the MYTH technology**

(A) A membrane bait protein (Neto2) is fused to C\textsubscript{ub} followed by an artificial transcription factor LexA-VP16, while another prey membrane (or cytoplasmic) protein is fused to the N\textsubscript{ub}G tag. When there is no interaction between the bait and the prey, there is no reconstitution of ubiquitin and no UBP-mediated cleavage of the transcription factor, thus resulting in no growth and a negative colorimetry assay.

(B) Upon interaction between bait and prey proteins, the ubiquitin reconstitution occurs leading to a proteolytic cleavage by UBPs and the subsequent release of the transcription factor. The factor enters into the nucleus and activates reporter genes by binding to the LexA operator sites (lexA ops) within their promoters. This results in \textit{HIS}^+/\textit{ADE2}+ and \textit{lac}Z yeast cells in growth assay and colorimetry assays.
1.3.2 Rationale to the use of MYTH strategy

It is well known that several synaptic proteins; particularly the ion channels and receptors, form multi-protein complexes to carry out specific cellular functions (Kennedy 2000; Scannevin and Huganir 2000). Traditionally, biochemical methods such as co-purification or co-immunoprecipitation from tissue samples and heterologous cell systems have been used to study the composition of protein complexes and identify direct protein interactions. However, to characterise the direct interacting partners of a specific protein of interest these methods generally require extensive optimization for each interaction, making them unsuitable for unbiased screens. These biochemical approaches are best suited to validate putative interactors obtained from other large-scale, unbiased mass-spectrometry (MS) and YTH screens – the two most popular approaches available to study protein:protein interactions occurring in the nervous system (Husi and Grant 2001). While the traditional YTH approach cannot be used to identify the protein interactions occurring at the membrane (as described in the Part 3 introduction), a limitation of MS screens is that these screens require elaborate isolation and purification of native protein complexes from the membrane (Iyer, Burkle et al. 2005; Anderson and Grant 2006).

MYTH is a recently developed approach to identify protein:protein interactions and is a modification of the traditional YTH, where split-ubiquitin with a cleavable transcription factor is tagged with the protein of interest. This single modification results in an important advantage - integral membrane proteins can be directly tagged with split-ubiquitin, thus allowing the identification of their interacting protein partners at the membrane (Stagljar and Fields 2002; Fetchko and Stagljar 2004; Iyer, Burkle et al. 2005). The previous approaches used in the lab to
identify Neto2 interacting partners did not allow for such an advantage, MYTH is one of the best approaches available to perform an unbiased protein: protein interaction screen for Neto2.

1.3.3 Specific objectives and rationale of the thesis

Objective

My overall objective was to identify novel membrane-bound interacting partners of the Neto2 protein in order to acquire a better understanding of the biology of Neto2.

Strategy

I used a recently developed yeast-based screen called MYTH that allows the identification of putative interacting partners of membrane proteins (Fetchko and Stagljar 2003; Gisler et al, 2008). I used a human adult whole brain cDNA library (purchased from Dual system Biotech) and a human embryonic whole brain cDNA library (obtained from our collaborator Dr. Igor Stagljar) to identify putative interacting partners. The rationale behind choosing these particular cDNA libraries is based on:

- My interest in understanding the role of Neto proteins in the embryonic and adult brain.
- Availability of libraries specifically for type I and type II transmembrane proteins. Both type I and type II membrane proteins have single-pass transmembrane domains, but differ in the orientation of their COO\(^{-}\) and NH\(_2\)\(^{+}\) termini. While type I proteins have their N-terminus facing the extra cellular space (or ER lumen) and C-terminus facing the cytoplasm, type II proteins have their have C-terminus facing the
extracellular space (or ER lumen) and the N-terminus facing the cytoplasm. The specificity of each library for these two types of membrane proteins allow the identification of putative Neto2-interacting partners specifically from these two libraries (explained in discussion and appendix III).

- Reliability of the cDNA libraries as observed from previous publications (Thaminy, Auerbach et al. 2003; Matsuda, Giliberto et al. 2005).

Rationale

It will be informative to identify additional interacting partners of Neto proteins other than those reported because, certain complex phenotypes in the Neto2 null mice cannot be explained by the known interactions alone. Furthermore, the inclusion of the CUB and/or LDL domains of Neto2 will enable elucidation of a broader interactome of the Neto2, and the anticipated partners may include components of neurotransmitter receptor complexes, ion channels, channel binding proteins, neurotransmitter transporter complexes, or other classes of proteins that could not be identified with the traditional Y2H methodology. It might provide more clues about the observed phenotypes in Neto2 null mice (like the aberrant neuronal excitability phenotype). Additionally, this study could also open new avenues in understanding the broader biological functions of Neto2 with the identification of additional primary phenotypes that have not been observed so far.
1.3.4 Advantages and limitations of MYTH

The most important advantage of the MYTH system is that it allows for the identification of the interacting partners of membrane proteins in their natural cellular location at the membrane. Therefore by using this approach, novel membrane-bound interacting partners for Neto2 can be identified. However, the ectopic expression of human Neto2 in the yeast system has its own limitations.

I. The strong exogenous promoter directing the Neto2-bait fusion expression in yeast may result in Neto2-bait protein over-expression. Self-activation of the over-expressed bait protein may lead to a high number of false positive interactions in the MYTH system. Another similar limitation in the MYTH system is the over-expression of prey proteins due to the use of an exogenous promoter for prey plasmid expression. Therefore, appropriate measures need to be taken to verify the expression of bait protein and to recognize self-activating bait complications.

II. The over-expression of bait/prey proteins might lead to false positive results due to non-specific interactions. This limitation should be avoided by using appropriate experiments and carefully designed control evaluations.

III. Some of the Neto2 interactions that might be mediated by post-translational modifications, such as glycosylation, cannot be identified by the MYTH system because yeast do not have the elaborate post-translational mechanisms of higher eukaryotes.

IV. The Cub-LexA-VP16 tag on the bait protein could cause steric hindrance for certain interactions, which thus cannot be identified through this system. Also, because Neto2
has a C-terminal class II PDZ ligand, and because this PDZ ligand is completely masked by the C ub-fusion tag, those interactions mediated through the Neto2-PDZ ligand cannot be identified in the MYTH system. However, this limitation is not anticipated to be a major issue as the traditional YTH has already led to the detection of C-terminal class II PDZ ligand-mediated interactions.

V. Additionally, there are certain limitations that are associated with the cDNA libraries used for the MYTH screen. As in most cDNA libraries, the cDNA clonal representations in the MYTH library might not be the actual representations of mRNA levels in the brain tissue from which they were produced. As a result, certain clones might be over-represented and others could be under-represented. Therefore, putative interactors identified using the MYTH system should be well validated by other biochemical approaches in vivo.

VI. Other limitations of this yeast-based approach are inherent to the model system. For example, mutations within the reporter genes could cause false positive results in the growth and lacZ assay. This can be prevented using fresh yeast strains from time to time. Also, leaky reporter genes might cause false positive results in the growth assay. This can be minimized by the use of appropriate chemical inhibitors that inhibit the leaky gene product.

Despite these limitations, MYTH allows the identification of protein partners of membrane proteins by not requiring the nuclear translocation of the bait-protein, but only a cleavage product from it. For the Neto2-MYTH screen, well-validated human whole brain-embryonic and adult cDNA libraries are available separately for type I and type II membrane
proteins. Additionally, these cDNA libraries will allow the identification of novel Neto2-interacting partners from multiple brain regions simultaneously. Therefore, coupled with additional biochemical approaches like co-immunoprecipitation from heterologous cell systems and tissue samples, MYTH is a powerful approach to identify putative membrane-bound Neto2-interactors.
Chapter 2: Materials and methods
Part 1 - Summary of materials

Appendix I is a compiled list of bacterial and yeast strains, plasmids, oligonucleotide primers and antibodies used; Appendix II is a compiled list of additional information on the MYTH bait and prey plasmids; Appendix III provides details about the cDNA libraries used for the MYTH screen; and Appendix IV contains all the standard protocols used.

The yeast strain THYAP4, MYTH empty plasmids, MYTH control plasmids, and human embryonic whole brain cDNA library used in the project was obtained from Dr. Stagljar’s lab. The plasmids has been constructed and verified by Victoria Wang, Dawn Edmonds and Jamie Snider from their lab. The Human adult whole brain cDNA library was purchased from Dualsystems Biotech, Switzerland. The Neto2-bait plasmid and Neto1-positive control prey plasmid were constructed using base vectors obtained from Dr. Stagljar’s lab as described in this chapter.
Part 2 - Construction of the Neto2 bait plasmid

2.2.1 Primer design for the Neto2- MYTH

Forward and reverse long primers were designed such that they contained 40 nucleotide (nt) homologous to the bait vector pAMBV, in-frame with the 20 nt homologous to the human Neto2 cDNA reading frame. In the forward primer, the homologous region within the bait vector was flanked with an MF–alpha signal sequence at the 5’end and the NcoI restriction site at the 3’end, while the homologous region within the Neto2 cDNA was chosen from the beginning of the first CUB domain, omitting the human signal sequence. In the reverse primer, the homologous region within the bait vector was flanked with the NcoI restriction site at the 5’end and Cub - VP16 fusion tag at the 3’end, whereas the homology chosen in the Neto2 cDNA was at its 5’end, omitting the human stop-codon. Thus the forward primer (5’ - CCG AAC CAG TGG CTG CAG GGC CGC CTC GGC CAA AGG CCT CAA AAA ACC CAA GAT GGA CAA – 3’) and the reverse primer (5’ -GAT CAA TCT TTG TTG ATC TGG AGG GAT CCC CCC CGA CAT GAA GTC AAT GGA TAT GGA TGC – 3’) were designed.

2.2.2 PCR amplification of the Neto2 cDNA using MYTH primers

Full length human Neto2 was initially PCR amplified from total retinal cDNA, cloned into pBluescript vector and sequenced by Zhenya Ivakine. Using the MYTH long primers, the human Neto2 cDNA was amplified such that its native signal sequence and stop codon were removed and a 40 nt stretch of homologous arms of the bait plasmid were included at its 5’ and 3’ ends. The PCR reaction was performed as described in (Iyer, Burkle et al. 2005). Briefly, the
reaction contained 10 µM of each primer, 25 mM of each dNTP, 5 ng of Neto2 cDNA template, 1 µl of high-fidelity Herculase polymerase (Stratagene) and 10 µl of 5X PCR buffer, made to a final volume of 50 µl with nucleic acid-free sterile ddH₂O. After a 2 min denaturation of the sample at 95°C, PCR was performed for 30 thermal cycles consisting of a short denaturation for 30 sec at 95°C, annealing for 1 min at 48°C and extension for 4 min at 72°C. A final extension was performed at 72°C for 4 min before the PCR products were incubated at 4°C. PCR products were analyzed using a 0.8 % agarose gel and they were gel-purified using a QIAquick Gel Extraction kit from Qiagen according to the manufacturer’s instructions. Purified PCR products were stored at -20°C prior to transformation.

**2.2.3 pNeto2-Cub-fusion bait construction by in vivo homologous recombination strategy**

1 µg of empty bait vector pAMBV was restriction digested using 5U/µl of NcoI restriction enzyme prepared in NEB 3 buffer (New England Biolabs), along with 2.5 µl of 10X NEB 3 buffer and made to a final volume of 25 µl with nucleic acid-free sterile ddH₂O. The reaction mixture was incubated at 37°C for 1 hr after which the enzyme was heat-inactivated at 75°C for 3 min. 2 µl of the digested DNA was run on a 0.8% agarose gel along with an undigested empty vector control to verify complete digestion of the empty bait vector. The reaction mixture was purified using a QIAgen purification kit from Qiagen according to the manufacturer’s instructions. Purified linearized vector was stored at -20°C prior to transformation.

Before the day of transformation, a single colony of fresh THYAP4 yeast strain was inoculated into 5 ml of YPAD media. The culture was grown overnight at 30°C, shaking at 225
rpm. The OD$_{600}$ of the overnight culture was measured and the culture was diluted in YPAD media to a final OD$_{600}$ of 0.3. For each transformation 2 ml of yeast culture was required. Therefore the overnight culture was diluted in an appropriate volume of YPAD media. The diluted culture was again incubated at 30°C with shaking to a final OD$_{600}$ of 0.8 (3-5 hrs). 2 ml of yeast cells per transformation reaction were then pelleted at 700g for 5 min at 4°C; resuspended and washed in 1 ml of sterile chilled-1X TAE buffer in a sterile 1.5 ml eppendorf tube. The cells were again pelleted at 700g for 5 min at 4°C and resuspended in 100 µl of sterile, chilled ddH$_2$O. The cells were incubated on ice while preparing the other reagents.

2mg/ml ssDNA was thawed in a boiling water bath for 5 min prior to use and the PEG/LiOAc master mix was freshly prepared. For each transformation reaction, 326 µl of the master mix, 500 ng of the purified Neto2 PCR product and 100 ng of linearized empty bait vector were added and the volume was made to 360 µl / reaction with sterile chilled ddH$_2$O in the tube containing 100 µl of yeast cells. Additionally, a similar reaction with 100 ng of linearized empty bait (without the PCR product); and a third reaction with 100 ng of undigested bait vector (without the PCR product) served as a negative control and transformation control respectively. All the transformation reactions were incubated in a 42°C water bath for 15 min.

All transformation reactions were pelleted at 700 g for 5 min at room temperature, and they were resuspended in 200 µl of ddH$_2$O. 25 µl and 50 µl of each transformation reaction were diluted in 0.9% NaCl to a final volume of 100 µl, which were plated onto SD-Leu plates. All the plates were sealed with parafilm and incubated at 30°C for 2 – 3 days. After growth, the colonies were counted in the control plates and the master plates to assess transformation efficiency of homologous recombination. After a successful transformation, eight isolated colonies from each plate containing putative recombinant Neto2-C$_{ub}$-fusion vector and 23 isolated colonies from the
negative control transformation plate were restreaked onto fresh SD-Leu plates; they were sealed and incubated at 30°C for 2 days. After growth, colony PCR was performed on each of these restreaked colonies to assess a successful transformation.

2.2.4 Identification of putative Neto2-Cub-fusion clones, sequencing, and verification

Colony PCR - For colony PCR, a forward primer was designed from within the first CUB domain of human Neto2 and a reverse primer was designed within the LexA domain of pAMBV; forward (5’ - ATT TGG GTT CGA ACC AGC AAT GGA – 3’) and reverse (5’ - TTT TCT GGC AAC AGT TCG ACT TTA TT – 3’) respectively. 10 µl of 2X Qiagen multiplex PCR buffer (containing DNA polymerase, dNTPs and reaction buffer) was mixed with 0.5 µl each of the 10 µM forward and reverse primers; and were made to a final volume of 20 µl with nucleic acid-free sterile ddH2O in PCR tubes. The tubes were incubated on ice until the start of the reaction. Each fresh colony of the putative Neto2-Cub-fusion clone was picked using a sterile micropipette tip and inoculated into the individual PCR tubes and mixed. Only miniscule amounts of the colony were sufficient for a successful PCR reaction. A clone from yeast bearing an empty bait plasmid was considered as a negative control.

Before the actual reaction, the reaction mixtures were heated in the PCR plate for 95°C for 15 min to break the yeast cell wall. 30 thermal cycles consisting of a short denaturation for 30 sec at 95°C, annealing for 1 min at 57°C and extension for 2 min at 72°C was performed. This was followed by a final extension for 4 min at 72°C, before the PCR products were incubated at 4°C. PCR products were analyzed using a 0.8 % agarose gel. Putative Neto2-Cub-fusion plasmids were rescued from the clones corresponding to a DNA band size of 1.6 kb.
Plasmid rescue from yeast and bacterial transformation – Putative Neto2-Cub-fusion plasmids were isolated using a QIAprep -Miniprep Kit from Qiagen. The manufacturer’s bacterial miniprep protocol was slightly modified and applied for yeast plasmid rescue. Fresh colonies were inoculated into 5 ml of SD-Leu media each. The cultures were grown for 20 – 24 hrs at 30˚C, shaking at 225 rpm. The cells were harvested and resuspended in P1 buffer containing RNaseA. 200 µl of glass beads were added and the cells were vortexed 5 times for 1 min each, on ice. After spinning, the clear supernatant was incubated with P2 buffer and the regular bacterial miniprep protocol was applied subsequently, to extract the yeast plasmid using QIAprep spin columns. At the final step DNA was eluted in 30 µl TE buffer. Upon isolating the yeast plasmid DNA, the quality and concentration of the yield were verified by running the samples on a 0.8% agarose gel and by spectrophotometry.

Since the yeast plasmid isolation procedures may not yield good quality plasmids sufficient in quantity for subsequent sequencing analyses, the isolated yeast plasmids were transformed into E. coli to obtain high quality pure plasmid in sufficient quantity for subsequent sequencing and analyses. 5 µl of the yeast plasmid from the above procedure was used to transform 25 µl of the bacterial E. coli strain DH5α by the heat-shock method. After a recovery of the transformants in 970 µl of LB media in 37 °C, 100 µl of each transformed culture was plated onto a single LB plate containing 30 µg/ml of kanamycin. After growth of the colonies, the plasmids were isolated using a standard QIAprep -Miniprep Kit from Qiagen according to the manufacturer’s instructions.

Sequencing of the clones containing the Neto2-fusion bait insert - A forward primer and a reverse primer were designed for sequencing and verifying that the Neto2 cDNA was in-frame with the pAMBV bait vector; forward (5’ – TTT CCT CGT CAT TGT TCT CGT TCC CT
TCT – 3’) and reverse (5’ - TTT TCT GGC AAC AGT TCG ACT TTA TT – 3’). 250 ng of the bacterial miniprep along with 35 ng of the custom primers made to 7.7 µl with TE buffer was sent for sequencing at the TCAG (The Centre for Applied Genomics)-DNA sequencing facility at The Hospital for Sick Children. After verifying that the Neto2 cDNA sequence was in-frame at its 5’ and 3’ end along with the pAMBV, the whole fusion plasmid was sequenced with four forward and four reverse primers spanning throughout the ORF of the fusion plasmid (refer to appendix I for the primer information). Upon obtaining the sequences, each was carefully examined for point mutations that might alter the reading frame of the Neto2-Cub-fusion bait plasmid. One clone containing the Neto2-Cub-fusion plasmid was identified with proper insert orientation devoid of any change in the nucleotides. This clone was used for the experiments performed to verify proper expression, localization, and functionality.

Part 3 - Verification of the bait protein expression and localization

2.3.1 Preparation of total protein extracts and verification of the Neto2-fusion protein expression

To identify whether the Neto2-fusion protein is expressed in the yeast transformants bearing the Neto2-fusion plasmid, total protein extracts were prepared and tested with different antibodies after the samples were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. A fresh Neto2-Cub bait-bearing yeast clone was inoculated into 5 ml of SD-Leu media and grown overnight at 30°C with shaking. In parallel, a fresh culture of empty bait-bearing yeast was grown as a negative control. The overnight culture was diluted into 20 ml of fresh SD-Leu media to an OD₆₀₀ of 0.2. Cells were grown at 30°C with shaking until an OD₆₀₀ of
0.6 – 0.8 was reached. 9 ml of the fresh culture was then fixed with 1ml of 100 % trichloroacetic acid (TCA). The culture tube was rotated for 15 min at room temperature. Cells were pelleted at 4000 rpm for 10 min at 4°C and they were resuspended in 5 ml of 1M Tris-HCl, pH 7.5 and washed. Cells were pelleted at 13,000 rpm for 1 min at 4°C and the supernatant was discarded. Cells at this stage were quickly frozen in liquid nitrogen and stored at -80°C until used. Before loading, the pellets were thawed on ice and resuspended in 50 µl 6X SDS-gel loading buffer (containing fresh DTT) along with 25 µl of glass beads. Cells were vortexed 6 times for 30 sec each, alternating with 30 sec on ice. Another 50 µl of 6X SDS-gel loading buffer was added. Samples were denatured in a boiling water bath for 5 min. The mixture was again vortexed 2 times for 30 sec. 10 µl each of the fusion protein extract and the negative control along with 10 µg of (positive control) crude synaptosomal extract from wild type mice were resolved using 8% SDS-PAGE as duplicates. The proteins were transferred onto a nitrocellulose membrane overnight at 4°C. After confirming the transfer of proteins onto the membranes using Ponceau-S staining, the membranes were washed in 1X TBST thoroughly before adding the blocking buffer. After 1 hr of blocking in 5 % blocking milk buffer, the duplicate blots were incubated with primary antibodies in 3% milk buffer (Rabbit anti-Neto2 – 1:1000; Rabbit anti-VP16 – 1:2000) for 2 hrs at room temperature. Following incubation, blots were washed with 1X TBST thrice, 10 min each, and then blots were incubated with HRP tagged anti-rabbit secondary antibody (1:5000) in 5 % milk buffer for 1 hr at room temperature. Following incubation, blots were washed with 1X TBST thrice, 10 min each and the blots were developed using the enhanced chemiluminescence system from Amersham.
2.3.2 Immunoprecipitation of the bait protein using Neto2-ectodomain specific antibody

To identify whether the ectodomain of the Neto2-bait protein is intact in the bait-bearing yeast, total protein extracts were prepared and the samples were immunoprecipitated with Neto2-ectodomain specific antibody, resolved by SDS-PAGE and blotted against the Neto2 intracellular region and the VP16 fusion tag of the Neto2-bait. Fresh cultures of the Neto2-Cub bait-bearing yeast clone and empty bait-bearing yeast were inoculated into 10 ml of SD-Leu media and grown overnight at 30°C with shaking. The yeast cells were grown overnight to an OD$_{600}$ of 0.5-1.0. The volume of culture corresponding to 20-OD$_{600}$ units was pelleted at 3000 rpm for 3 min. After washing the pellets in 2 ml of TAE, the cells were pelleted down at 3000 rpm for 3 min and spheroplasted with 60 µl of 10 mg/ml zymolyase in 2 ml SK buffer for 1 h at 30°C. Spheroplasts were pelleted at 6500 rpm for 30 sec and washed 3 times with SK buffer. Spheroplasts were resuspended in 1 ml of immunoprecipitation-lysis buffer in the presence of protease inhibitors (appendix IV). Cells were homogenized 5 times in a tight glass-potter. At 4°C, the cells were vortexed vigorously for 30 sec followed by chilling on ice for 30 sec, which was repeated 10 times. The supernatant was transferred to an eppendorf tube. The homogenates were centrifuged at 2000 rpm for 5 min at 4°C to remove the broken cells. After removing 20 µl of the homogenate, the rest was incubated overnight at 4°C with 5 µl of Neto2 ectodomain-specific antibody. These samples were then incubated with 30 µl of a 50 % slurry of Sepharose-gamma bind beads for 30 min at 4°C. Later, the mixture was centrifuged at 2500 rpm for 1 min at 4°C to collect the beads and the unbound fraction was stored. The beads were washed 3 times, 10 min each with cold IP lysis buffer (without SDS) containing protease inhibitors. The homogenate, bound, and unbound fractions for the Neto2-bait bearing sample and the negative control sample were denatured with 6X SDS loading buffer and resolved using 8% SDS-PAGE.
The proteins were transferred onto a nitrocellulose membrane overnight at 4°C. The blots were probed with Neto2-cytosolic and VP 16 primary antibodies as described above and processed.

2.3.3 Preparation of membrane protein extracts and verification of the Neto2-fusion protein localization

A fresh Neto2-C_{ab} bait-bearing yeast clone was inoculated into 10 ml of SD-Leu media and grown overnight at 30°C with shaking. In parallel, a fresh culture of empty bait-bearing yeast was grown for negative control and a fresh culture of artificial bait CD4 as a positive control for membrane localization. The yeast cells were grown overnight to an OD_{600} of 0.5-1.0. The yeast homogenates were prepared as described above. After removing the broken cells, cleared homogenate was transferred carefully to a new eppendorf tube. 400 μl was saved as H fraction and 500 μl was centrifuged at 13000 rpm for 10 min at 4°C to obtain the S13 and P13 fractions. The S13 fraction was then centrifuged at 50000 rpm for 20 min at 4°C in a Beckman table top centrifuge using a TLA100.3 rotor to obtain the S200 (cytosolic proteins) and the P200 fraction (membrane proteins). P13 and P200 fractions were resuspended in 500 μl and 400 μl respectively with lysis buffer containing protease inhibitors.

50 μl of 6X SDS-gel loading buffer was added to the samples. While the H fraction and S200 fractions were denatured in a boiling water bath for 5 min, the P200 fraction was incubated at 37°C for 20 min for denaturation. The mixtures were again vortexed 2 times for 30 sec. 50 μl each of the fusion protein extract, the positive control, and the negative control extracts were resolved using 8% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane overnight at 4°C. The blots were probed with Neto2-cytosolic and VP 16 primary antibodies as described above and processed.
Part 4 - Verification of the Neto2-bait protein folding using a functional assay

2.4.1 $N_{ub}G / N_{ub}I$ test for the Neto2 bait-bearing yeast

Several 100 mm diameter SD-trp-leu, SD-trp-leu-his and SD-trp-leu-his-ade selection plates were initially prepared for the assay. Colonies of fresh, Neto2 bait-bearing yeast strain were inoculated in 50 ml YPAD and grown overnight with shaking at 30°C. The OD$_{600}$ of the overnight culture was between 0.6 – 0.8. Cells were pelleted for 5 min at 2500x g and resuspended in 5 ml ddH$_2$O and the whole transformation procedure was performed on ice. PEG/LiOAc master mix sufficient for 10 transformations was prepared and 300 µl added to each tube containing 1.5 µg of the control plasmids pOst1- $N_{ub}I$, PFur4- $N_{ub}I$, pOst1- $N_{ub}G$ and PFur4- $N_{ub}G$ along with the empty prey vector pPR3N (obtained from Dr. Stagljar’s lab). 100 µl of the resuspended yeast cells was added to each tube, followed by incubating at 42°C water bath for 45 min. The cells were then pelleted for 5 min at 700x g. Each pellet was dissolved in 150 µl of 0.9% NaCl and 50 µl of each transformation was plated onto 100mm diameter selection plates – SD-trp-leu, SD-trp-leu-his and SD-trp-leu-his-ade. The plates were sealed with parafilm and incubated at 30°C for 4 days. After growth, the transformation efficiency was calculated.

In this co-transformation experiment, growth of the transformants on SD-trp-leu plates indicate that the clones have both bait and the prey plasmids reflected by their ability to synthesize tryptophan (using the prey plasmid) and leucine (using the bait plasmid) on medium lacking these amino acids. Additionally growth of these clones on SD-trp-leu-his-ade plates and a white colour of the colony indicates the activation of the reporter genes $HIS3$ and $ADE2$.
following bait:prey interaction whereas absence of growth on this selection plate or appearance of a red colony indicates the absence of a bait:prey interaction (discussed elaborately in 2.6.2).

Spot dilution test - Isolated colonies from the different control co-transformations were picked and resuspended in 20 µl of 0.9% NaCl. 5 µl of this resuspension were plated at different dilutions ranging from 1:10 to 1:10000 on SD-trp-leu-his-ade to verify the $N_{ub}G / N_{ub}I$ test results. The plates were sealed with parafilm and incubated at 30°C for 2 days and verified for growth.

Part 5 - Pilot screening and optimization of the screening conditions

2.5.1 Large scale transformation of the empty prey plasmid into Neto2 bait-bearing yeast and 3-aminotriazole titration

Several 150 mm diameter SD-trp-leu-his and SD-trp-leu-his-ade selection plates supplemented with 5, 10, 25, 50 or 100 mM 3-AT were initially prepared for the pilot screen. Colonies of fresh, Neto2 bait-bearing yeast strain were inoculated in 10 ml SD-leu medium and grown overnight with shaking at 30°C. The OD$_{600}$ of the overnight culture was measured and the amount required for 22.5 OD$_{600}$ units was calculated. This amount of the overnight culture was transferred into a 50 ml Falcon tube and the cells were pelleted at 700x g for 5 min. The pellet was resuspended in 10 ml of prewarmed (30°C) 2X YPAD medium and transferred into a 1-liter conical flask. 150 ml of prewarmed 2X YPAD was added into the flask, and the OD$_{600}$ of the resulting culture was measured to ensure that it was 0.15. The cells were grown with vigorous shaking (225rpm) to an OD$_{600}$ of 0.6 – 0.7 at 30°C. After appropriate growth, the culture was split into three 50 ml falcon tubes and the cells were pelleted for 5 min at 700x g at 4°C.
The pellets were resuspended in 30 ml sterile ddH$_2$O and again pelleted for 5 min at 700x g at 4˚C and placed on ice. Fresh ssDNA, LiOAc/TAE master mix and PEG/LiOAc master mixes were prepared and each pellet was resuspended in 600 µl of LiOAc/TAE master mix. To each reaction 7 µg of empty prey plasmid pPR3N, 100 µl of ssDNA and 2.5 ml of PEG/LiOAc master mixes were added and the mixture was thoroughly vortexed for 1 min.

The tubes were incubated in a 30 °C water bath for 45 min, briefly vortexing them every 15 min. Then, 160 µl of DMSO was added to each tube and incubated in a 42˚C water bath for 20 min. After heat-shock, the cells were pelleted at 700x g for 5 min, and each pellet was resuspended in 3 ml of 2X YPAD medium. All the resuspended cells were then pooled and were allowed to recover at 30 °C for 90 min with slow shaking (150 rpm). Finally the cells were pelleted at 700x g for 5 min and resuspended in 3.6 ml of 0.9% NaCl. 300 µl of the cells were spread onto each of the selection plates (150 mm SD-trp-leu-his and SD-trp-leu-his-ade). 1:100, 1:1000, 1:10000 dilutions in 0.9% NaCl were prepared from the remaining cell suspension and 100 µl of each dilution was plated onto 100mm SD-trp-leu plates to calculate the transformation efficiency. All plates were sealed with parafilm and incubated at 30 °C for 2 days for the small plates and 5 - 7 days for the large plates. After growth, the transformation efficiency was calculated from the number of colonies on the SD-trp-leu plates:

Transformation efficiency (clones/g DNA) = \[ \frac{\text{Total number of transformants}}{21 \mu g} \]

Total number of transformants = (no of colonies on SD-trp-leu plates) \times (dilution factor) \times (10) \times (3.6)
Part 6 - cDNA library screening using the Neto2 bait

2.6.1 Large scale transformation of a human adult / human embryonic, whole brain cDNA library into the Neto2 bait-bearing yeast

Large-scale transformation of the cDNA library into Neto2 bait-bearing yeast was performed using a similar protocol as described above for the pilot screen. The important changes that were made from that protocol were that overnight culture corresponding to 30 OD$_{600}$ units were grown in 300 ml of 2X YPAD medium at 30°C, until they reach an OD$_{600}$ of 0.6 - 0.7. After appropriate growth, the culture was split into six 50 ml Falcon tubes and they were prepared for transformation as described above. The entire transformation reaction was scaled up for six reactions, each using 7 µg of human adult, whole brain-pPR3N cDNA library (or human embryonic, whole brain- pPR3N cDNA library). After the transformation reaction, cells from the six reactions were resuspended in 7.2 ml of 0.9% NaCl. 300 µl of the cells were spread onto each of the 22 selection plates (150 mm SD-trp-leu-his-ade). 1:100, 1:1000, and 1:10000 dilutions in 0.9% NaCl were prepared from the remaining cell suspension and plated 100 µl of each dilution onto 100mm SD-trp-leu plates to calculate the transformation efficiency. All plates were sealed with parafilm and incubated at 30 °C for 2 days for the small plates and 5 - 7 days for the large plates. After growth, the transformation efficiency was calculated from the number of colonies on the SD-trp-leu plates.

Transformation efficiency (clones/g DNA) = \frac{\text{Total number of transformants}}{42 \mu g} 

Total number of transformants = (no of colonies on SD-trp-leu plates) \times (\text{dilution factor}) \times (10) \times (7.2)
2.6.2 Selection of clones in the growth assay (white-pink selection)

Due to the presence of the ADE2 reporter gene in the THYAP4 yeast strain, yeast colonies carrying this reporter will display different colors ranging from dark red to white depending on whether an interacting protein pair is present or not. When the ADE2 reporter gene is not transcribed (i.e. when no protein interaction takes place between the Neto2-bait and a prey), the adenine synthesis pathway is blocked and a red colored intermediate accumulates, turning the cells red. This may be initially visible as a slightly pink color and can turn into dark red upon longer storage of the yeast. If the Neto2-bait and prey interact, the ADE2 reporter gene is activated and the adenine synthesis pathway is unblocked, and there is no accumulation of the red colored intermediate. Thus, the color of yeast clones containing a protein-protein interaction can appear from a color range of faintly pink (a weak interaction corresponding to slow growth on a SD-trp-leu-his-ade selection plate) to white (a strong interaction corresponding to fast growth on a SD-trp-leu-his-ade selection plate). After 7 days of incubation, faintly pink to white colonies were picked from the master SD-trp-leu-his-ade plate and resuspended in 25 µl of 0.9% NaCl. 5 µl of each of these resuspended clones was spotted onto fresh 100 mm SD-trp, SD-trp-leu, SD-trp-leu-his and SD-trp-leu-his-ade selection plates. All plates were sealed with parafilm and incubated at 30 °C for 2 days. The size and colours of the clones were observed and tabulated.

2.6.3 Selection of clones in the lacZ assay (blue-white selection)

In addition to the growth reporter genes HIS3 and ADE2 the THYAP4 yeast strain also contains the color reporter lacZ gene. The lacZ gene encodes the bacterial enzyme β-
galactosidase, which converts the substrate X-gal into a blue compound. Yeast cells expressing the enzyme β-galactosidase therefore turn blue when incubated with X-gal reagent. Colonies that show a rapid and intense blue coloration indicates the presence of a strong interaction while a slow and faint blue coloration indicates a weak interaction. The white and faint-pink colonies from the above selection assay were again resuspended in 15 µl of 0.9% NaCl. 5 µl of each of these clones were spotted onto fresh 100 mm SD-trp-leu-his-ade selection plates containing 100 mg/ml X-Gal as duplicates. All plates were sealed with parafilm and incubated at 30 °C for 2 days. The rate of appearance of blue colouration and the strength of blue colouration were observed and tabulated. All clones positive in the lacZ assay were further restreaked and maintained on fresh SD-trp-leu and SD-trp-leu-his-ade selection plates.

Part 7 - Identification of the positive clones from the reporter assays

2.7.1 Colony PCR for the reporter assay-positive clones

For colony PCR, forward and reverse primers were designed such that they were flanking the insert region in the NubG and HA tag regions within the pPR3-N library vector; forward (5’ – GTC GAA AAT TCA AGA CAA GG – 3’) and reverse (5’ – AAG CGT GAC ATA ACT AAT TAC – 3’) respectively. 10 µl of 2X Qiagen multiplex PCR buffer (containing DNA polymerase, dNTPs and reaction buffer) was mixed with 0.5 µl each of the 10 µM forward and reverse primers; and were made to a final volume of 20 µl with nucleic acid-free sterile ddH₂O in PCR tubes. The tubes were incubated on ice until the start of the reaction. The fresh clones positive for both selection assays (containing the putative-interacting protein pairs) were picked using a sterile micropipette tip and inoculated into individual PCR tubes and mixed.
Before the actual reaction, the reaction mixtures were heated in the PCR plate for 95˚C for 15 min, to break the yeast cell wall. 30 thermal cycles consisting of a short denaturation for 30 sec at 95˚C, annealing for 1 min at 42˚C and extension for 2 min at 72˚C was performed. This was followed by a final extension for 4 min at 72˚C, before the PCR products were incubated at 4˚C. PCR products were analyzed using 0.8 % agarose gel. Putative prey plasmids corresponding to DNA band size of 0.9 – 1.9 kb were excised and gel-purified using a QIAquick Gel Extraction kit from Qiagen, according to the manufacturer’s instructions.

2.7.2 Sequencing of the clones and BLAST analysis

The purified PCR products correspond to the putative interactors of the Neto2-bait. A forward primer and a reverse primer were designed for sequencing and identifying the putative Neto2-interacting prey plasmid; forward (5’ – AAT GTA AGC GTG ACA TAA CTA ATT ACA TGA C – 3’) and reverse (5’ – ATC GAC AAC GTT AAG TCG AAA ATT CAA GAC – 3’). 50 - 100 ng of the purified PCR product along with 35 ng of the custom primers made to 7.7 µl with TE buffer was sent for sequencing at the TCAG (The Centre for Applied Genomics)-DNA sequencing facility at The Hospital for Sick Children. The sequences obtained were matched against the Human Genome using Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) and the University of California Santa Cruz (UCSC) Genome browser and the results were tabulated. The frequent false positives from both MYTH libraries, as reported from previous screens, were identified and they were not analyzed further. For all the remaining clones, the sequences were analyzed to check whether the cDNA insert was in-frame with the reading frame of the MYTH prey vector.
2.7.3 Prey plasmid recovery from the putative interacting bait:prey-bearing yeast and retransformation in *E. Coli*

Putative Neto2-interacting prey plasmids (after disregarding the frequent false positives) were rescued from yeast by using modified Qiagen bacterial miniprep protocol as described earlier. After isolation, they were transformed into DH5α cells and plated onto LB plate containing 100 µg/ml of ampicillin. After growth of the colonies, the plasmids were isolated using a standard QIAprep -Miniprep Kit from Qiagen according to the manufacturer’s instructions.

**Part 8 - Controls for the Neto2-MYTH screen**

2.8.1 Construction of a positive control prey plasmid (Human *Neto1*) for the Neto2 bait

Earlier, other experiments performed in our lab indicated that Neto2 heterodimerizes with Neto1 in both HEK293 cells and *in vivo* (Tang, unpublished). Therefore Neto1 integrated into the MYTH-prey plasmid pPR3C (MYTH prey plasmid for type I membrane proteins) was chosen as a positive control for the Neto2-bait.

Forward and reverse long primers were designed such that they contained 40 nt homologous to the prey vector pPR3C, in-frame with the 20 nt homologous to the human *Neto1* cDNA reading frame as described previously. Thus the forward primer (5’ – GAG TGG CCA TTA CGG CCC GGG AAA AAA CAT GTC GGC CGC ATG ATC CAT GGG CGC AGC GTG – 3’) and the reverse primer (5’ – AGC GTA ATC TGG AAC ATC GTA TGG GTA CAT
ATC GAT AAG GAC CCT AGT TGT GTT GTA TTC – 3’

were designed to insert human Neto1 (devoid of native signal sequence and stop codon) between the \( N_{ub}G \) tag and HA tag in the prey vector pPR3C.

Full length human Neto1 was initially PCR amplified from total retinal cDNA, cloned into pBluescript vector and sequenced (using standard protocols, not described here). The PCR reaction was performed as described earlier during the Neto2-bait construction, with the exception of annealing the PCR products at 61°C. After the reaction, products were purified as described earlier. Empty prey vector pPR3C was restriction digested using EcoRI restriction enzyme (New England Biolabs); and the linearized prey vector was purified as described earlier.

Homologous recombination in yeast was applied to integrate the linearized empty pPR3C with the human \( Neto1 \)-purified PCR product. The transformants were plated onto SD-Trp plates and incubated. After growth of colonies, putative recombinant \( pNeto1 - N_{ub}G \) - fusion clones were identified using a colony PCR approach and the positive clones were verified using sequencing as described earlier. The following primers were designed and used for sequencing; forward (5’ – TAT CTC GAA GCA CAC GAA ACT TTT TCC TTC CTT – 3’) and reverse (5’ – TAT AAT GTT ACA TGC GTA CAG GCG TCT GTA CAG – 3’). This verified \( pNeto1 - N_{ub}G \) clone inserted into the pPR3C vector was used as a positive control for the Neto2-C\( _{ub} \) bait.

2.8.2 Construction of a negative control bait plasmid (Human \( CD4 \)) for the cDNA prey

An artificial bait comprising the transmembrane segment of Human \( CD4 \) - integrated with pAMBV was constructed and transformed into THYAP4 by Jamie Snider from Dr.
Stagljar’s lab. This served as a negative control bait to detect the non-specifically interacting prey proteins obtained from the MYTH screen.

Part 9 - Identification of true positives in the Neto2 MYTH screen

2.9.1 Bait dependency test

This test was performed to identify the genuinely interacting prey isolated from the MYTH screen from the non-specifically interacting prey. For this, the isolated prey plasmids from the MYTH screen were reintroduced into fresh yeast bearing Neto2-bait or CD4 artificial bait. The clones showing positive in the growth assay and in the lacZ assay following transformation into negative control bait-bearing yeast were considered to be non-specifically interacting prey and they were ignored. The clones showing positive in the growth assay and in the lacZ assay following retransformation with the Neto2-control bait-bearing yeast were considered as genuine Neto2-putative interactors in the MYTH system.

Several 100 mm diameter SD-trp-leu and SD-trp-leu-his-ade (containing 25 mM 3-AT and 100 mg/ml X-Gal) selection plates were initially prepared for the test. Colonies of fresh, Neto2 bait-bearing yeast and Alg3 bait-bearing yeast were separately inoculated as two cultures into 50 ml of YPAD and they were grown overnight with shaking at 30˚C. The OD$_{600}$ of the overnight cultures were between 0.6 – 0.8. Cells were pelleted for 5 min at 700x g and resuspended each in 5 ml ddH$_2$O and the whole transformation procedure was performed on ice. 1.5 µg of isolated prey plasmids, including the related prey plasmid Neto1- N$_{ub}$G and the unrelated prey plasmid pFUR4- N$_{ub}$G were added as duplicates in separate eppendorf tubes. PEG/LiOAc master mix sufficient for 40 transformations was prepared and 300 µl was added to
each tube containing the prey plasmids to be transformed. 100 µl of the resuspended Neto2 bait-bearing cells were added to the tubes containing prey plasmids. 100 µl of the resuspended CD4 artificial bait-bearing cells were added to the duplicate tubes containing prey plasmids. Tubes were thoroughly vortexed for 30 sec and all transformation reactions were incubated in a 42°C water bath for 45 min. The cells were then pelleted for 5 min at 700x g. Each pellet was dissolved in 200 µl of 0.9% NaCl and each transformation reaction was plated onto 100mm SD-trp-leu and SD-trp-leu-his-ade (containing 25 mM 3-AT and 100 mg/ml X-Gal) selection plates, 100 µl each. The plates were sealed with parafilm and incubated at 30°C for 4 days. Growth on high stringency SD-trp-leu-his-ade with 25mM 3-AT, and appearance of blue colouration was observed for all the clones and tabulated.

2.9.2 Re-sequencing and verification of the prey plasmids

Putative Neto2 bait-interating prey plasmids were recovered from the clones that were positive in the bait-dependency test. The plasmids were retransformed into DH5α cells and the plasmids were extracted by standard Qiagen bacterial miniprep protocols, as described earlier. The recovered plasmids were sent for re-sequencing using the following primer set; forward (5’ – AAT GTA AGC GTG ACA TAA CTA ATT ACA TGA C – 3’) and reverse (5’ – ATC GAC AAC GTT AAG TCG AAA ATT CAA GAC – 3’) as described above. The sequences were matched against the Human Genome using BLAST software from NCBI and the UCSC Genome browser and the results were tabulated.
Part 10 - Possible outcomes of the Neto2 MYTH screen based on the choice of cDNA libraries

An important feature of the oligo dT primed N\textsubscript{ub}G-X, (adult and embryonic) cDNA libraries are that they contain predominantly full length genes, increasing the chances of identifying interactions mediated through a large portion of the protein. Another feature of the two libraries employed is that the oligo dT priming of the total RNAs allows the cDNA library to be biased towards the 3’end of the coding sequence (which is the C-terminus of the encoded protein). This is advantageous for membrane proteins that have internal membrane-anchor sequences near the C-terminus of the protein, like type II membrane proteins. These proteins are oriented in such a way that their N-terminus faces the cytosol due to the absence of an N-terminal ER signal sequence. Therefore, the specific orientation of N\textsubscript{ub}G-HA fused with the N-terminal portion of the prey protein will allow the N\textsubscript{ub}G tag to face the cytoplasm, similar to the C\textsubscript{ub}-tagged Neto2 bait. (Recollect that it is important in the split-ubiquitin system for the N\textsubscript{ub} and C\textsubscript{ub} tags to face the same membrane compartment for them to successfully reconstitute upon bait: prey interaction). This method of cDNA synthesis and the orientation of the N\textsubscript{ub}G tag do not affect cytoplasmic proteins, but affects the type I membrane protein enrichment in this cDNA library. This is because the 5’ ends of very long transcripts and hence the amino terminus of the type I membrane proteins (including their ER signal sequences) may not be covered during the oligo dT primed - cDNA synthesis and thus they may be underrepresented. Therefore, this particular pPR3N - N\textsubscript{ub}G-X cDNA library is more suitable for finding integral membrane proteins that does not have a N-terminal signal sequence (like the type II membrane and other multi-pass membrane proteins topologically similar to the type II membrane proteins) as well as the cytosolic interacting partners of Neto2.
Chapter 3: Results and discussion
Part 1 - The Neto2-C\textsubscript{ub} bait plasmid was generated by yeast homologous recombination

Yeast homologous recombination was applied to integrate the linearized MYTH bait-vector with a Neto2 PCR product containing homologous arms (Figure 3.1a). Since the MYTH bait plasmid contains the \textit{LEU2} gene for auxotrophic selection in media lacking leucine, successful transformation was revealed by the growth of transformants on SD-leu selection plates. This was further confirmed by colony PCR for the putative Neto2-C\textsubscript{ub}-fusion clones, in which the forward and reverse primers were designed to match the insert and vector regions respectively. DNA bands corresponding to the amplified insert and vector regions indicated that the bait vector contains the \textit{Neto2} reading frame insert (Figure 3.1b). As a final confirmation, the putative clones were sequenced and confirmed to posses the \textit{Neto2} coding region in-frame with the ORF of the C\textsubscript{ub}-fusion plasmid. It was also confirmed through sequencing that the Neto2-C\textsubscript{ub}-fusion bait plasmid did not have any mutations that would affect function (Figure 3.1c). This sequence-verified bait clone was then further verified for expression, localisation, and functionality of fusion protein before conducting the MYTH screen.
Figure 3.1 – Generation of pNeto2-\(C_{ub}\) fusion plasmid

a) Strategy for homologous recombination to insert human Neto2 cDNA into pAMBV; b) Analysis of colony PCR products to identify putative Neto2-\(C_{ub}\) fusion plasmids; c) The human Neto2 was inserted in-frame with the \(C_{ub}-\text{LexA-VP16}\) – ORF to generate a Neto2-\(C_{ub}\) fusion bait protein.
Part 2 - The Neto2-C\textsubscript{ub} bait protein is expressed within the yeast membrane

After confirming the sequence of the Neto2-fusion plasmid, the expression and localization of the fusion protein were confirmed by multiple approaches. Initially, the expression of the Neto2 fusion protein in total yeast lysates was examined using an antibody directed against the VP16 portion of the tag and an antibody directed against the terminal 70 amino acids at the carboxyl cytoplasmic tail of Neto2. Extracts from yeast bearing empty bait plasmid were used as a negative control, while whole brain extract from wild type mice was used as a positive control for the Neto2 antibody. While the expected molecular weight of the fusion protein was around 93kD, the prominent band observed in the fusion protein lane was around 70 kD. A shift in the molecular weight of the fusion protein was observed from the actual molecular weight of 60kD for wildtype Neto2, while the fusion protein was absent in the negative control lysates (Figure 3.2a). These results indicated that the Neto2-C\textsubscript{ub}-fusion protein was expressed in the yeast cells bearing the Neto2-C\textsubscript{ub}-fusion plasmid and corresponding antibodies could detect the endodomain of the fusion protein. Since the human Neto2 was ectopically expressed in yeast, the fusion protein may have been cleaved within the yeast cells by proteases and may no longer be intact. This could have also happened during the sample preparation due to the action of yeast proteases that were not adequately inhibited by the protease-inhibitor cocktail used in the extraction buffer.

To detect whether the Neto2-fusion protein has an intact ectodomain as well, the yeast lysates were immunoprecipitated with Neto2-ectodomain recognizing antibody and probed with the endodomain recognizing VP16 and Neto2-cytoplasmic antibodies. Both the VP16 tag and Neto2 endodomain were detected in the yeast extracts from the Neto2-bearing clone, while they
were absent in the empty plasmid bearing negative control clone (Figure 3.2b). This indicated that the fusion protein has both the ectodomain and endodomain of Neto2 including the C-terminal VP16 tag. A faint band of around 100kD was visible slightly above the expected molecular weight of 93kD, and this band may correspond to the full-length Neto2-fusion protein. It is also possible that the degradation products might have associated together to produce a band of this molecular weight. However, the prominent band observed in this experiment was around 70kD, and it is again likely that the ectodomain of the fusion protein at the N-terminus got cleaved. Even if this was the case and if the CUB1 domain was cleaved, the fusion protein would still be able to mediate protein:protein interactions through its CUB2 domain and LDLa motif. Therefore the same Neto2-bait clone was used for further experiments.

To determine whether the expressed fusion protein was localized to the membrane, a different approach was undertaken. Here, subcellular fractionation of yeast homogenate was performed to separate the soluble cytoplasmic and membrane components of the extracts followed by probing using VP16 antibody. The Neto2-fusion bait indicated the presence of VP16 tag in the membrane extract fraction but not in the cytoplasmic extract fraction. This result indicated that the Neto2-fusion protein is localized to the membrane (Figure 3.2c). A parallel approach was undertaken to determine the localization of the Neto2-fusion protein by immunofluorescence, but the results were inconclusive and the data are not shown.
**Figure 3.2 – Verification of expression and localization of Neto2-C<sub>ub</sub> fusion protein**

a) Verification of Neto2-fusion protein expression from yeast total lysates using Neto2-endodomain specific and VP16 specific antibody.

b) Immunoprecipitation of Neto2-fusion protein using Neto2-ectodomain specific antibody.

These experiments indicate that the Neto2-C<sub>ub</sub> fusion bait protein is expressed in yeast and its ectodomain and endodomains are detectable by their respective antibodies. Bands that are marked with an asterisk in the fusion-protein lane are considered as the Neto2 bait-fusion protein.
c) Verification of Neto2-fusion protein localization by using subcellular fractionation and detection by VP16 specific antibody; bands marked with an asterisk above 60kD are considered as the Neto2 bait-fusion protein bearing the VP16 tag.

**Figure 3.2 – Verification of expression and localization of Neto2-C\textsubscript{ub} fusion protein**

**C**

Blot - Anti-VP16 antibody

1 2 1 2 1 2

- 75 kD
- 55 kD
- 40 kD
- 25 kD

Crude fraction Membrane fraction Cytoplasmic fraction

Lane 1 - Negative control (Empty plasmid bearing yeast extract);
Lane 2 - Neto 2 fusion protein (expected molecular weight of fusion protein is 90 kD);
(molecular weight of VP16 tag is around 16kD)
Part 3 - The Neto2-Cub-fusion protein is folded and functional in the NubG / NubI test

To test whether the membrane localized fusion protein is correctly folded and functional, the Neto2-bait bearing yeast strain was co-transformed individually with each of the 4 control prey plasmids pFur4-NubG, pFur4-NubI, pOst1-NubG, or pOst1-NubI encoding the yeast plasma membrane prey protein Fur4 and the yeast endoplasmic reticulum prey protein Ost1. Co-expression of the Neto2-bait with NubI plasmids should result in the reconstitution of split-ubiquitin due to the strong affinity of wildtype NubI for the Cub, and a subsequent activation of reporter genes followed by growth on SD-trp-leu-his-ade selective plates. On the other hand, co-expression of bait with NubG plasmids should not result in reconstitution of split-ubiquitin and no activation of reporter genes since NubG has no affinity for Cub. Thus, NubI serves as a positive control and NubG serves as a negative control in this experiment. Since the Neto 2 fusion protein is ectopically expressed and tagged, it may not be properly folded. However, detection of an interaction in a NubG / NubI test would indicate that atleast a potion of fusion protein is correctly folded and functional (Fetchko and Stagljar 2004; Iyer, Burkle et al. 2005) and usable in a library screen.

Growth of red colonies was evident in the baits transformed with negative control NubG plasmids, indicating that the Neto2- Cub bait protein does not non-specifically interact with the yeast membrane proteins Fur4 and Ost1 due to bait over-expression. Also, red colonies were observed in the baits transformed with the, empty prey plasmid pPR3N, indicating that the bait-protein does not self-activate. Growth of white colonies was observed in the baits transformed with positive control NubI plasmids, indicating that the bait has passed through the secretory
pathway, where the Neto2-Cub-fusion protein has interacted with NubI of the control proteins Ost1 and Fur4 at the ER and plasma membrane (the basis for the red/pink – white selection explained in Part 5 of this chapter). This experiment clearly demonstrates that the Cub tag of the fusion protein is properly folded and functional (Figure 3.3a).

Additionally, the Neto2-bait bearing clone was co-transformed with positive control Neto1-prey plasmid and growth of white colonies was observed in SD-trp-leu-his-ade selective plate indicating that the Neto2-bait and the Neto1-prey interact. This experiment clearly demonstrates that the ectodomain of the Neto2-fusion protein is also properly folded and functional (Figure 3.3b).
Figure 3.3 – Verification of folding and functionality of the Neto2-Cub fusion protein

pNeto2-Cub bearing yeast was co-transformed with different control plasmids to assess the proper folding and functionality of the fusion protein. Growth of colonies in SD-WL plate indicate the presence of bait and prey plasmids while growth of colonies in SD-WLAH plate indicate a positive interaction between the bait and prey protein.

a) Co-transformation with negative control plasmids bearing NubG does not result in a growth in the SD-WLAH plates indicating that the bait is not overexpressed; Co-transformation with positive control plasmids bearing NubI result in a growth in the SD-WLAH plates indicating that the Cub tag of the bait is properly folded and functional. This also indicates that bait protein has passed through the secretory pathway as indicated by interaction with ER protein Ost1 and the PM protein Fur4.

b) Co-transformation with the positive control plasmid Neto1-NubG result in a growth in the SD-WLAH plates indicating that the ectodomain of Neto2-Cub bait is properly folded and functional.
Part 4 - 25 mM of 3-aminotriazole reduces the background colonies in the pilot screen

After ensuring that the Neto2-Cub bait was functional, the basic screening conditions were optimized in a pilot screen. Here, the actual screening conditions were simulated except that the empty library vector pPR3N was used instead of the cDNA library. After a large scale transformation of the empty prey plasmid into the bait-bearing yeast cells, growth of colonies were observed on selective plates of increasing stringency (SD-leu-trp > SD-leu-trp-his > SD-leu-trp-his-ade). Since the bait is co-expressed with empty NabG, the colonies seen in the pilot screen are background colonies due to leaky HIS3 gene expression. To eliminate false positives due to leaky HIS3 gene expression, increasing concentrations of 3-aminotriazole (3-AT, a competitive inhibitor of the HIS3 gene product) were added to the SD-leu-trp-his-ade plates. While 50 mM 3-AT in the selection plate completely abolished all growth, 25 mM 3-AT considerably reduced the number of background colonies when compared to the plates without any 3-AT. These finding indicate that the 50mM 3-AT concentration is too stringent to allow any histidine synthesis even during true bait: prey interaction. Therefore a level of 25 mM 3-AT was chosen to be optimal for library screening (Figure 4).
Figure 3.4 – 3-aminotriazole titration for reducing the background colonies

Shown here is the 3-AT titration following a co-transformation with the control plasmids used in the $N_{ub} G / N_{ub} I$ test. While 50 mM 3-AT completely abolished the growth on SD-WLAH plate, 25 mM 3-AT eliminated the background growth (with $N_{ub} G$ plasmid), but allowed the growth of a meaningful interaction (with $N_{ub} I$ plasmid). A similar result was obtained during the large-scale empty prey plasmid (pPR3N) co-transformation with the Neto2-bait bearing yeast. Therefore SD-WLAH plates were supplemented with 25 mM 3-AT during library transformation.
Part 5 - cDNA library screens using the Neto2-bait yielded several positive clones

After the screening conditions were optimised, a human adult and a human embryonic whole-brain cDNA library were transformed into the Neto2 bait-bearing yeast in separate experiments. A transformation efficiency of approximately 4 million -trp-leu co-transformants was achieved for both libraries as observed from their respective SD-trp-leu plates. This transformation efficiency was comparable to previous split-ubiquitin screens with other baits performed in the Stagljar lab and indicated that all individual clones in the cDNA library were evaluated at least 2 times considering that the libraries had approximately 2 million independent clones.

First reporter assay - After library transformation, visible colonies started to appear as early as the third day and they were allowed to grow for at least a week before picking them for further analyses. These colonies growing on SD-trp-leu-his-ade indicated that they were able to synthesize histidine and adenine due to the activation of the reporter genes HIS3 and ADE2. The HIS3 marker is known to be a very sensitive reporter even for low level of transcriptional activation driven by a low number of cleaved VP16 transactivators as may occur with weak interactions. On the other hand, it is also a leaky reporter gene as a bait with a very low level of self-activation might also show auxotrophic growth leading to a most common concern for false positives. Therefore background growth due to leaky HIS3 expression was suppressed by the addition of 25 mM 3-aminotriazole (3-AT) to the SD-trp-leu-his-ade plates. Along with HIS activation, a second ADE reporter gene (activated by the same ADH promoter element as the HIS3 gene), permit clone selection based on colony colour. The absence of bait: prey interaction
results in the accumulation of a colour absorbing intermediate due to the blockade of adenine synthesis and hence the colonies appear red upon storage. In contrast, a potentially strong interaction would remove the adenine synthesis blockade resulting in white colony colour even upon storage; and a weak interacting pair would result in a whitish-pink colony colour upon storage. This white pink selection along with auxotrophic growth on the SD-trp-leu-his-ade plates was used to select the colonies from the master screening plates. A total of 215 colonies for the adult library and 125 colonies for the embryonic library (white to faint pink) were picked from the master plates. They represented the putative strong and intermediate Neto2-interactors from the first reporter assay (Figure 3.5a).

**Second reporter assay** – A second reporter assay was applied for further increasing the stringency above that used for white-pink selection. This assay used the *lacZ* reporter gene, which was activated by a second *ADH* promoter element (at a position different from the *HIS3, ADE2* reporter genes). This *lacZ* reporter gene is not as sensitive as *HIS3*. Hence, *lacZ* transcription requires stronger activation of VP16 transactivator expression driven by a meaningful interaction than in the absence of a bait:prey interaction. The blue colouration shown by the clones bearing an interaction pair (in the presence of X-gal reagent) is thus a direct transcriptional read-out of the strength of interaction. Therefore the *lacZ* assay fine-tunes the distinction between potential interacting pair-bearing clones and the clones that do not have an interaction pair or a very weak interaction by the presence or absence of blue coloration respectively. Out of 215 adult clones that were positive in the growth assay, only 69 were positive during the X-gal assay; and out of 125 embryonic clones that were positive in the growth assay, only 46 were positive during the X-gal assay ranging in colour from faint to dark
blue (Figure 3.5a). This indicated that a substantial number of clones were false positives in the white-pink selection. All the lacZ positive clones were further analysed.

**Part 6 - True Neto2-bait-dependent prey interactions were identified**

Identification of HIS3\(^+\)-ADE2\(^+\)-lacZ\(^+\) clones - Colony PCR was performed to amplify the cDNA insert within the prey vector for all HIS3\(^+\)-ADE2\(^+\)-lacZ\(^+\) clones. This yielded products corresponding to DNA band sizes of 0.9 – 1.9Kb (Figure 3.5b). Upon sequencing of the PCR products and subsequent analysis using BLAST software, the results revealed that there were many clones which were identified as frequent false positives as reported by Dualsystems Biotech (including proteolipid protein and ubiquitin). Further literature searches revealed that a few clones corresponded to hypothetical proteins. With the exception of these clones, a bait-dependency test was performed on the remaining positive clones to confirm the final putative Neto2-interactors identified through the MYTH system.

**Bait-dependency test** – This was the final confirmatory test to identify the true Neto2-bait-dependent-preys as opposed to potentially sticky-preys that may non-specifically interact with unrelated bait. After plasmid rescue and the reintroduction into fresh Neto2-bearing bait, only 21 clones from the adult library and 7 clones from the embryonic library were positive in the white-pink and blue-white assays. The remaining clones were negative in the lacZ assay indicating that the lacZ gene may have been mutated to give false positive results in the same assay previously performed for these clones. In parallel, the Neto1-N\(_{ub}\)G - positive control prey showed strong blue coloration (and thus a true interaction), and the pFUR4-N\(_{ub}\)G – negative
control prey did not grow on the stringent plate (Figure 3.5c) indicating that the assay was robust.

In parallel experiments the rescued plasmids of what were considered positive clones were reintroduced into fresh cultures of yeast with artificial bait CD4- bearing negative control bait. Of these clones, only one clone corresponding to the Nogo receptor 3-prey from the embryonic library was positive in the lacZ assay. This result indicated that this particular prey interaction occurred non-specifically with the unrelated bait (Figure 3.5c). The negative control artificial bait contained only the transmembrane domain (TMD) of the human CD4 molecule fused with the Cub tag. Therefore any prey protein interacting with this artificial prey might be due to prey over-expression (a sticky prey), or a non-specific hydrophobic TMD – mediated interaction, or a physiologically irrelevant interaction. In this case, it was found that the NogoR3 prey clone rescued from the cDNA library corresponded to a segment outside the ORF of human Nogo3 cDNA (within the 3’UTR) and thus interpreted as not corresponding to a physiologically meaningful product. Therefore this clone was considered to be a false positive and was disregarded.
Figure 3.5 – Identification of putative Neto2 interacting clones from the MYTH screen

a) Representative results from the two reporter assays employed to identify the positive clones; White-pink selection involves the activation of first reporter genes \( ADE2 \) and \( HIS3 \). Blue-white selection involves the activation of second reporter gene \( lacZ \). While the first reporter is more sensitive for low amounts of VP16 transactivator, the second reporter requires a stronger activation, thereby providing stringency to the detection of true Neto2:prey interactions.

b) Colony PCR performed for the \( HIS3^{+} - ADE2^{+} - lacZ^{+} \) clones yielded DNA bands corresponding to their cDNA insert sizes. These bands were excised, gel-purified, their DNA sequences obtained and aligned to human sequences for identification.
c) Bait-dependency test – This is the final confirmatory test for identifying true interactors through the MYTH system.

Isolated preys were reintroduced into fresh yeast bearing Neto2-bait. While most of the clones were HIS+ADE+lacZ+, adult clone 22 did not pass the test indicating that it is a false positive.

The clones were also co-transformed into yeast bearing CD4-negative control bait. All clones except the embryonic clone 32 were negative in this co-transformation. A strong interaction with the negative control bait indicated that this prey clone binds nonspecifically.

The remaining prey clones were re-sequenced and verified with their corresponding human cDNA sequences. 21 clones from the adult library screen corresponded to 9 putative Neto2 interactors and 6 clones from the embryonic library screen corresponded to 4 Neto2 putative interactors. (shown in the figure are only the individual clones corresponding to the 9 and 4 Neto2 putative interactors).
Part 7 - Multiple false positives were eliminated at different stages during the Neto2-MYTH screen

False positives are proteins that behave as spurious interactors with the Neto2 bait protein in the MYTH screen, but do not correspond to a physiologically meaningful interaction. Due to different reasons these interactions will lead to a His\(^+\) and lacZ\(^+\) result, independent of a true interaction \textit{in vivo}. This section details the multiple reasons that might lead to a false positive interaction in a MYTH screen, and the different approaches taken to eliminate them. The most common causes for false positives in the MYTH system are described below. All of these are common to the traditional YTH as well.

- \textit{Bait over-expression}

  This is the most common cause for false positive results. Since the MYTH system uses an exogenous \textit{ADH} promoter within the \textit{pAMBV}-bait plasmid to drive the expression of the Neto2-C\textsubscript{ub}-fusion protein, the expression of the fusion protein might not be as tightly regulated as if it were driven by an endogenous promoter. This might lead to an over-expression of Neto2, which in turn could lead to non-specific interactions. This type of false positive was identified in the Neto2 MYTH screen by co-transforming the Neto2-bait bearing strain with yeast proteins fused with N\textsubscript{ub}G as negative controls. The yeast plasma membrane protein Fur4 and the ER protein Ost1 are not expected to have an affinity for the Neto2 bait. Also, the N\textsubscript{ub}G fusion tags do not interact with the bait- C\textsubscript{ub} tag, unless the bait and the prey show affinity towards one another. In an ideal scenario, where the bait protein is not over-expressed, the transformants should not grow on a SD-trp-leu-his-ade selection plate whereas the transformants will activate the reporter genes
if the bait were over-expressed. During the screen, the transformants did not grow on the selection plate after co-transformation, indicating that the screen was not affected by Neto2-bait protein over-expression.

An additional line of evidence to show that the Neto2-fusion protein was not over-expressed came from another co-transformation experiment with the empty prey plasmid pPR3N. Over-expressed bait also may have the property of self-activation and expression of reporter genes. In this experiment, even in the absence of prey protein, there was no growth on the selection plates indicating that the Neto2 bait did not self activate.

- **Bait folding and functionality**

Another important point to consider is due to the ectopic expression of the human protein in yeast. If the human Neto2-fusion protein is not properly folded in yeast, it might not only be non-functional, but it might also lead to non-specific interactions that are not physiologically relevant. Also, the native Neto2 protein is fused with an artificial tag that might also lead to misfolding. Therefore, during the Neto2 MYTH screen, I tested control prey proteins to assess whether the Neto2 fusion protein is properly folded and functional. From other experiments conducted in our lab, it was demonstrated that Neto2 heteromerizes with Neto1 in both a heterologous mammalian cell expression system (HEK293 cells) and endogenously in the mouse brain. Therefore, a \( pNeto1-N_{ub}G \) -fusion prey plasmid was constructed to serve as a positive control for the interaction with the Neto2 bait. During co-transformation of the \( pNeto1- N_{ub}G \) prey plasmid with the Neto2- \( C_{ub} \) bearing yeast, \( HIS^+ \) and \( ADE^+ \) transformants were observed. Also, these
transformants developed a deep blue colouration indicating a strong interaction consistent with our lab’s findings of interaction. These results further indicate that the ectodomain of the fusion protein was properly folded and functional since the Neto1:Neto2 interaction is ectodomain-mediated (Tang, unpublished).

Additionally, growth of white colonies was observed in the baits transformed with positive control $N_{ub}I$ plasmids, indicating that the bait has passed through the secretory pathway, where the Neto2-$C_{ub}$-fusion protein has interacted with $N_{ub}I$ of the control proteins Ost1 and Fur4 at the ER and plasma membrane. This experiment further demonstrated that the $C_{ub}$ tag of the fusion protein is also properly folded and functional.

- **Leaky HIS reporter gene**

  The *HIS3* reporter gene always shows a basal level expression in yeast strain THYAP4. This allows the growth of even wildtype colonies on the selection plates lacking histidine thus increasing the concern of false positive results. Therefore during the pilot screen with empty prey plasmid, the selection plates SD-trp-leu-his-ade were supplemented with different concentrations of 3-aminotriazole, a competitive inhibitor for the *HIS3* gene product (Imidazoleglycerol-phosphate dehydratase) that plays a vital role in yeast histidine biosynthesis. Increasing concentrations of 3AT reduced the number of background colonies during the pilot screen. While 50 mM 3AT completely abolished all background colonies, a level of 25mM 3AT substantially reduced the background colonies, at the same time allowing sufficient *HIS3* gene expression for growth assays. 25 mM 3AT containing SD-trp-leu-his-ade plates were thus used during the cDNA library screening.
• **Mutations in the reporter genes HIS, ADE, lacZ**

Because the Neto2 bait-bearing yeast strain was subjected to repeated plating and growth cycles during the MYTH screen, it was also possible that reversion mutations within the reporter genes might trigger their expression even during the absence of meaningful bait: prey interactions. In order to negate this sort of false positive situation, the putative Neto2-interacting prey plasmids were rescued from the yeast and reintroduced back into fresh Neto2 bait-bearing yeast (restreaked from the glycerol stock) to assess reporter gene activation in independent bait-dependency tests. Only 21 clones (corresponding to 9 putative interactors) out of 69 clones from the adult library and 6 clones (corresponding to 4 putative interactors) out of the 46 clones from the embryonic library were positive in the lacZ assays during the bait-dependency test, from a total of 115 clones that originally passed the reporter assays. This indicated that several bait: prey interactions identified through the reporter assays were caused due to reversion mutations in the reporter genes.

• **Prey over-expression**

Just like bait-over-expression, the exogenous promoter in the prey plasmid can also drive prey over-expression, resulting in a non-specific interaction of a prey protein. To test whether the 14 rescued prey plasmids were positive in the reporter assays due to prey-over-expression; they were reintroduced into yeast cells bearing the artificial bait plasmid pCD4- C ub. This bait has been previously tested and is currently used in the Stagljar lab as a standard negative control-bait plasmid. While 13 clones failed to grow upon the prey plasmid co-transformation into CD4-bait bearing cells, one prey
corresponding to human NogoR3 was positive for the reporter assays. While the other 13 clones did not non-specifically interact with the negative control bait, the clone corresponding to the human NogoR3 showed a suspicious non-specific interaction.

Further verification of the false positive result of this clone revealed that the cDNA insert of this clone contained sequences outside the open reading frame (ORF) of the human NogoR3 gene but within its 3’UTR. Thus, the corresponding fusion-prey product did not appear to be physiologically meaningful and therefore it was concluded that this false positive result was not due to prey over-expression, but due to the physiologically irrelevant prey. Other prey-insert sequences were also matched with the human cDNA sequences to identify similar results for the other clones. It was found that the prey-insert corresponding to the remaining 13 clones included the ORF of their corresponding human genes and that they were in-frame with the ORF of the prey vector.

Although several false positive results were eliminated during the Neto2 MYTH screen, it should again be emphasised that the screen only identifies putative interactors, which may or may not turn out to be true interactors under physiological conditions. This is largely because the interactions identified in the yeast system are entirely due to ectopic expression of the human proteins that do not directly reflect correct cell type expression in the same sub-cellular location under physiological conditions. Therefore it becomes extremely important to validate any putative interactions with both in vitro and in vivo methods by using other biochemical tools such as co-immunoprecipitation following the heterologous expression of the interacting proteins in mammalian cells, and co-immunoprecipitation from endogenously expressed proteins from the appropriate mammalian tissues where they might physiologically interact.
Part 8 - MYTH identifies 13 novel putative Neto2 interacting proteins

3.8.1 Results from the Neto2-adult cDNA library screen

Nine putative Neto2-interactors (corresponding to 21 clones) that were positive in all the MYTH selection assays (white-pink assay, blue-white assay and bait-dependency test) were obtained from the human adult, whole brain cDNA library (Table 3.1). Clones corresponding to three proteins namely, the VAMP-associated protein B (VAPB), glutamate transporter EAAT3-associated protein (GTRAP3-18) and emopamil binding protein (EBP) were considered to be putative strong Neto2-interactors. Clones corresponding to VAPB were the strongest hits in terms of the growth of the clones and intensity of blue colour in the lacZ assay. Also, 10 identical clones of VAPB were identified in two separate library screens. The clone determined to encode GTRAP3-18 also showed rapid growth in the growth assay and rapid appearance of blue coloration in the lacZ assay. Sequencing results revealed that a full length human GTRAP3-18-cDNA was integrated into the NubG prey vector. The clone corresponding to EBP also showed rapid growth in the growth assay and rapid appearance of blue coloration in the lacZ assay. Two identical clones of EBP were identified from two independent library screens and the sequencing results revealed that full length human EBP-cDNA was integrated into the NubG prey vector.

The remaining clones were considered to be putative, weaker Neto2 interactors as indicated by the strength of their interaction in the growth and lacZ assays. These were chimerin-isoform-b, protein phosphatase 1B, BCL2/adenovirus E1B 19kD-interacting protein 3-like, transmembrane protein 59-like 1, rhomboid domain containing 2 isoform-a, and zinc finger protein with UFM1-specific peptidase domain.
### Table 3.1 – Putative Neto2-interactors from the adult library

<table>
<thead>
<tr>
<th>Name of the protein</th>
<th>No of Clones</th>
<th>Clone number(s)</th>
<th>Amino acids fused to Neto2</th>
<th>NCBI Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAMP-associated protein B (Vesicle trafficking)</td>
<td>10*</td>
<td>7, 9, 17, 18 (screen 1)</td>
<td>aa 183 - 243 (243)</td>
<td>NT_011362</td>
</tr>
<tr>
<td>Glutamate transporter EAAC1 – associated protein</td>
<td>1</td>
<td>25 (screen 2)</td>
<td>aa 1 - 188 (188)</td>
<td>NT_022459</td>
</tr>
<tr>
<td>Chimerin1 – isoform b</td>
<td>1</td>
<td>7 (screen 2)</td>
<td>aa 194 - 459 (459)</td>
<td>NT_005403</td>
</tr>
<tr>
<td>Protein phosphatase 1B</td>
<td>1</td>
<td>35 (screen 2)</td>
<td>aa 379 - 479 (479)</td>
<td>NT_022184</td>
</tr>
<tr>
<td>Emicopamil binding protein</td>
<td>2*</td>
<td>1 (screen 1)</td>
<td>aa 1 - 160 (160)</td>
<td>NT_079573</td>
</tr>
<tr>
<td>BCL2/adenovirus E1B 19kD-interacting protein 3-like</td>
<td>1</td>
<td>30 (screen 2)</td>
<td>aa 19 - 194 (194)</td>
<td>NM_004052</td>
</tr>
<tr>
<td>Transmembrane protein 59 – like 1</td>
<td>1</td>
<td>21 (screen 2)</td>
<td>aa 187 - 342 (342)</td>
<td>NT_011295</td>
</tr>
<tr>
<td>Rhomboid domain containing 2 isoform a</td>
<td>1</td>
<td>24 (screen 1)</td>
<td>aa 1 - 230 (364)</td>
<td>NT_007933</td>
</tr>
<tr>
<td>Zinc finger with UFM1-specific peptidase domain</td>
<td>3*</td>
<td>2, 4 (screen 1)</td>
<td>aa 311 - 579 (579)</td>
<td>NT_025741</td>
</tr>
</tbody>
</table>

* Indicate that the clones were obtained from two independent screens
- Screen 1 was performed with expanded human adult whole brain cDNA library
- Screen 2 was performed with the master stock of human adult whole brain cDNA library
A brief overview of the putative interactors is given here. Detailed descriptions of the two most interesting hits VAPB and GTRAP3-18 and the rationale for choosing these molecules for initial studies are given in the next chapter:

- **VAMP-associated protein B (VAPB)**

  The protein encoded by this gene is a 27.2 kDa, conserved type II membrane protein found in the plasma membrane and intracellular vesicle membranes of neurons. *VAPB* mRNA is expressed in multiple adult-mouse brain regions like the hippocampus, cerebellum and spinal cord motor neurons, where *Neto2* mRNA is also expressed. This neuronal protein is known be present at the presynapse (where *Neto2* is also present), particularly at the spinal cord motor neuron presynapse in mouse and at the glutamatergic neuromuscular junction presynapse in flies, and plays multiple roles in glutamatergic neurotransmission. A single missense mutation within the human *VAPB* gene, involving a conserved proline residue at position 56 (P56S), within the protein – protein interacting MSP domain was identified to be associated with three forms of familial motor neuron disease (Nishimura, Mitne-Neto et al. 2004; Kanekura, Nishimoto et al. 2006). The *VAPB* clones identified from the adult library correspond to amino acids 183 – 243 (comprising the coiled-coil and the transmembrane domains) of the human *VAPB* gene product.

- **Glutamate transporter EAAT3 – associated protein (GTRAP3-18)**

  The protein encoded by this gene is a 21.6 kDa, four-transmembrane domain protein found in the plasma membrane and intracellular vesicle membranes of neurons. *GTRAP3-18* mRNA is expressed abundantly in different regions of the developing and adult-mouse brain regions. The mRNA expression of *GTRAP3-18* in the adult mouse brain closely
resembles Neto2 mRNA expression in the cortex, hippocampus and cerebellum, and this neuronal protein is present at the postsynaptic membrane of glutamatergic and GABAergic neurons in these regions. GTRAP3-18 is known to regulate glutamatergic neurotransmission by modulating the trafficking and substrate affinity of the neuronal glutamate transporter EAAT3, an important excitatory amino acid-transporter that is present at the postsynaptic neuronal membrane (Lin, Orlov et al. 2001; Ruggiero, Liu et al. 2008). The GTRAP3-18 clone identified from the adult library corresponds to the full-length human GTRAP3-18 gene product (amino acids 1 – 188).

• **Chimerin isoform 1 b (Chn1b)**

The protein encoded by this gene is a 53.2 kDa, neuronal GTPase-activating protein. This cytoplasmic protein is known to interact with NR2A-NMDAR subunits and to play an important role in regulating dendritic spine morphology. Also this protein interacts with EphrinA4 receptor and plays an important role in EphA4-mediated axon pathfinding. Heterozygous missense mutations in this gene have been identified in Duane's retraction syndrome 2, a congenital oculomotor disorder (Miyake, Chilton et al. 2008). The Chn1 clone identified from the adult library corresponds to amino acids 194 – 459 (comprising the protein kinase-c-like and the Rho-GTPase activating protein (RhoGAP) domains) of the human Chn1 gene product.

• **Protein phosphatase, magnesium dependent, 1B (PPM1B)**

The protein encoded by this gene is a 52.6 kDa, Ser/Thr protein phosphatase that is present in many cell types including neurons. This phosphatase has been shown to dephosphorylate cyclin-dependent kinases (CDKs) and also regulates NF-kappa B
activity and thus may be involved in cell cycle control (Cheng, Kaldis et al. 2000; Prajapati, Verma et al. 2004). Other proteins of this family are known to be negative regulators of cell stress response pathways. The PPM1B clone identified from the adult library corresponds to amino acids 379 – 479 (uncharacterised region) of the human PPM1B gene product.

- **Emopamil binding protein (EBP)**

  The protein encoded by this gene is an 18.3 kDa, four-transmembrane protein found in the ER membranes of multiple cell types including neurons and astrocytes. This protein is known to play a crucial role in cholesterol biosynthesis by catalyzing the sterol-isomerase reaction. Missense mutations in this gene have been identified in X-linked dominant Conradi-Hunermann syndrome which is characterised by defective cholesterol biosynthesis (Ikegawa, Ohashi et al. 2000). The EBP clone identified from the adult library corresponds to the full-length human EBP gene product (amino acids 1 – 160).

- **BCL2/adenovirus E1B 19kD-interacting protein 3-like (BNIP3)**

  The protein encoded by this gene is a 21.5 kDa, type II membrane protein that plays a role during oxidative stress and other proapoptotic cellular functions along with Bcl2. Other BH3 domain family proteins are known to be intracellular death-ligands critical for initiating apoptosis (Lomonosova and Chinnadurai 2008; Burton and Gibson 2009). The BNIP3 clone identified from the adult library corresponds to amino acids 19 – 194 (comprises the BH3 and the transmembrane domains) of the human BNIP3 gene product.
• **Transmembrane protein 59 – like 1 (TMEM59L)**

The protein encoded by this gene is a 37.6 kDa, predicted membrane glycoprotein protein of uncharacterised function. The *TMEM59L* clone identified from the adult library corresponds to amino acids 187 – 342 (uncharacterised region) of the human *TMEM59L* gene product.

• **Rhomboid domain containing 2 isoform a (RHBDD2)**

The protein encoded by this gene is a 39.2 kDa, predicted multi-pass membrane protein of uncharacterised function. The *RHBDD2* clone identified from the adult library corresponds to amino acids 1 – 230 (uncharacterised, predicted transmembrane region) of the human *RHBDD2* gene product. In a recent study the RHBDD2 mRNA and protein expression were found to be significantly elevated in breast carcinomas (Abba, Lacunza et al. 2009). Other rhomboid family members are widely conserved proteins that are known to function as serine proteases and are also known to regulate molecules involved in apoptosis.

• **Zinc finger protein with UFM1-specific peptidase domain (ZUFSP)**

The protein encoded by this gene is a 65.9 kDa protein of uncharacterised function. The *ZUFSP* clone identified from the adult library corresponds to amino acids 311 – 579 (comprises the uncharacterised peptidase domain) of the human *ZUFSP* gene product. Other members of this family are known to be cysteine peptidases required for the processing and activation of ubiquitin fold modifier 1 (UFM1) and their zinc-finger domains have been shown to be involved in protein: protein interactions (Kang, Kim et al. 2007)
3.8.2 Results from the Neto2-embryonic cDNA library screen

Four putative unique Neto2-interactors (corresponding to 6 clones) that were positive in all the MYTH selection assays were obtained from the human embryonic whole brain cDNA library. Three clones corresponding to transmembrane protein 14A (TMEM14A) led to it being considered as a putative strong Neto2-interactor. The three identical clones corresponding to the TMEM14A were amongst the strongest hits in terms of the rapid growth of the clone and rapid appearance of blue colour in the \( \text{lacZ} \) assay. The remaining clones were considered as putative weak-Neto2 interactors as indicated by their relatively weaker interaction based on the growth and \( \text{lacZ} \) assays. These were transmembrane protein 53, protein di-sulphide isomerasae and the BCL2/adenovirus E1B 19kD-interacting protein 3-like protein.

Table 3.2 - Positive hits from the embryonic library

<table>
<thead>
<tr>
<th>Name of the protein</th>
<th>No of Clones</th>
<th>Clone number(s)</th>
<th>Amino acids fused to ( N_{\text{Ub}} )</th>
<th>NCBI Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein di-sulphide isomerasae</td>
<td>1</td>
<td>10</td>
<td>aa 1 - 226 (440)</td>
<td>NT_005334</td>
</tr>
<tr>
<td>BCL2/adenovirus E1B 19kD-interacting protein 3-like</td>
<td>1</td>
<td>8</td>
<td>aa 51 - 194 (194)</td>
<td>NM_004052</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of the protein</th>
<th>No of Clones</th>
<th>Clone number(s)</th>
<th>Amino acids fused to ( N_{\text{Ub}} )</th>
<th>NCBI Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmembrane protein 14A</td>
<td>3</td>
<td>2, 3, 4</td>
<td>aa 1 - 99 (99)</td>
<td>NT_007592</td>
</tr>
<tr>
<td>Transmembrane protein 53</td>
<td>1</td>
<td>22</td>
<td>aa 62 - 277 (277)</td>
<td>NT_032977</td>
</tr>
</tbody>
</table>

A brief overview of the positive hits is given here:
• **Transmembrane protein 14A (TMEM14A)**

The protein encoded by this gene is a 10.7 kDa, predicted membrane protein of uncharacterised function. The *TMEM14A* clone identified from the embryonic library corresponds to the full length human *TMEM14A* gene product (amino acids 1 – 99).

• **Transmembrane protein 53 (TMEM53)**

The protein encoded by this gene is a 31.6 kDa, predicted membrane protein of uncharacterised function. The *TMEM53* clone identified from the embryonic library corresponds to amino acids 62 – 277 of the human *TMEM53* gene product.

• **Protein di-sulphide isomerasae, family A, member 6 (PDIA6)**

The protein encoded by this gene is a 48.1 kDa, ER resident proteins that catalyzes the formation, reduction, and isomerization of disulfide bonds in proteins and play a role in folding of disulfide-bonded proteins (Hayano and Kikuchi 1995). The *PDIA6* clone identified from the embryonic library corresponds to amino acids 1 – 226 (comprises the di-sulphide isomerase domain) of the human *PDIA6* gene product.

• **BCL2/adenovirus E1B 19kD-interacting protein 3-like (BNIP3)**

Refer to above section from the adult screen
Part 9 - Criteria used to choose molecules from the MYTH screen for subsequent analyses

It should be remembered that MYTH screening results suggest only a possible interaction in vivo, and systematic studies must subsequently be conducted to determine which putative interactors actually associate with Neto2 in vivo. The strong and multiclone interactors in the screen may not necessarily be real interactors in vivo and weak, infrequent interactors should not necessarily be ignored. However, strong and multiclone interactors should be the subjects of initial work.

Initial studies will be focused on those molecules that are known to have well-established neuronal functions, particularly at the synapse. Particularly, those transmembrane proteins that are known to play crucial roles during glutamate-mediated neurotransmission at the synapse will be of immediate interest. Additionally, the molecules that are strongly associated with human neurological disease conditions / mouse phenotypes will be of particular interest. I will later focus on those molecules with less established neuronal functions, particularly the subsets that are not neurospecific. I will give less attention to those molecules with no established neuronal function or unknown function. I will not study molecules that have been reported to be frequent non-specific interactors in the MYTH screening system.
Chapter 4: Conclusions and future directions
Part 1 - A brief summary of the project and major findings from the Neto2-MYTH screen

Neto2, a novel type I neuronal transmembrane protein was recently reported as an auxiliary partner for the kainate-type glutamate receptor subunits (Zhang, St-Gelais et al. 2009). Other unpublished results from our lab (reviewed earlier) indicated that Neto2 can interact with multiple protein partners during the regulation of different neuronal functions including neuronal excitability and synaptic transmission. In order to acquire a broader understanding of the biological roles of Neto2 in neurons, and to gain additional knowledge about its currently known functions, Membrane Yeast Two Hybrid screens were performed to identify putative membrane-bound interacting partners of Neto2.

The Neto2-MYTH screens were conducted using first a human adult, and then a human embryonic cDNA library. Thirteen novel putative Neto2 interactors were identified. Of the thirteen putative Neto2 interactors six proteins have a defined biological function, while seven proteins are uncharacterised. All six proteins with known functions participate in different roles including protein folding, trafficking, and post-translational protein modification. Four proteins namely, VAPB, GTRAP3-18, Chn1b, and PPM1b have neuron-specific functions whereas the proteins EBP and PDIA6 have functions in multiple cell types.

Of the 13 putative Neto2 interactors, clones corresponding to five molecules occurred more than once during the screen. Ten identical clones of VAPB, three identical cones of ZUSFP and two identical clones of EBP were identified in two separate screens from the adult library, while three identical clones of TMEM14A were identified in the screen of the embryonic library. Interestingly, two different clones corresponding to overlapping regions of BNIP3 were
identified independently from both the adult and embryonic cDNA library screens indicating that this molecule may be a true Neto2-interactor. Apart from the three soluble proteins Chn1b, PDIA6 and PPM1B, the remaining ten proteins identified in the screens were transmembrane proteins; of these three clones correspond to full-length transmembrane proteins (*GTRAP3-18, EBP* and *TMEM14A*).

In view of the specific rationale of the project - to identify novel membrane-bound Neto2-interacting partners in the nervous system, two proteins generate particular interest; VAPB and GTRAP3-18. While VAPB is a type II membrane protein, GTRAP3-18 is a four-transmembrane domain protein found in the plasma membrane, ER membrane, and other vesicular membrane compartments of neurons. In particular, VAPB and GTRAP3-18 are both known to be present at the synapse and to interact with multiple synaptic proteins. Both proteins are involved in the trafficking of specific proteins between the ER and other secretory compartments and also to the plasma membrane. While the *Drosophila* homologue of VAPB is known to regulate postsynaptic glutamate receptor clustering, murine GTRAP3-18 is known to alter glutamate reuptake by the glutamate transporter EAAT3 from the synapse. Thus both molecules are known to regulate glutamatergic neurotransmission. Since Neto1 and Neto2 are already known to play important roles in regulating the function of NMDA and kainate type glutamate receptors, the study of Neto2-VAPB and Neto2-GTRAP3-18 interactions, once validated *in vivo*, might lead to a deeper understanding of how the Neto proteins (particularly Neto2) fine tune glutamatergic neurotransmission. Literature reviews of VAPB and GTRAP3-18 are discussed in the next section.
Part 2 - The most promising putative-Neto2 interactors for follow-up studies

In this section I will review the features of VAPB and GTRAP3-18 and their functional relevance with Neto2 that makes them interesting candidates for further study.

4.2.1 VAMP-associated protein B (VAPB)

VAPB is a potential candidate for further studies for the following reasons:

It is the strongest, multicloned interactor identified in 2 independent MYTH screens conducted for the Neto2 bait using the type II adult cDNA library. VAPB is an evolutionarily conserved protein, which is abundantly present in multiple brain regions. *VAPB* mRNA is widely expressed in the mouse nervous system, in the spinal cord, cortex, hippocampus, and cerebellum (Kanekura, Nishimoto et al. 2006; Teuling, Ahmed et al. 2007), where *Neto2* is also highly expressed. The protein encoded by this gene is reported to be abundant at the presynapses (like Neto2); particularly at the presynapses of murine spinal-motor neurons and also at the presynaptic boutons of the *Drosophila* glutamatergic neuromuscular junction, and VAPB has also been reported only once at the postsynapse (Collins, Husi et al. 2006; Chai, Withers et al. 2008).

Mutations in the human *VAPB* gene have been identified in three forms of familial Motor neuron disease including late onset spinal muscular atrophy (SMA) as well as typical and atypical amyotrophic lateral sclerosis (ALS) (Nishimura, Mitne-Neto et al. 2004; Kanekura, Nishimoto et al. 2006). Moreover, the *Drosophila* homologue of VAPB has been shown to regulate glutamatergic neurotransmission by different mechanisms. It regulates glutamate release
at the presynapse through its interactions with the vesicle associated membrane protein /synaptobrevin (VAMP) (Skehel, Martin et al. 1995). VAPB also regulates the presynaptic bouton size by modulating microtubule assembly, postsynaptic glutamate receptor clustering and the postsynaptic abundance of specific glutamate receptor subunits at the fly neuromuscular junction (Pennetta, Hiesinger et al. 2002; Chai, Withers et al. 2008). While the presynaptic function of Neto2 has not been studied before, it has been shown to interact with the GluR6 subunit of the kainate-type glutamate receptor in the cerebellum and to modulate glutamatergic neurotransmission by modulating channel function of the kainate receptor subunits (Zhang, St-Gelais et al. 2009). Additionally, initial studies from our lab also suggest that Neto2 along with Neto1 might regulate the abundance of postsynaptic kainate receptor subunits within the PSDs in the hippocampus and cortex (Tang, Unpublished). These studies indicate that Neto2 might also play multiple roles during glutamatergic neurotransmission.

4.2.2 Current knowledge about VAPB

Human VAPB is a 27kD, type II integral membrane protein that belongs to a family of proteins found across species from yeast to mammals. It shares 63% amino acid identity and hetero dimerizes with a closely related protein, VAPA, in mammals. VAPB has a cytoplasmic N-terminal major sperm protein (MSP) domain, a central coiled-coil domain, and a C-terminal, membrane anchored transmembrane domain (Figure 4.1 a, b) (Weir, Klip et al. 1998; Lev, Ben Halevy et al. 2008). In both humans and mice, VAPB is highly expressed in the motor neurons of the spinal cord and is moderately abundant in the cortex, hippocampus and cerebellum (Figure 4.1 c) as well as in heart, kidney and liver. It is predominantly localized to the ER, golgi membrane and neuromuscular junctions in motor neurons. It has been mostly shown to be a
component of the presynapse and in one study as a component of the postsynaptic density (Weir, Klip et al. 1998; Collins, Husi et al. 2006; Teuling, Ahmed et al. 2007; Chai, Withers et al. 2008; Emes, Pocklington et al. 2008; Lev, Ben Halevy et al. 2008).

VAPB is known to play important roles in many cellular processes including glutamatergic neurotransmission through specific interacting partners. It is known to play important roles in vesicular protein transport between the ER and golgi that is mediated by the coat-protein (COPI) (Soussan, Burakov et al. 1999). Also, it plays an important role in maintaining the lipid compositions of the ER and golgi membranes by interacting with the lipid binding and lipid transfer proteins Nir2, oxysterol binding protein (OSBP), and ceramide transfer protein (CERT) (Peretti, Dahan et al. 2008). The VAPB homologue in Aplysia was originally identified as a binding partner of the vesicle associated membrane protein (VAMP / synaptobrevin), a protein known to be involved in neurotransmitter release (Skehel, Martin et al. 1995). In Drosophila, loss-of-function mutations of the VAPB homologue result in an increase of presynaptic-bouton size associated with an increased clustering of postsynaptic glutamate receptor subunit-IIA (Pennetta, Hiesinger et al. 2002; Chai, Withers et al. 2008). Moreover, over-expression of the VAPB homologue leads to a decrease in presynaptic-bouton size and a reduction in cluster volume of several glutamate receptor subunits by altering microtubule assembly (Pennetta, Hiesinger et al. 2002; Chai, Withers et al. 2008). These studies indicate that VAPB plays a crucial role in synaptic homeostasis by coordinating changes between both the pre and post synapse.

A single missense mutation within the human VAPB gene, substituting a conserved proline residue at position 56 with a serine (P56S) within the protein:protein interacting MSP domain, has been identified in three forms of familial motor neuron disease (Nishimura, Mitne-
Neto et al. 2004; Kanekura, Nishimoto et al. 2006). Studies from animal models and cultured motor neurons reveal that it is a dominant negative mutation and that it depletes wild type VAPB protein from its normal cellular localization, forming insoluble aggregates (Kanekura, Nishimoto et al. 2006; Teuling, Ahmed et al. 2007). P56S VAPB has been shown to affect lipid metabolism and lipid transport between the ER and golgi compartments (Peretti, Dahan et al. 2008). It is also known that this mutation attenuates the activity of ATF6, a resident ER stress signaling transcription factor, thereby leading to an increased unfolded protein response (UPR) (Gkogkas, Middleton et al. 2008). These studies demonstrate that VAPB participates in multiple cellular processes and the mechanism responsible for motor neuron degeneration may be due to a complex interplay between different cellular pathways.
Figure 4.1 – a) VAPB domain organisation (The VAPB clone identified from the adult library corresponds to amino acids 183 – 243); b) VAPB topology; c) VAPB mRNA expression by in situ hybridization in adult mouse brain (http://www.brain-map.org/)
4.2.3 Glutamate transporter EAAT3-associated protein (GTRAP3-18)

GTRAP3-18 is a potential candidate for further studies for the following reasons:

It is a strong, full-length putative interactor identified during the Neto2-MYTH screen using the human adult cDNA library. GTRAP3-18 is an evolutionarily conserved neuronal protein that is abundantly present in multiple brain regions that have high densities of glutamatergic and GABAergic neurons, particularly at their postsynaptic membrane. GTRAP3-18 mRNA is widely expressed in the mouse nervous system particularly in the cortex, hippocampus, cerebellum, and basal ganglia (Butchbach, Lai et al. 2002; Inoue, Akiduki et al. 2005), and it is expressed both during development and in the adult brain. Neto2 mRNA is also expressed both in the developing and in adult brain, in similar brain regions as GTRAP3-18 mRNA. (Figure 4.2 a, b, c)

GTRAP3-18 is known to directly associate with the neuronal protein of the excitatory amino acid transporter family, EAAT3 (also called as excitatory amino acid carrier-1, EAAC1 in humans) to regulate its trafficking and glutamate affinity (Lin, Orlov et al. 2001; Ruggiero, Liu et al. 2008). EAAT3 is an important neuronal glutamate transporter that buffers synaptic-glutamate levels following the release of glutamate from the presynapse (Nieoullon, Canolle et al. 2006), thus modulating glutamate-mediated neurotransmission. Additionally, EAAT3 regulates GABA synthesis and also functions as a glutamate-gated Cl⁻ channel (Eskandari, Kreman et al. 2000; Koch, Brown et al. 2007), thus regulating GABAergic-inhibitory neurotransmission. Therefore GTRAP3-18 might play important roles during EAAT3-mediated glutamatergic and GABAergic neurotransmission. While the role of the Neto2:GluR6 interaction modulating glutamate-mediated neurotransmission is reviewed earlier (section 1.2.2), an interaction of Neto2 with KCC2 (a major ion pump required for Cl⁻ homeostasis in neurons)
regulates the chloride equilibrium and membrane excitability in cultured hippocampal neurons (Ivakine et al, Unpublished). Therefore Neto2 is also thought to perform multiple functions during excitatory and inhibitory neurotransmission like GTRAP3-18.

4.2.4 Current knowledge about GTRAP3-18

GTRAP3-18 is a 21.6 kDa, four-transmembrane protein expressed in the neuronal membrane and ER membrane in mice and humans (Lin, Orlov et al. 2001; Inoue, Akiduki et al. 2005; Schweneker, Bachmann et al. 2005; Akiduki, Ochiishi et al. 2007; Ruggiero, Liu et al. 2008). This molecule was identified as a negative regulator of the glutamate-uptake activity of the excitatory amino acid transporter 3 (EAAT3) (Lin, Orlov et al. 2001) that acts by decreasing the glutamate affinity of EAAT3. Further studies indicated that GTRAP3-18 delays the ER-exit of EAAT3 and other members of the excitatory amino acid transporter family (Ruggiero, Liu et al. 2008), thus playing an important modulatory role in glutamatergic neurotransmission.

The time point and expression of GTRAP3-18 in the brain is similar with the expression patterns of EAAT3. Both EAAT3 and GTRAP3-18 mRNA is expressed early during development (peaks at around P5) and also in the adult brain across multiple regions, particularly regions of high densities of glutamatergic and GABAergic neurons in the hippocampus, cerebellum, caudate putamen, and amygdala (Inoue, Akiduki et al. 2005; Akiduki, Ochiishi et al. 2007; Tzingounis and Wadiche 2007; Ruggiero, Liu et al. 2008). It has also been shown that EAAT3 is the earliest expressed neuronal glutamate transporter and thus GTRAP3-18, along with EAAT3, is thought to play important roles in the developing and in the adult brain (Nieoullon, Canolle et al. 2006; Tzingounis and Wadiche 2007).
Apart from regulating glutamate concentration at synapses, EAAT3 is also known to regulate GABA synthesis. Homo-trimeric and homo-pentameric EAAT3 also functions as an glutamate-gated chloride channel (Eskandari, Kreman et al. 2000; Amara and Fontana 2002; Koch, Brown et al. 2007). While the precise role of GTRAP3-18 in EAAT3-mediated inhibitory neurotransmission has not been studied, it might indirectly participate during this process because these functions are also dependent on glutamate binding to EAAT3.
Figure 4.2 – a) GTRAP3-18 domain organisation - (The GTRAP3-18 clone identified from the adult library corresponds to amino acids 1 – 188); b) GTRAP3-18 topology; c) GTRAP3-18 mRNA expression by in situ hybridization in adult mouse brain (http://www.brain-map.org/)
Part 3 - Proposed experiments for Neto2 and VAPB

The following experimental plan would help us understand the biological relevance of Neto2-VAPB interaction. Initial studies would focus on characterizing the interaction between Neto2 and VAPB, and the following questions could be addressed:

a) Do VAPB and Neto2 physically interact in vivo?

Co-immunoprecipitation (CoIP) experiments using wildtype and Neto2 null mice tissues, particularly from the spinal cord, hippocampus and cerebellum (where both proteins are expressed) would help us find whether the interaction is physiologically relevant. Also, CoIP experiments using a heterologous cell system (HEK293 cells) by transfecting those with Neto2 and VAPB cDNA could be performed to confirm whether the interaction is direct. Deletion constructs could be generated for VAPB, to determine the minimal domains required for an interaction with Neto2. Previously validated Neto1 association with Neto2 or GluR6 association with Neto2 would serve as a positive control for the Neto2 interaction in these experiments.

b) In which sub-cellular location do they co-localize?

Using primary neuronal cultures of spinal cord motor neurons / hippocampus / cerebellum obtained from Neto2 null and WT mice, co-immunostaining could be performed to identify whether Neto2 and VAPB co-localise, using markers specific for different sub-cellular locations. Using the same approach, it could also be determined whether the two proteins co-localize at the presynapse or at the postsynapse. These results could be confirmed by performing CoIP from crude synaptosomes, presynaptic vesicles and postsynaptic density components from Neto2 null mice and WT mice. In these experiments VAMP-2 could be used as a marker for synaptic
vesicle at the presynapse, PSD-95 for postsynaptic density, microtubule associated protein1B for microtubule association, and calnexin for ER membrane.

Depending upon in which sub-cellular location the association between the two molecules is observed, the following questions could be addressed:

c) Does the absence of Neto2 affect any of the known cellular functions of VAPB?

Since VAPB regulates the presynaptic microtubule assembly and glutamate release, disruption of microtubule assembly along with changes in the size and number of synaptic vesicles could be visualized using electron microscopy approach in multiple brain regions like spinal cord, hippocampus and cerebellum of the Neto2 null animals. Additionally, glutamate release assay could be performed in the motor neurons of Neto2 null animals to confirm whether the presynaptic glutamate release is aberrant.

d) Does the loss of VAPB function affect the trafficking / surface expression of Neto2 and / or kainate receptor subunits? Does the loss of both VAPB and Neto2 function affect the kainate receptor trafficking?

The above questions could be addressed by using primary neuronal cultures of spinal cord motor neurons / hippocampus / cerebellum obtained from Neto2 null and WT mice. By applying RNAi approach VAPB mRNA in both Neto2 null and WT cultures could be knocked down and subsequently the surface expression, sub-cellular location of Neto2 and different subunits of KAR could be studied using immunostaining. Additional experiments could be performed to study whether VAPB sub-cellular location is altered in the Neto2 nulls.
If the kainate receptor trafficking / abundance / surface expression is disrupted in the *VAPB* knockdown experiments in motor neuronal cultures, then its biological consequences and corresponding neurophysiological changes (changes in glutamate mediated currents) could be studied. Our lab collaborates with Dr. Michael Salter, and Dr. Melanie Woodin to study the electrophysiology of these neurons following such changes in the receptor density at the neuronal surface.

Additionally, due to a significant amino acid identity between Neto1 and Neto2, it would be important to determine whether Neto1 could also interact with VAPB. Neto1 has been shown to associate with different NMDAR subunits and loss of *Neto 1* leads to a decrease in the NR2A level (a subunit of NMDA receptor) (Ng et al., 2009) and also a decrease in the GluR6 (a subunit of kainate receptor) (Tang, Unpublished) in the hippocampal PSD fraction. It would be interesting to study the consequence of Neto1-VAPB interaction with respect to glutamate receptor trafficking, surface expression and abundance.
**Part 4 - Proposed experiments for Neto2 and GTRAP3-18**

The following experimental plan would help us understand the biological relevance of Neto2-GTRAP3 interaction. Initial studies would focus on characterizing the interaction between Neto2 and GTRAP3-18, and the following questions could be addressed:

a) Does GTRAP3-18 and Neto2 physically interact *in vivo*?

b) In which sub-cellular location do they co-localize?

Similar experimental approaches could be used to address these questions as described for the characterization of the Neto2-VAPB interaction. After validating the Neto2-GTRAP3 interactions *in vivo* the other important questions to be addressed are:

a) Does the loss of Neto2 affect the surface expression GTRAP3-18 and EAAT3?

HEK293 cells expressing GTRAP3-18 / EAAT3 with or without the Neto2 co-expression, followed by biotinlation studies would reveal whether the absence of Neto2 affect the surface expression of GTRAP318 / EAAT3 in these non-neuronal cells. Additionally, subcellular fractionation of the hippocampus / cerebellar lysates from the Neto2 null and wildtype mice, could be used to study whether the surface abundance of GTRAP3/18 / EAAT3 in the neuronal membrane is altered using specific antibodies against them.

b) Does the loss of Neto2 affect the glutamate-uptake function of GTRAP3-18 and EAAT3?

The glutamate affinity for EAAT3 can be measured during the presence and absence of Neto2, co-expressed in HEK293 cells. Additionally the glutamate affinity can be measured in the presence of both Neto2 and GTRAP3-18 in HEK293 cells.
c) Does the loss of Neto2 affect the anion conductance function of GTRAP3-18 and EAAT3?

The anion conductance of the EAAT3 can be electrophysiologically measured co-injecting Neto2 cRNA and with EAAT3 cRNA in Xenopus laevis oocytes.

Part 5 - Conclusions

The Neto2-MYTH screen was successfully completed leading to the identification of several novel, putative-Neto2 interactors. Particularly, if the Neto2-VAPB and Neto2-GTRAP3-18 interactions are confirmed in vivo, this would open new avenues in the current understanding about the biology of Neto2 particularly during synaptic transmission in the central nervous system (Figure 4.3)
Figure 4.3 – Possible novel functions for Neto2 during the fine tuning of glutamatergic neurotransmission

a) Neto2 association with the GluR6 type kainate receptor complex is already known to modulate the glutamatergic neurotransmission by altering the channel properties of the receptor.

b) If the Neto2 association with VAPB is physiologically true, it may be involved during the glutamate release by regulating the microtubule assembly at the presynapse.

c) If the Neto2 association with GTRAP3-18 is physiologically true, it may be involved during the glutamate clearance by regulating the function of glutamate transporter EAAT3 at the postsynapse.
Appendices
Appendix I - List of strains, antibodies, plasmids and primers

List of yeast and bacterial strains used

Yeast strain THYAP4 was obtained from Dr. Igor Stagljar

Bacterial strains DH5α, XL1-Blue were purchased from Stratagene Inc.

List of antibodies used

1. Rabbit Anti-Neto2; in-house antibody raised against the intracellular carboxyl 70 amino acids of mouse Neto2
2. Mouse Anti-Neto2; raised against the ectodomain of mouse Neto2
3. Rabbit Anti-VP16; Raised against synthetic peptide corresponding to amino acids 464-476 of the herpes simplex virus VP16 protein (Sigma)
4. HRP conjugated mouse anti-rabbit secondary antibody (Sigma)
5. Alexa fluor 488 conjugated goat anti-rabbit secondary antibody (Invitrogen)

List of plasmids and cDNA used

All MYTH plasmids were obtained from Dr. Igor Stagljar

Human Neto2 cloned into pBluescript was obtained from Zhenya Ivakine

1. pAMBV (bait vector)
2. pPR3-N (prey/ type II library vector)
3. pPR3-C (prey/type I library vector)
4. pOst1- NubI (unrelated prey, positive control for bait over-expression, ER)
5. pOst1- NubG(unrelated prey, negative control for bait over-expression , ER)
6. pFur4- NubI(unrelated prey, positive control for bait over-expression, PM)
7. pFur4- NubG(unrelated prey, negative control for bait over-expression, PM)
8. pCD4 (unrelated artificial bait, control for prey over-expression)
9. Human Neto2 cDNA (ensembl gene ID ENSG00000171208)

10. Human Neto1 cDNA (ensembl gene ID ENSG00000166342)

11. Human adult, whole brain-cDNA library – pPR3N (specific for type II membrane proteins and cytosolic proteins) was purchased from Dual Systems Biotech

12. Human embryonic, whole brain-cDNA library – pPR3N (specific for type II membrane proteins and cytosolic proteins) was originally from Dual Systems Biotech, was obtained from Dr. Igor Stagljar.

List of additional primers used for Neto2- Cαβ-fusion plasmid sequencing*

1. Forward primer 1 - (5’ – TTT CCT CGT CAT TGT TCT CGT TCC CTT TCT – 3’)
2. Forward primer 2 - (5’ – ATG AAG AGC TTG AAG GAC TGG GAT TTC GAG – 3’)
3. Forward primer 3 - (5’ – TTC AGG GAT TGT CTT GGT CCT TCT CAT TA – 3’)
4. Forward primer 4 - (5’ – TTC GAT CTC ATC CGT GAT CAC ATC AGC CAG – 3’)
5. Reverse primer 1 - (5’ – TTC TAC CTG ACT AGA GCG CAC TAT TCC ATC – 3’)
6. Reverse primer 2 - (5’ – ATG GAA CTG AGG TTG GTC CTG CTT T – 3’)
7. Reverse primer 3 - (5’ – AAC CCT TCT TCC TCT TCC TGC AAC AGA CGG AT – 3’)
8. Reverse primer 4 - (5’ – ATA AAT AGG GAC CTA GAC TTC AGG TTG TCT – 3’)

*Vector NTI software (version 10) was used for designing primers and construction of the fusion plasmids
Appendix II – Additional details about the MYTH bait vector pAMBV and prey vector pPR3-N

The Bait vector pAMBV ($ADH1$-MFα-Bait-$C_{ub}$-LexA-VP16) contains a weak $ADH1$ promoter, which drives low levels of heterologous protein expression. Specific for type I transmembrane proteins, pAMBV contain the yeast MFα signal sequence at the N terminus, resulting in an improved targeting of the heterologous bait protein to the yeast membrane. Following the signal sequence is the multiple cloning site (MCS) for introducing the gene of interest ($Neto2$). Following the MCS is the C-terminus of Ubiquitin ($C_{ub}$) and the artificial transcription factors LexA-VP16. pAMBV is a centromeric plasmid containing an autonomously replicating sequence (ARS) origin of replication and one centromeric locus (CEN), which results in one to two copies of the plasmid per cell. These low-copy-number plasmids autonomously replicate in both $E. coli$ and $S. cerevisiae$ and contain the $KanR$ and $LEU2$ genes for selection of plasmid-bearing cells on medium containing kanamycin in bacteria) or lacking leucine in yeast respectively.

The prey vector pPR3-N ($ADH$-$N_{ub}$G-HA-prey) allows cloning of a library of prey genes in frame with $N_{ub}$G at the N-terminus of the protein (for type II membrane and cytoplasmic proteins). It expresses the fusion protein from the strong constitutive promoter $ADH1$ and is replicated by the high-copy-number origin of replication. They are selected for by the $AmpR$ and $TRP1$ genes, allowing growth on medium containing ampicillin (bacteria) or lacking tryptophan (yeast), respectively. The use of the $AmpR$ marker for $E. coli$ facilitates the re-isolation of these vectors after screening with respect to the bait vector, which has a $KanR$ marker.
Multiple cloning site

Xba I Sfi I
TC TAG AGC GCC ATT AGC GCC AAA CTC GCT GCC GCC GCC C3A ACC AGT GCC TGC

Sfi I Stu I Nco I
AGC GCC GCC TCG GCC AAA GCC CTC CAT GGC TAT ATC TGC AGG AAT TCG ATA TCA

AGC TTA TCG ATA CCG TCG ACC ATG --> Cub

Bait vector features (Dualsystems biotech)

<table>
<thead>
<tr>
<th>Position</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start: 107, End: 1581</td>
<td>ADH1 promoter</td>
</tr>
<tr>
<td>Start: 1709, End: 1844</td>
<td>Cub, ubiquitin (amino acids 34-76)</td>
</tr>
<tr>
<td>Start: 1868, End: 2474</td>
<td>LexA DNA binding domain (amino acids 1-200)</td>
</tr>
<tr>
<td>Start: 2491, End: 2729</td>
<td>Herpes simplex VP16 transactivator (amino acids 464-476)</td>
</tr>
<tr>
<td>Start: 2730, End: 2983</td>
<td>CYC1 terminator</td>
</tr>
<tr>
<td>Start: 3701, End: 5929</td>
<td>LEU2 auxotrophic marker</td>
</tr>
<tr>
<td>Start: 6078, End: 7110</td>
<td>KanR resistance gene</td>
</tr>
<tr>
<td>Start: 8193, End: 8917</td>
<td>CEN/ARS - Origin of replication</td>
</tr>
</tbody>
</table>
Prey vector features (Dualsystems biotech)

Position                  Feature
Start: 62                CYC1 promoter
Start: 364               NubG, ubiquitin amino acids 1-38
Start: 481               HA epitope tag
Start: 622               CYC1 terminator
Start: 1598              TRP1 auxotrophic marker
Start: 2855              2micron origin of replication
Start: 4335              AmpR resistance gene
Start: 5328              pBluescript origin of replication
Appendix III - Brief description of the MYTH cDNA libraries

*Human adult and Human fetal whole-brain cDNA-library (Dualsystems biotech)*

The employed human adult brain NubG-X split-ubiquitin cDNA library was purchased from Dual systems Biotech and the human fetal brain NubG-X split-ubiquitin cDNA library was kindly provided by Dr. Igor Stagljar, and they were constructed as follows: Split-ubiquitin prey expression vectors are based on plasmid pPR3N that contain the NubG fused with HA coding sequence. Total RNA was isolated from the brain of a Caucasian-female donor aged 59 years (who died of tracheal cancer). Total RNA was isolated from normal fetal brains pooled from 21 spontaneously aborted male/female Caucasian fetuses that were aged between 26-40 weeks. cDNA synthesis was performed using oligo dT priming during which Sfi I restriction sites were introduced. The cDNA were directionally cloned into the pPR3N vector using Sfi I site such that, the NubG-HA coding sequence is oriented in-frame with the 5' end of the cDNA. These libraries have a complexity of 2 x 10^6 independent clones and average insert sizes of 1.6 kb. These pPR3N - NubG-X cDNA libraries are more suitable for finding integral membrane proteins that does not have a N-terminal signal sequence (like the type II membrane proteins as well as other multi-pass membrane proteins topologically similar to the type II membrane proteins) as well as the cytosolic interacting partners of Neto2.
Appendix IV - Standard reagent recipes

10X TAE buffer, pH 7.5
Mix 20 ml 1M Tris-HCl (pH 7.5) and 4 ml of 0.5M EDTA (pH 8.0), add ddH₂O to a final volume of 200 ml. Autoclave at 121°C for 15 min, store at room temperature.

10X PBS, pH 7.5
Dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of NaH₂PO₄ and 2.4 g of KH₂PO₄ in 800 ml of ddH₂O. Adjust the pH to 7.5 with HCl and adjust the volume to 1 liter with ddH₂O. Autoclave at 121°C for 15 min, and store at room temperature.

YPAD plates and media
Mix 10 g Bacto Yeast Extract, 20 g Peptone, 20 g glucose, 40 mg Adenine Sulphate (add 20 g of Bacto Agar for plates). Add ddH₂O to a final volume of 1000 ml, mix, and autoclave at 121°C for 15 min. Pour plates while the agar is still warm (∼50°C). Store the media at room temperature and the plates at 4°C.

2X YPAD media
Mix 20 g Bacto Yeast Extract, 40 g Peptone, 40 g glucose, 40 mg Adenine Sulphate. Add ddH₂O to a final volume of 1000 ml, mix, autoclave at 121°C for 15 min and store at room temperature.

LB plates and media
Mix 10g Tryptone, 5g Bacto Yeast Extract, 10g NaCl (add 20 g of Bacto Agar for plates). Add ddH₂O to a final volume of to 1000 ml, mix, autoclave at 121°C for 15 min. Pour plates while the agar is still warm (∼50°C). Store the media at room temperature and the plates at 4°C.

Lysis buffer for yeast immunoprecipitation
Mix 0.5ml of 1M Tris-HCl, pH 7.4, 0.3 ml of 5M NaCl, 20 µl of 0.5M EDTA, 100 µl of 1% triton-X-100, 100 µl 100mM of NaVO₄, 50 µl of 20% SDS. Adjust the final volume to 10 ml with ddH₂O and store at -4°C. Dissolve 1 tablet of 10X Complete protease inhibitor cocktail tablets (inhibits serine proteases, cysteine proteases and metalloproteases) from Roche prior to use.
6X gel-loading buffer for SDS-PAGE
Mix 7.2 ml of 1M Tris-HCl, pH 6.8, 2.4 g of SDS, 2.82 ml of 87 % glycerol, 6 mg of bromophenol blue and 2.4 ml of β-mercapto ethanol. Adjust the final volume to 20 ml with ddH₂O and store at -20°C. Add 20 μl of 1M DTT to 80 μl of 6X buffer prior to gel loading.

10% acrylamide SDS-PAGE gel
For resolving gel, mix 2.5 ml of 1.5 M Tris-HCl buffer, pH, 8.8, 2.5 ml 40 % acrylamide-bisacrylamide mix (29:1), 100 μl of 10 % SDS, 100 μl of 10 % APS and 4 μl of TEMED. Mix it well with 4.8 ml of ddH₂O. For stacking gel, mix 1.815 ml of water, 750 μl of 1M Tris-HCl buffer, pH, 6.8, 375 μl 40 % acrylamide-bisacrylamide mix, 30 μl of 10 % SDS, 30 μl of 10 % APS and 3 μl of TEMED.

10 X SDS-PAGE running buffer
Dissolve 30.3 g Tris base, 144.4 g of glycine, 10 g of SDS in 900 ml of ddH₂O and adjust the volume to 1 liter.

5X Transfer Buffer for western blotting
Dissolve 14.5 g of glycine, 29 g of tris base and 1.5 g of SDS in 900 ml ddH₂O and make up the volume to 1 liter. During western transfer, mix 200 ml of 5X TB with 200 ml of methanol and adjust the volume to 1 liter with ddH₂O.

5X TBST (for 2 liters)
Dissolve 80 g of NaCl, 2 g of KCl, 30 g of Trizma base and 10 ml of Tween-20 in 1800 ml ddH₂O and make up the volume to 2 liters.

Blocking buffer for western blotting
Dissolve 0.5 g of skim milk powder in 10 ml of 1X TBST to make 5 % blocking buffer for pre-incubation of blot prior to addition of primary antibody. Dissolve 0.3 g of milk powder in 10 ml of 1X TBST to prepare 3 % buffer for incubations with primary antibody. Use 5 % buffer for secondary antibody incubations.
10X General Yeast dropout amino acid mix

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>In mg (for 1 liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Isoleucine</td>
<td>300</td>
</tr>
<tr>
<td>L-Valine</td>
<td>1500</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>300</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1500</td>
</tr>
<tr>
<td>L-Phenyalanine</td>
<td>500</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>2000</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>300</td>
</tr>
<tr>
<td>L-Uracil</td>
<td>200</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>200</td>
</tr>
</tbody>
</table>

After dissolving, make the final volume to 1000 ml with ddH₂O, autoclave at 121°C for 15 min and store the media at 4°C.

Yeast Synthetic Dropout plates and media (SD-)

Mix 6.5 g Bacto Yeast Nitrogen Base and 20 g D-Glucose (add 20 g of Bacto Agar for plates).

To make a dropout media / plate, include the specific amino acid (from below) along with 100ml of amino acid dropout mix (from above)

| L-Histidine      | 200                 |
| L-Leucine        | 1000                |
| Adenine Sulfate Dihydrate | 400           |
| L-Tryptophan     | 400                 |

After dissolving, make the final volume to 1000 ml with ddH₂O, autoclave at 121°C for 15 min. Pour plates while the agar is still warm ≈50°C. Store the media at room temperature and plates at 4°C.
Yeast Synthetic Dropout plates with X-Gal

Mix SD media with agar and dropout mix as indicated; and make the final volume to 900 ml with ddH$_2$O, autoclave at 121°C for 15 min, cool to ≈50°C. In a separate bottle autoclave 7 g of Sodium Phosphate (dibasic) and 3 g of Sodium Phosphate (monobasic) in 100 ml ddH$_2$O and cool to ≈50°C. Mix the two solutions and add 0.8 ml of 100 mg/ml X-Gal (in N,N-dimethyl formamide). Pour plates and store the plates at 4°C.

Yeast Synthetic Dropout plates with 3-aminotriazole (3-AT)

Mix SD media with agar and dropout mix as indicated; and bring the final volume to 1000 ml with ddH$_2$O, autoclave at 121°C for 15 min, cool to ≈50°C. Add 3-AT according to the concentrations of 1, 2.5, 5, 10, 25, 50, 75 and 100 mM. Prepare in small volumes (five - 90 mm plates for each concentration) for the initial titration and later in large – 150 mm plates for pilot and library screening.

1M Lithium Acetate, pH 7.5

Dissolve 20.4g of LiOAc in ddH$_2$O, adjust pH to 7.5 with 5% acetic acid and make up the final volume to 200 ml. Mix, autoclave at 121°C for 15 min and store at room temperature.

0.9 % NaCl

Dissolve 0.9 g NaCl in 100 ml ddH$_2$O. Sterilize by autoclaving at 121°C for 15 min; store at room temperature.

50% Poly Ethylene Glycol (PEG) 3350

Dissolve 50 g of PEG 4000 in ddH$_2$O to a final volume of 100 ml. Mix, autoclave at 121°C for 15 min. Aliquot in sterile 15 ml tubes, parafilm the cap, and store at 4°C.

Single-stranded DNA from salmon sperm (2mg/ml)

Dissolve 200 mg salmon sperm DNA sodium salt in 100 ml ddH$_2$O. Dissolve the chunks of DNA by drawing up and down a few times with a 25ml sterile plastic pipette. Stir for 2–3 h to completely dissolve the DNA and sterilize by autoclaving at 121°C for 15 min; aliquot and store at -20°C. Thaw required volumes of ssDNA for 5 min in a 100°C just before use.
PEG/LiOAc/ssDNA master mix (small-scale transformation)
Mix 240 µl 50% PEG 3350, 36 µl 1M LiOAc and 50 µl 2mg/ml ssDNA in a 1.5 ml eppendorf tube. This 326 µl total volume is used for a single transformation. Freshly prepare before use.

TAE/LiOAc master mix (large-scale transformation)
Mix 1.1 ml of 1M LiOAc, 1.1 ml of 10X TAE buffer and 7.8 ml of sterile ddH2O. Freshly prepare before use.

PEG/LiOAc/ssDNA master mix (large-scale transformation)
Mix 1.5 ml of 1M LiOAc, 1.5 ml of 10X TAE buffer and 12 ml of 50% PEG in a 50 ml falcon tube. This 15 ml total volume is used for a single transformation. Freshly prepare before use.

Sorbitol/Phosphate (SK) buffer
Mix 30 ml of 2M sorbitol, with 6.4 ml of 0.2 M of K2HPO4 and 6.4 ml of KH2PO4 in 12.525 ml of ddH2O. Autoclave at 121°C for 15 min and store at 4°C.

Zymolyase solution (25mg/ml in S/K buffer)
Mix 50 mg Zymolyase powder in 1.2 ml S/K buffer, 20 µl β-mercapto ethanol; and bring the volume to 2 ml with ddH2O. Aliquot and store at -20°C.

DAPI reagent
10 mg/ml of DAPI was dissolved in 1X PBS; aliquoted and stored in -20°C. 1:5000 dilution of stock DAPI in 1X PBS was used as a working reagent.
References


